Involvement of PI3K-Dependent Signal Transduction During the Integrated Control of Basal and Ligand-Biased Pituitary Cell Functions

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Physiology, Cell & Developmental Biology

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Abstract

Within multicellular organisms, individual cells must communicate with one another in order to coordinate physiological functions. One of the ways this is achieved is through the production and release of chemical messengers, called hormones. Hormones alter the functions of their target cells by binding to receptors that are often present on the cell-surface to regulate the activity of intracellular messaging proteins that control cellular responses. This process is collectively referred to as signal transduction. The ability of receptors to engage distinct signal transduction mechanisms following hormone binding allows for the hormone-selective regulation of cells and intracellular functions. Recent studies reveal that closely related hormones can selectively activate a distinct subset of signal transduction responses through shared receptors in a process called biased signalling. Interestingly, multiple variants of a single hormone are found in almost all organisms; however, the extent to which naturally occurring hormone variants utilize biased signalling during the integrated control of cell physiology is poorly understood.

To explore the possibility of signalling bias within natural hormone families, my thesis work characterizes the signal transduction mechanisms utilized by two endogenous variants of gonadotropin-releasing hormone (GnRH; called GnRH2 and GnRH3) using a pituitary cell primary culture system established in a classical vertebrate model of hormonal communication, the goldfish (*Carassius auratus*). In general and across vertebrates, GnRHs reliably stimulate the release of luteinizing hormone (LH) and growth hormone (GH) from pituitary gonadotropes and somatotropes, respectively, to coordinate reproduction and somatic growth. Using hormone release and cellular hormone content as physiologically relevant endpoints, results demonstrate that different

natural variants of GnRH utilize overlapping, and yet distinct, intracellular signal transduction mechanisms to control diverse aspects of pituitary cell function. In particular, I identify signal transduction mediated by the phosphoinositide 3-kinase (PI3K) superfamily as important for the integrated regulation of hormone release and production. These studies include the first evidence of biased intracellular regulation of the classical receptor-coupled class I PI3K isoforms, as well as demonstrate heterogeneity in the transduction systems involved downstream of PI3K-dependent signalling within goldfish gonadotropes and somatotropes. Lastly, results indicate that the highly-conserved mitogenic Raf-MEK-ERK cascade, as well as common PI3K-dependent signal transduction elements, likely interact to selectively affect GnRH-stimulated as well as basal hormone release and total availability in a cell type-, time-, and GnRH-dependent manner.

Overall, the results presented in this thesis represent the first comprehensive examination of PI3K-dependent signalling in any pituitary cell type, including the only characterization of isoform-selective involvement of PI3Ks during regulated pituitary hormone exocytosis. The experiments performed also identify class I PI3Ks as evolutionarily conserved signalling targets of the G protein-coupled receptor superfamily and outline, for the first time, the versatility of PI3Ks as an intracellular platform for mediating biased cellular responses. Taken together, data presented in this thesis provide several novel findings that illustrate that the biased actions of individual GnRH variants leads to differences in their ability to control acute and long-term hormone release responses, as well as facilitate the selective regulation of cellular hormone availability. More generally, these studies represent the first steps in understanding how the

complexity of biased signalling contributes to the endocrine control of reproduction and growth by selectively controlling pituitary cell functions. Furthermore, by using a basal vertebrate model to study biased signalling by natural hormone variants at the intracellular level, my doctoral research has provided important evolutionary insights into the molecular mechanisms that couple receptor activation to biased intracellular signal transduction responses and reveal how this phenomenon can regulate cell functions that can ultimately impact whole-organism physiology.

Preface

This thesis is the original work by Joshua George Pemberton. The research project, of which this thesis is a part, received research ethics approval from the University Research Office 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 Pemberton, J.G., Stafford, J.L., Yu, Y., and Chang, J.P., 2011, "Differential involvement of phosphoinositide 3-kinase in gonadotrophin-releasing hormone actions in gonadotrophs and somatotrophs of goldfish, *Carassius auratus*" Journal of Neuroendocrinology, vol 23, 660-674; and Pemberton, J.G., Orr, M.E., Stafford, J.L., and Chang, J.P., 2014, "PI3K signalling in GnRH actions on dispersed goldfish pituitary cells: relationship with PKC-mediated LH and GH release and regulation of long-term effects on secretion and total cellular hormone availability" General and Comparative Endocrinology, vol 205, 268-278. Chapter 4 has been published in part as Pemberton, J.G., Stafford, J.L., Yu, Y., and Chang, J.P., 2011, "Differential involvement of phosphoinositide 3-kinase in gonadotrophin-releasing hormone actions in gonadotrophs and somatotrophs of goldfish, *Carassius auratus*" Journal of Neuroendocrinology, vol 23, 660-674; and Pemberton, J.G., Stafford, J.L., and Chang, J.P. , 2015, "Ligand-selective signal transduction by two endogenous GnRH isoforms involves biased activation of the class I PI3K catalytic subunits p110β, p110δ, and p110γ in pituitary gonadotropes and somatotropes" Endocrinology, vol 156, 218-230. Chapter 6 is published as Pemberton, J.G., Orr, M.E., Stafford, J.L., and Chang, J.P., 2014, "PI3K signalling in GnRH actions on dispersed goldfish pituitary cells: relationship with PKC-mediated LH and GH release and regulation of long-term effects on secretion and total cellular hormone availability" General and Comparative Endocrinology, vol 205, 268-278. Chapter 7 has been published as Pemberton, J.G., Orr, M.E., Booth, M, and Chang, J.P., 2013, "MEK1/2 differentially participates in GnRH actions on goldfish LH and GH secretion and hormone protein availability: acute and long-term effects, *in vitro*" General and Comparative Endocrinology, vol 192, 149-58.

I was responsible for the data collection and analysis, as well as the manuscript composition. Drs. John P. Chang (supervisor) and James Stafford (supervisory committee member) provided conceptual guidance and manuscript composition editing. Dr. Yi Yu assisted with training me on Fura-2 Ca^{2+}-imaging techniques. I supervised and helped undergraduate students Mr. Michael E. Orr (summer and project student), Mr. Federico Sacchi (summer and project student), Mr. Nicholas Churchill (summer student), and Mr. Malcolm Laraque (project student) in executing static incubation studies included in this thesis and performed the radioimmunoassays for the samples and analyzed the collected data. Mr. Morgan E. Booth was a project student who provided the acute MEK1/2 inhibition data that I reanalyzed and formatted for graphic presentation. Dr. Y. Yu and Mr. M.E. Orr and Booth also provided input into co-authored manuscripts.

Acknowledgements

I would like to thank: my family and friends, the shoulders of giants (*in general*), Charles Darwin, Richard (*Dick*) Peter, John Chang, James Stafford, Declan Ali, Patrick MacDonald, Glen Van Der Kraak, Chris Morris, Tim Prinsen, Tom Dennehy, Danny Boily, the members of the Wu-Tang Clan (R.I.P. the *Ol' Dirty Bastard* a.k.a. *Dirt McGirt* a.k.a. *Ason Unique* a.k.a. *Big Baby Jesus*), Yvonne Strahovski, the Edmonton Oilers, the Edmonton Eskimos, the University of Alberta Golden Bears Football Team, the Harry Ainlay Titans Senior Men's Football Team, the New England Patriots, Subway, Edo Japan, Tim Hortons, the staff at the SugarBowl, and all of the goldfish (*Carassius auratus*) who sacrificed their lives for the good of science.

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List of Abbreviations

$[Ca^{2+}]_i$	intracellular free Ca^{2+} concentration
7TM	seven transmembrane topology
AA	arachidonic acid
AC	adenylyl cyclase
ADP	adenosine diphosphate
Akt	Protein Kinase B
Akt_i VIII	selective allosteric Akt inhibitor
ANOVA	analysis of variance
Arf	ADP-ribosylation factor
ARNO	ADP-ribosylation factor nucleotide-binding-site opener
Arp2/3	actin-related protein complex
ATM	Ataxia telangiectasia mutated kinase
ATPase	adenosine trisphosphatase
ATR	ataxia telangiectasia and Rad3-related protein kinase
BSA	bovine serum albumin
BTK	Bruton's Tyrosine Kinase
BYL719	selective inhibitor of the class IA PI3K p110α catalytic subunit
Caff	caffeine; activator of intracellular Ca^{2+} release
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic-3′-5′-adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CGI1746	selective BTK inhibitor
CK2	casein kinase 2
CZC24832	selective inhibitor of the class Ib PI3K p110γ catalytic subunit
DA	dopamine
DAG	1,2-diacylglycerol
DIC	differential interference contrast
DiC8	PKC activator; other intracellular targets are known

DMSO	**dimethyl sulphoxide**
DNA-PK	**DNA-dependent protein kinase**
DNAse	**deoxyribonuclease**
DRD1	**D1-type dopamine receptor**
DRD2	**D2-type dopamine receptor**
EGFR	**epidermal growth factor receptor**
ERK	**extracellular signal-regulated protein kinase**
G Protein	**guanine nucleotide-binding protein**
GAPs	**GTPase-activating proteins**
GDC0941	**pan-class I PI3K inhibitor**
GDP	**guanosine diphosphate**
GEFs	**guanine nucleotide exchange factors**
GfA GnRHR	**goldfish gonadotropin-relesing hormone receptor type A**
GfB GnRHR	**goldfish gonadotropin-relesing hormone receptor type B**
GH	**growth hormone**
GHRH	**growth hormone-releasing hormone**
GnIH	**gonadotropin-inhibitory hormone**
GnRH	**gonadotropin-releasing hormone**
GnRH2	**[His5,Trp7,Tyr8]GnRH; Type II GnRH; (c)GnRH-II**
GnRH3	**[Trp7,Leu8]GnRH; Type III GnRH; (s)GnRH**
GnRHR	**gonadotropin-releasing hormone receptor**
GPCR	**G protein-coupled receptor**
GRK	**G protein-coupled receptor kinase**
GRL	**ghrelin**
GRP1	**general receptor for phosphoinositides 1**
GSK2334470	**selective PDK1 inhibitor**
GSK3	**glycogen synthase kinase 3**
GTH	**gonadotropin**
GTP	**guanosine-5′-trisphosphate**
GTPase	**guanine trisphosphatase**
Gα	**G protein alpha subunit**

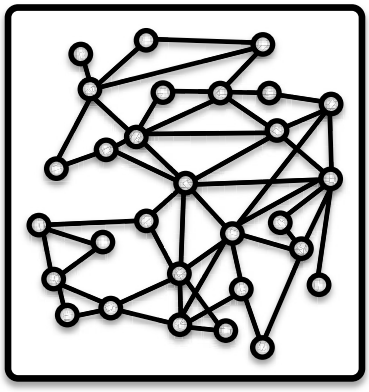
Gαβγ	**heterotrimeric G protein complex**
Gβ	**G protein beta subunit**
Gβγ	**heterodimeric complex of G protein beta and gamma subunits**
Gγ	**G protein gamma subunit**
HRP	**horseradish peroxidase**
IC87114	**selective inhibitor of the class IA PI3K p110δ catalytic subunit**
INK128	**TOR kinase inhibitor**
Ins(1,4,5)P_3	**inositol 1,4,5-trisphosphate**
IP_3 R	**inositol 1,4,5-trisphosphate receptor**
JNK	**cJun N-terminal kinase**
Kiss	**kisspeptin**
LH	**luteinizing hormone**
LHRH	**luteinizing hormone-releasing hormone; Type I GnRH; mGnRH**
LY294002	**pan-PI3K inhibitor; PIKK-selective**
MAPK	**mitogen-activate protein kinase**
MEK	**extracellular signal-regulated protein kinase kinase**
mGnRH	**mammalian GnRH; Type I GnRH; also LHRH**
MLCK	**myosin light-chain kinase**
mLST8	**mammalian lethal with SEC thirteen 8**
MMP	**matrix metalloproteinase**
mRNA	**messenger ribonucleic acid**
NO	**nitric oxide**
NOS	**nitric oxide synthase**
NP-40	**nonylphenoxypolyethoxylethanol**
NPY	**neuropeptide Y**
NSC8668	**Gallein; allosteric inhibitor of Gβγ interactions with effectors**
P-Rex1	**PtdIns(3,4,5)P_3-dependent Rac exchange factor 1**
PACAP	**pituitary adenylate cyclase-activating polypeptide**
PD98059	**selective MEK1/2 inhibitor**
PDE	**pdohphodiesterase**
PDK1	**phosphoinositide-dependent protein kinase 1**

PH	**pleckstrin homology; lipid-binding domain**
PI3K	**phosphoinositide 3-kinase**
PI4K	**phosphatidylinositol 4-kinase**
PIF	**PDK1-interacting fragment**
PIKK	**phosphoinositide 3-kinase related kinase**
PIP5K	**phosphatidylinositol-4-phosphate 5 kinases**
PIT-1	**antagonist of PtdIns(3,4,5)P_3-PH domain interactions**
PKA	**protein kianse A; cAMP-dependent protein kinase**
PKC	**protein kinase C**
PKG	**protein kinase G; cGMP-dependent protein kinase**
PLA_2	**phospholipase A2**
PLC	**phospholipase**
PLK	**polo-like kinase**
PRAS40	**proline-rich Akt substrate of 40 kDa**
PRL	**prolactin**
PtdIns	**phosphatidylinositol**
PtdIns(3,4,5)P_3	**phosphatidylinositol 3,4,5-trisphosphate**
PtdIns(3,4)P_2	**phosphatidylinositol 3,4-bisphosphate**
PtdIns(3)P	**phosphatidylinositol 3-phosphate**
PtdIns(4,5)P_2	**phosphatidylinositol 4,5-bisphosphate**
PtdIns(4)P	**phosphatidylinositol 4-phosphate**
PTEN	**phosphatase and tensin homolog deleted on chromosome ten**
Raf	**serine/threonine-specific kinases**
Rapamycin	**allosteric inhibitor of TORC1**
RAPTOR	**regulatory-associated protein of TOR**
Ras	**Ras superfamily small GTPase**
RasGRP	**Ras guanyl nucleotide releasing proteins**
RBD	**Ras-binding domain**
RBL-2H3	**rat basophilic leukemia mast cell line**
Rheb	**RAS homolog enriched in brain**
Rho	**Rho family small GTPase**

RIA	**radioimmunoassay**
RICTOR	**rapamycin-insensitive companion of TOR**
RSK	**ribosomal S6 kinase**
RTK	**receptor tyrosine kinases**
Ryn	**ryanodine**
RyR	**ryanodine receptor**
S6K	**S6 kinase**
SDS	**sodium dodecyl sulfate**
SFK	**Src family tyrosine kinase**
sGC	**soluble guanylyl cyclase**
SGK	**serum- and glucocorticoid-induced protein kinase**
SH1	**Src homology 1; tyrosine kinase catalytic domain**
SH2	**Src homology 2; protein interaction domain**
SH3	**Src homology 3; protein interaction domain domain**
SIN1	**SAPK-interacting protein 1**
SST	**somatostatin**
TBS	**Tris buffered saline**
TBST	**Tris buffered saline supplemented with 0.1% Tween-20**
Tg	**thapsigargin**
TGX221	**selective inhibitor of the class IA PI3K p110β catalytic subunit**
TOR	**target of rapamycin**
TORC1	**target of rapamycin complex 1**
TORC2	**target of rapamycin complex 2**
TPA	**PKC activator; other intracellular targets are known**
TRP	**transient receptor potential**
TSC	**tuberous sclerosis complex**
U0126	**selective MEK1/2 inhibitor**
Vps	**vacuolar protein-sorting defective**
VSCC	**voltage-sensitive Ca2+ channel; L-type**
wortmannin	**pan-PI3K inhibitor; PIKK-selective**

Chapter One:

General Introduction



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

In order for complex multicellular organisms to survive and reproduce, they need to coordinate physiological processes across multiple tissues throughout ontogeny. For this to happen, not only do the activities of individual cells have to be properly regulated, but cells must also communicate with one another. One of the ways that cellular communication is achieved involves the use of secreted chemical messengers, called hormones. In general, hormone-producing cells are part of a complex communication network called the endocrine system and hormones ultimately alter the functions of their target cells by binding to hormone-specific recognition sites called receptors. For peptide hormones, these receptors are often present on the cell surface and receptor activation regulates the activity of one or more conserved families of intracellular messaging proteins that ultimately control the structural and chemical responses of cells in a process called signal transduction. The ability of receptors to engage distinct signal transduction mechanisms following hormone binding allows for the hormone-selective regulation of cell functions and contributes to the endocrine control of physiological responses (Taniguchi et al., 2006; Kholodenko et al., 2010; Purvis and Lahav, 2013).

Interestingly, multiple structural variants of a single hormone are found in almost all of the vertebrate classes, as well as higher invertebrates, examined (Hoyle, 1998; Holmgren and Jensen, 2001; Roch et al., 2014; On et al., 2015). Recent studies have demonstrated that closely related hormone variants can selectively activate a distinct subset of signal transduction responses through shared receptors in a process called biased signalling (Rajagopal et al., 2010). However, the extent to which biased signalling by naturally occurring hormone variants can contribute to the integrated regulation of physiological systems is poorly understood. To explore the possibility of signalling bias

within natural hormone systems, my thesis research has focused on characterizing the signal transduction mechanisms utilized by two endogenous isoforms of gonadotropin-releasing hormone (GnRH) using a well-established vertebrate model of hormonal communication, the goldfish (*Carassius auratus*).

In goldfish, GnRH2 (Type II; [His^5,Trp^7,Tyr^8]GnRH, chicken (c)GnRH II) and GnRH3 (Type III; [Trp^7,Leu^8]GnRH, salmon (s)GnRH) have both been shown to stimulate maturational gonadotropin (luteinizing hormone, LH) and growth hormone (GH) release from pituitary gonadotropes and somatotropes, respectively, in the general regulation of reproduction and somatic growth. Upon delivery from hypothalamic nerve terminals, GnRH2 and GnRH3 bind GnRH receptors (GnRHRs) that are coupled to heterotrimeric guanine nucleotide-binding proteins (G protein-coupled receptors; GPCRs) located on the cell surfaces of gonadotropes and somatotropes (Cook et al., 1991; Habibi and Peter, 1991). However, surprisingly, GnRH2 and GnRH3 utilize similar, as well as distinct, post-receptor signal transduction mechanisms to stimulate LH and GH release (Chang et al., 2009, 2012). These observations suggest the presence of biased signalling in the regulation of gonadotrope and somatotrope functions by each of the endogenous peptide isoforms of GnRH found in goldfish. The primary goal of this thesis was to explore biased GnRHR signalling in more detail, as well as understand the integrated control of gonadotrope and somatotrope cell functions by endogenous variants of neurohormones. In doing this research, I have focused on characterizing the signalling role of the phosphoinositide 3-kinase (PI3K) superfamily of lipid kinases in mediating GnRH actions and as central regulators of endocrine cell functions in general.

In the following sections of this introductory chapter, instead of attempting to

comprehensively review the vast literature available on receptor biology or intracellular signalling, I will focus on integrating and synthesizing ideas or concepts based on recent advances in the understanding of biased signalling and demonstrate the importance of goldfish pituitary cells as a model for the multifactorial control of endocrine cell functions. Furthermore, I will also highlight PI3K-dependent intracellular signal transduction as a major component in the regulation of cellular functions and examine the potential role for PI3Ks in mediating post-receptor biased signalling.

1.1 G protein-coupled receptors

GPCRs are a versatile family of physiologically important transmembrane signalling proteins that sense extracellular molecules such as hormones and neurotransmitters (Venkatakrishna et al., 2013). GPCRs share a common seven-transmembrane (7TM) topology and control cellular responses by activating a diverse array of intracellular signal transduction cascades via effector proteins, such as heterotrimeric G proteins and/or arrestins (Venkatakrishna et al., 2014; Figure 1.1). With nearly 800 members identified in the human genome, GPCRs represent the largest family of proteins involved in signal transduction across biological membranes (Audet and Bouvier, 2012). Although the GPCR superfamily recognizes a great diversity of extracellular ligands, there are conserved structural elements present within the 7TM superfold (Venkatakrishna et al., 2013; Katritch et al., 2013). However, based on the sequence similarities of the 7TM domain, GPCRs are mainly classified into five subfamilies. These are the *Rhodopsin* family (Class A GPCRs), the *Secretin* and *Adhesion* families (Class B GPCRs), the *Glutamate* family (Class C GPCRs), and the

Frizzled/TAS2 family (Class F GPCRs; Katritch et al., 2013). Throughout the superfamily, all GPCRs operate as a transduction unit containing the transmembrane-spanning receptor capable of binding to the extracellular signal, a heterotrimeric G protein (Gαβγ), and signalling effectors that promote intracellular changes leading to a cellular response (Audet and Bouvier, 2012).

Ligand binding to the orthosteric binding pocket created at the extracellular surface of GPCRs, and including certain extracellular loops of the 7TM regions, activates GPCRs and results in the stimulation of a nucleotide exchange reaction for the associated Gα subunit (Venkatakrishna et al., 2014). Therefore, signalling by GPCRs depends on the ability of Gα to cycle between an inactive GDP-bound conformation that is primed for interaction with an activated receptor, and an active GTP-bound conformation that can modulate the activity of downstream effector proteins (Oldham and Hamm, 2008). Following agonist binding and nucleotide exchange, the heterotrimeric Gαβγ complex is thought to dissociate into free Gα and Gβγ subunits (Oldham and Hamm, 2008).

Similar to the classification of GPCRs, G protein heterotrimers are divided into four main classes based primarily on the sequence similarity of the Gα subunits: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Oldham and Hamm, 2008). These distinct classes of Gα subunits have been shown to couple to conserved intracellular signalling effectors, including: adenylyl cyclases (ACs; $G\alpha_s$ and $G\alpha_{i/o}$) and phosphodiesterases (PDEs; $G\alpha_{i/o}$), phospholipase Cβ (PLC; $G\alpha_{q/11}$), protein kinase Cs (PKCs; $G\alpha_{q/11}$), calcium (Ca^{2+}; $G\alpha_{q/11}$), as well as members of the Rho-family of small GTPases ($G\alpha_{12/13}$; Marinissen and Gutkind, 2001). Interestingly, independent of signalling downstream of the Gα subunits, Gβγ heterodimers also directly associate with downstream effectors and can regulate

distinct aspects of cellular physiology (Khan et al., 2013). Taken together, heterotrimeric G proteins are versatile signalling complexes that can coordinately control many canonical signal transduction cascades and therefore comprise the fundamental unit of GPCR-controlled signalling networks (Marinissen and Gutkind, 2001; Figure 1.2).

1.2 Biased signal transduction

Agonist-bound GPCRs have also been shown to signal in a G protein-independent manner through interactions with other intracellular effectors, for example, through the coordinate actions of G protein-coupled receptor kinases (GRKs) and arrestins (Ritter and Hall, 2009). G protein-independent signalling has distinct biochemical and functional consequences from those mediated by heterotrimeric G proteins (Rajagopal et al., 2010). Interestingly, some GPCR-ligand systems preferentially signal through either Gαβγ- or arrestin-mediated pathways (Rajagopal et al., 2010). This unique behavior for displaying bias towards one signal transduction pathway over another is referred to as biased agonism or signalling (Rajagopal et al., 2010; Figure 1.3). Understanding the functional implications of biased signalling has obvious clinical relevance due to the potential therapeutic impact of designing synthetic ligands that interact with a specific GPCR in manner that results in the biased regulation intracellular effectors that maximize beneficial on-target effects while mitigating undesirable off-target effects (Kenakin and Christopoulos, 2013). However, whether biased signalling in endogenous GPCR-ligand systems is involved in selectively altering intracellular signal transduction profiles in natural systems remains largely uncharacterized.

Interestingly, basal vertebrate models have become increasingly important for understanding the co-evolution of ligands and their receptors (Darlison and Richter, 1999; Conlon and Larhammar, 2005; Roch et al., 2014). In particular, studies using teleost fishes have identified the presence of multiple molecular variants for many regulatory peptides that serve important functions within the endocrine system; although the functional significance of many of these novel hormone variants is unknown. On the other hand, the goldfish (*Carassius auratus*) have emerged as one of the best-characterized models for studying the isoform-specific functions of neuropeptide variants in the control of cellular functions. Briefly, results from the goldfish model have focused on understanding the regulation of pituitary cell functions by multiple stimulatory and inhibitory regulators at the intracellular level and demonstrate that this multifactorial control contributes to the physiological control of growth and reproduction, including the regulatory effects of several GPCR-activating endogenous neurohormone isoform systems (Chang et al., 2009, 2012; Figure 1.4). Therefore, understanding the integrated regulation of pituitary cell activities in goldfish provides an excellent model for investigating how biased signalling in endogenous GPCR-ligand systems contributes to the integrated control of physiological functions. To better understand this relationship, I will briefly review the regulatory mechanisms responsible for the control of pituitary cell functions with a special focus on teleost fishes.

1.3 Neuroendocrine control of growth and reproduction

In vertebrates, the pituitary gland serves as the primary source of hormones responsible for the regulation of growth and reproduction. The major pituitary hormones

controlling these processes include LH, which stimulates gonadal steroid production, and GH, which enhances somatic growth and metabolism as well as potentiates the gonadal steroidogenic responses to LH (Van der Kraak et al., 1990; Le Gac et al., 1992). In turn, pituitary gonadotrope and somatotrope functions, including hormone production and release, are controlled primarily by neuroendocrine factors produced by hypothalamic neurons. In most vertebrates, hypothalamic hormone-releasing and release-inhibitory factors are secreted at the level of the median eminence into the hypothalamohypophysial portal blood system, which delivers them to the pituitary cells (Guillemin, 1978). However, in teleost fishes, the median eminence is absent; instead, neurons containing these hypophysiotropic factors directly innervate the pituitary, terminating directly on their pituitary target cells and/or in the vicinity of these cells (Peter et al., 1990; Anglade et al., 1993; Figure 1.5). In addition to regulation by hypothalamic neurohormones, feedback from peripheral endocrine regulators or the presence of local factors secreted from neighboring pituitary cells can also regulate gonadotrope and somatotrope activity (Denef, 2008; Le Tissier et al., 2012). Overall, determining how different pituitary cell regulators influence gonadotrope and somatotrope functions is an integral part of understanding the physiological control of growth and reproduction.

Across vertebrates, the hypothalamic neuropeptide GnRH acts as the primary stimulator of LH synthesis and release, while GH-releasing hormone (GHRH) is thought to be the major stimulator of GH release (Guillemin et al., 2011; Chang et al., 2009, 2012). In teleosts such as the goldfish, the stimulatory effect of GHRH on GH secretion appears to be less prominent than in higher vertebrates and in contrast, GnRH is also a reliable and effective stimulator of GH secretion (Marchant et al., 1989; Canosa et al.,

2007). Taken together, understanding how naturally occurring isoforms of GnRH regulate the activity of the gonadotropes and somatotropes of goldfish provides an ideal physiological model for studying the involvement of biased signalling in control of basic cellular processes that contribute to the integrated control of growth and reproduction.

1.4 Gonadotropin-releasing hormone

GnRHs are decapeptides that serve as central regulators of reproduction. Across all vertebrate classes, 18 molecular forms of GnRH have been identified based on primary structure, phylogenetic analysis, and gene synteny (Roch et al., 2011, 2014). The vertebrate peptides are divided into three major structural families: type I GnRH or GnRH1, including the classical mammalian (m)GnRH or luteinizing hormone-releasing hormone; type II GnRH or GnRH2, including cGnRH-II; and type III GnRH or GnRH3, including sGnRH (Roch et al., 2011, 2014). Interestingly, the presence of multiple GnRH variants in the brain, as well as in peripheral tissues, is a common feature among chordates (Lethimonier et al., 2004; Roch et al., 2011, 2014). GnRH2 is the most ubiquitous form of GnRH, found in teleost fishes as well as higher mammals, and is thought to represent the ancestral vertebrate peptide (Lethimonier et al., 2004). In addition to GnRH2, most vertebrates also possess a species-specific GnRH isoform that is typically present in the mediobasal hypothalamus and is associated with the classical hypophysiotropic function of GnRHs (Lethimonier et al., 2004). In mammals, the hypothalamic form is GnRH1; however, GnRH3 peptides are unique to teleost fishes and have been shown to serve as the dominant hypophysiotropic form in many species (Lethimonier et al., 2004).

In goldfish, two endogenous GnRH isoforms (GnRH2 and GnRH3) are expressed in the brain (Yu et al., 1988, 1998; Figure 1.6). GnRH3-positive cell bodies are found in the preoptic area that are normally associated with neuroendocrine regulation; whereas cell bodies of GnRH2 neurons reside in extra-pituitary sites, primarily in the midbrain tegmentum (Yu et al., 1988, 1998). Interestingly, both GnRH2 and GnRH3 are secreted from neuronal terminals within the anterior pituitary and both GnRHs directly stimulate the release of LH from gonadotropes and GH release from somatotropes (Chang et al., 1990; Yu et al., 1998). Outside of the pituitary, GnRH2 and GnRH3 have been implicated in a wide variety of physiological roles in goldfish, including: regulation of spawning behavior (Volkoff and Peter, 1999), feeding behavior (Hoskins et al., 2008; Matsuda et al., 2008), ovulation (Peter and Yu, 1997), oocyte meiosis (Pati and Habibi, 2000), follicular steroidogenesis (Pati and Habibi, 2000), and spermatogensis (Andreu-Vieyra et al., 2004). It is also evident from these studies that, across tissues, GnRH2 and GnRH3 exhibit functional selectivity in terms of their physiological roles, with GnRH2 preferentially serving as an anorexigenic regulator of feeding behavior within the brain (Hoskins et al., 2008; Matsuda et al., 2008) and GnRH3, but not GnRH2, modulating LH-induced oocyte meiosis within the ovary (Pati and Habibi, 2000). However, subtle differences in the intracellular actions of GnRH2 and GnRH3 likely facilitate additional functional diversity of these compounds within target tissues common to both peptides. In particular, studies using primary cells and membrane preparations isolated from the goldfish pituitary demonstrate that both GnRH2 and GnRH3 bind and activate the same population of cell-surface receptors (GnRHRs; Murthy and Peter, 1994; Illing et al., 1999) that belong to the class A subfamily of GPCRs (Millar et al., 2004). Taken

together, these findings make goldfish pituitary cells an ideal model for examining the differential actions of endogenous GnRH isoforms at the intracellular level.

1.5 Potential for biased signalling downstream of GnRHRs

Recent advances in structural biology, and in particular macromolecular crystallography, have identified key intermediate conformations of GPCRs during receptor activation (Katritch et al., 2013; Figure 1.7). In addition, ligand-specific induction of conformational rearrangements within GPCRs can exist amongst functionally similar ligands (Kahsai et al., 2011). The observed heterogeneity in the conformational landscape allows for the selective engagement of specific transducers and regulatory proteins (Nygaard et al., 2013). Furthermore, recent studies suggest that loose allosteric coupling of the agonist-binding site and the G protein-coupling interface helps to shape the complex intracellular signalling behavior observed for GPCRs (Manglik et al., 2015). Therefore, GPCRs can be thought of as oscillating between a series of conformational intermediates and the stability of the energy landscape associated with the receptor is influenced by both extracellular ligands and intracellular effector proteins that bind to the receptor (Wisler et al., 2014). Consequently, distinct structurally-related ligands can regulate the activity of a shared GPCR by shifting the conformational equilibrium to a specific receptor state(s) that selectively regulate the activity of a subset of signal transduction effectors without activating others (Wisler et al., 2014). The heterogeneity displayed in the downstream signalling effectors activated by ligand interactions with GPCRs can be achieved by selectively modulating Gαβγ-dependent and/or Gαβγ-independent transduction events in order to coordinate ligand-biased

signalling responses. In particular, evidence suggesting that GnRHRs can adopt ligand-specific conformations and exhibit biased signalling comes from studies done in both mammalian and teleost models using GnRH structural analogs.

1.5.1 Evidence from mammalian studies

Mammals such as humans, mice, and rats express only a single functional GnRHR type (Roch et al., 2014), yet studies in mammals have shown that the efficacy of GnRH isoforms varies across GnRHR-mediated signalling pathways (Maudsley et al., 2004; López de Maturana et al., 2008; Millar et al., 2008). In addition, mutations of the GnRHR at residues that do not form direct ligand-binding contacts, particularly in the extracellular domains, differentially alter the binding affinity and/or potency of the receptor for distinct ligands (Lu et al., 2005, 2007; Pfleger et al., 2008). These results are consistent with the hypothesis that binding of structurally distinct GnRH ligands stabilize different receptor conformations to selectively activate intracellular signalling effectors.

1.5.2 Evidence from studies in goldfish

The majority of studies examining the structure-activity relationship between GnRH and GnRHRs in teleosts come from the goldfish. Early work in this species focused on understanding whether more than one population of GnRHRs, perhaps being preferential or selective for GnRH2 or GnRH3 respectively, contributed to the control of hormone release responses from pituitary cells. Receptor binding studies using crude pituitary membrane preparations identified two classes of binding sites: high affinity and low capacity sites, in addition to low affinity but high capacity sites (Habibi et al., 1987,

1990). Importantly, results consistently indicate the presence of only one class of high affinity binding sites (Habibi and Peter, 1991), and both GnRH2 and GnRH3 act through high-affinity binding sites to stimulate LH and GH release (Peter et al., 1991; Habibi et al., 1992). GnRH2 and GnRH3 also displace a bound ^{125}I-labelled GnRH3 analog from crude pituitary membrane preparations (Habibi et al., 1987, 1989; Habibi and Peter, 1991), as well as an avidin gold-labelled biotinylated GnRH3 analog from the surfaces of immunohistochemically identified gonadotropes and somatotropes (Cook et al., 1991). Furthermore in hormone release studies, single pulses of GnRH2 do not stimulate additional increases in LH or GH release during continuous treatment with equimolar doses of GnRH3; similarly, GnRH3 fails to stimulate additional hormone release in the presence of GnRH2 when tested in cell column perifusion experiments (Murthy et al., 1994a). Likewise, LH release responses to maximally effective doses of GnRH2 and GnRH3 are not additive in static incubation studies (Chang et al., 1993). Taken together, these studies demonstrate that both endogenous GnRH isoforms interact with high affinity GnRHRs on both gonadotropes and somatotropes, and suggest that both GnRH2 and GnRH3 act through the same population of receptors.

Interestingly, different structural analogs of GnRH exert direct, but differential abilities to stimulate acute LH and/or GH release (Murthy et al., 1994a). GnRH analogs also exhibit significant differences in potency, both within and between cell types (Murthy et al., 1994b). These data strongly suggest that the structural features of unique GnRH ligands can interact with a single population of GnRHRs to differentially control the intracellular signalling responsible for the control of LH and GH release responses; but also that GnRHRs on gonadotropes and somatotropes are functionally different.

The cloning of goldfish GnRHRs from brain and pituitary tissues has helped to clarify some of the structure-activity properties of GnRHRs, but this has also added more unanswered questions, as well as some confusion to the situation. Two goldfish GnRHRs both belonging to the mammalian GnRHR family, and referred to as the GfA and GfB GnRHR, share 71% identity but display marked differences in ligand selectivity (Illing et al., 1999). In particular, the GfA GnRHR retains an affinity for GnRH agonists with substitutions at position 8 of the GnRH decapeptide, whereas the GfB GnRHR is unable to recognize GnRH agonists with substitutions of His or Tyr at position 8 (Illing et al., 1999). In terms of the endogenous agonists, both GfA and GfB GnRHRs have a greater sensitivity to GnRH2 (ED_{50} = 0.03 ± 0.01 and 3.4 ± 2.0 nM, respectively); whereas their sensitivity to GnRH3 was about 100- and 16-fold less, respectively (Illing et al., 1999). These findings by Illing and colleagues are largely in agreement with earlier studies demonstrating that relative to GnRH3, GnRH2 has a higher binding affinity in pituitary membrane preparations (Habibi, 1991). In static cultures of dispersed goldfish pituitary cells, GnRH2 is also more potent in releasing LH, whereas both GnRH2 and GnRH3 were equipotent in stimulating GH release (Chang et al., 1990). Therefore, on the basis of the relative potencies for GnRH2 and GnRH3, the GfA GnRHR is arguably most similar to the LH-releasing GnRHR (Illing et al., 1999). Alternatively, GnRH analogs with substitutions of His, Leu, or Tyr at position 8 do not affect GH release, but significantly decrease LH release in perifused goldfish pituitary fragments (Habibi et al., 1987). Consequently, on the basis of sensitivity to substitutions at position 8, the GfB GnRHR is thought to correlate with the physiological activity of the GH-releasing GnRHR (Illing et al., 1999). In contrast to these functional data, *in situ* hybridization results suggest that the

mRNA expression of the GfB GnRHR overlaps with both LH- and GH-immunoreactive cells to a large extent, while mRNA expression of the GfA GnRHR coincides more with LH- than GH-immunoreactive cells (Illing et al., 1999). Therefore, in terms of mRNA expression, it appears that instead of only having one subtype of GnRHR on each pituitary cell type, both gonadotropes and somatotropes express both GfA and GfB GnRHRs. On the other hand, the *in situ* hybridization and hormone immunoreactivity observations were not performed on the same histological sections, but rather, on adjacent sections thus hampering the ability to accurately determine the localization of GfA and GfB GnRHRs. Furthermore, the presence of mRNAs encoding for GfA and GfB GnRHR does not necessarily mean that receptor proteins are properly folded and trafficked into the cell membrane. It should also be mentioned that these ligand sensitivity and affinity measurements of the goldfish GnRHRs were made by monitoring inositol phosphate production in COS-1 cells transiently transfected with either the GfA or GfB GnRHR. However, inositol phosphate production is indicative of only one of the many transduction elements mediating GnRH-stimulated effects in goldfish pituitary cells and inositol phosphate turnover may not functionally participate in GnRH2 actions in some cases (see Section 1.6 below). Thus, the assignments of GfA and GfB GnRHR as the functional equivalent of the LH- and GH-releasing receptors, respectively, may also have limitations.

Nevertheless, when results from physiological studies using the goldfish pituitary cell culture system are viewed together, the available data strongly suggest that the native GnRH2 and GnRH3 isoforms utilize the same population of GnRHRs to stimulate hormone release responses from goldfish somatotropes and gonadotropes. Furthermore,

there are obvious differences in the structure-activity relationship observed between GnRH analogs and the GnRHRs present on gonadotropes and somatotropes. These differences strongly suggest that goldfish GnRHRs adopt ligand-selective conformations to differentially control hormone release responses. It is also possible that the obvious differences in ligand selectivity observed between gonadotropes and somatotropes are due, at least in part, to the differential localization of the GfA and GfB GnRHRs to individual cell types, but this remains to be confirmed.

1.6 Intracellular signal transduction pathways stimulated by GnRH2 and GnRH3 in goldfish pituitary cells

Canonical signalling by the mammalian GnRHRs in pituitary gonadotropes is dependent upon the activation of the $G\alpha_{q/11}$ family of Gα proteins (Stanislaus et al., 1997; Millar et al., 2004). In extrapituitary sites, the actions of GnRHs are thought to occur through pharmacologically distinct mechanisms that involve the coupling of the GnRHR to additional families of Gα subunits, including $G\alpha_s$ and $G\alpha_{i/o}$ (Millar et al., 2008; Naor, 2009; Naor and Huhtaniemi, 2013). Although the coupling of a single GPCR to multiple Gα proteins is not uncommon, photolabelling using a nonhydrolyzable GTP analog suggests that mammalian GnRHRs exclusively couple to $G\alpha_{q/11}$ and not $G\alpha_s$, $G\alpha_{i/o}$, or $G\alpha_{12/13}$ (Grosse et al., 2000). As a result, it is clear that multiple GnRHR-mediated signalling cascades can be activated downstream of a single receptor-G protein interface in mammals (Grosse et al., 2000; White et al., 2008). Interestingly, studies using goldfish pituitary cells have produced similar results.

$G\alpha_{q/11}$ stimulate intracellular signalling responses by activating isoforms of PLCβ which cleave the polar head group of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to produce two important intracellular second messengers: inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG; Bunney and Katan, 2010; Fukami et al., 2010). Ins(1,4,5)P_3 stimulates the mobilization of Ca^{2+} from intracellular stores by binding to specific Ins(1,4,5)P_3 receptors (IP_3Rs), while DAG binds to and activates several families of effector proteins including multiple isoforms of PKC (Bunney and Katan, 2010; Fukami et al., 2010). Work in goldfish pituitary cells has shown that GnRH actions on LH and GH release are dependent on the activity of PKCs and intracellular Ca^{2+} signalling, which is generated via both extracellular Ca^{2+} entry and by the mobilization of Ca^{2+} from intracellular stores, thus supporting GnRHR coupling to $G\alpha_{q/11}$ and activation of PLCβ (Chang et al., 1991; Jobin and Chang 1992a,b; Johnson et al., 1999). Conversely, direct coupling to $G\alpha_s$ and $G\alpha_i$ subunits in the goldfish pituitary system can be ruled out. GnRH2- and GnRH3-induced LH and GH release are independent of AC activity and cyclic-3′-5′-adenosine monophosphate (cAMP) production (Wong et al., 1994; Jobin et al., 1996), and treatment with the selective $G\alpha_i$ inhibitor pertussis toxin stimulates acute hormone secretion from goldfish gonadotropes and somatotropes; indicating an inhibitory influence of $G\alpha_i$ subunits on pituitary cell responses (Chang et al., 1993). Taken together, as in mammals, it is likely that goldfish GnRHRs activate signal transduction responses primarily through the activation of $G\alpha_{q/11}$ family G proteins. However, detailed studies of the downstream signalling components have revealed unique differences in the transduction mechanisms utilized by the endogenous GnRH isoforms (Chang et al., 2009, 2012).

In both gonadotropes and somatotropes, the actions of both GnRH2 and GnRH3 on hormone release responses involves increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that are achieved by both increased entry of Ca^{2+} from extracellular sources and Ca^{2+} mobilization from intracellular stores. Extracellular Ca^{2+} entry involves L-type voltage-sensitive Ca^{2+} channels (VSCCs) and, as expected, GnRH-induced mobilization of intracellular Ca^{2+} in part involves activation of PLCs and the generation of the Ins(1,4,5)P_3 (Jobin and Chang, 1992; Chang et al., 2009, 2012). The spatiotemporal complexity of changes in $[Ca^{2+}]_i$, in part, allows for the differential control of pituitary cell functions (Johnson et al., 1999; Johnson and Chang, 2000). Furthermore, multiple pharmacologically distinct intracellular Ca^{2+} stores are present in both gonadotropes and somatotropes and are differentially involved in agonist- and function-specific actions. Briefly, in terms of the control of LH release, only GnRH3 utilizes caffeine- and xestospongin-/Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores; whereas ryanodine (Ryn)-sensitive (but caffeine-independent) stores participate in GnRH2-stimulated responses (Johnson et al., 2000). In somatotropes, although GnRH3 utilizes Ins(1,4,5)P_3- and caffeine-, but not Ryn-sensitive stores to stimulate GH release, GnRH2 actions involve both caffeine- and Ryn-sensitive elements (Johnson et al., 2000). Intracellular Ca^{2+} stores that are involved in GnRH actions are also replenished by Ca^{2+}-ATPases sensitive to the inhibitors BHQ and CPA in gonadotropes, but not in somatotropes; however, these Ca^{2+} stores are thapsigargin (Tg)-insensitive in both cell-types (Johnson et al., 2000, 2002). On the other hand, mitochondrial Ca^{2+} buffering only modulates GnRH3, but not GnRH2 actions on GH release (Johnson and Chang, 2005). Outside of the regulation of $[Ca^{2+}]_i$, GnRH2 and GnRH3 also utilize PKC- and calmodulin kinase (CaMK)-dependent

signalling to stimulate LH and GH release (Jobin et al., 1992, 1993, 1996). Signalling through extracellular signal-regulated protein kinase kinase 1 and 2 (MEK1/2), a member of the mitogen-activated protein kinase (MAPK) superfamily, has also been shown to participate in GnRH2 and GnRH3 stimulation of LH release (Klausen et al., 2008; Chang et al., 2009). In addition, nitric oxide (NO) also mediates GnRH3-induced LH and GH release, as well as GnRH2-stimulated GH secretion (Urestsky et al., 2003; Meints et al., 2012); while GnRH3 action on LH release involves the mobilization of arachidonic acid (AA) through activation of phospholipase A2 and the subsequent metabolism of AA through the lipoxygenase pathway (Chang et al., 1991; Chang et al., 1995).

Taken together, these studies demonstrate marked complexity in the control of Ca^{2+}-dependent signalling downstream of GnRHRs and identify the differential usage of Ca^{2+} stores, as well as NO- and AA-dependent signalling, as potential signalling components that are subject to biased GnRHR-mediated regulation (Figure 1.8). However, outside of PKCs and Ca^{2+}, it is unclear how these transduction components interact with canonical signalling components of $G\alpha_{q/11}$-coupled GPCRs. Furthermore, while the importance of the complex spatiotemporal features observed for the $[Ca^{2+}]_i$ dynamics are apparent, these changes are distal to the potential conformational heterogeneity of the GnRHRs. To better understand the control of biased signalling within goldfish gonadotropes and somatotropes, one must attempt to examine intracellular effectors that are proximal to receptor activation. In addition, due to a lack of evidence for promiscuous coupling of goldfish GnRHRs to multiple Gα families, it is likely that GnRH-selective control of intracellular signalling requires Gα-independent transduction mechanisms.

The transduction mechanisms contributing to the differential use of intracellular signalling by GnRH2 and GnRH3, including how dissimilar Ca^{2+} signals are produced, are not fully understood and several important signal transduction systems have yet to be investigated in goldfish gonadotropes and somatotropes. In particular, among the canonical transduction cascades that have been described, the highly conserved and specialized PI3K superfamily of lipid kinases serve as important mediators of receptor-dependent signalling in a variety of neural, endocrine, and immune cell types. In general, the diversity of the signal transduction responses controlled by PI3K-dependent signalling make the PI3K superfamily an attractive target for integrating the multifactorial control of endocrine cell functions, such as in goldfish gonadotropes and somatotropes (Hirsch et al., 2007). Interestingly, PI3K-dependent signalling has been shown to be important in the regulation of endocrine secretion, particularly from pancreatic islets (MacDonald et al., 2004; Pigeau et al., 2009; Kaneko et al., 2010; Kolic et al., 2013, 2014). However, the involvement of PI3Ks in the control of agonist-stimulated hormone secretion and synthesis in primary pituitary cells has not been studied in any system. On the other hand, PI3K-dependent signalling has been implicated in GnRH-stimulated effects in various tumors and immortalized cell lines (see Section 1.8 for a more detailed discussion). In addition, as discussed below in Section 1.7, some isoforms of PI3K are important signalling targets of Gβγ heterodimers and therefore may form an integral component of Gα-independent transduction pathways downstream of GPCR activation. Taken together, these observations suggest that PI3Ks may participate in the control of GnRH2- and GnRH3-selective actions in goldfish pituitary cells.

1.7 Phosphoinositide 3-Kinases

PI3Ks act at nearly all cellular membranes and regulate functions as diverse as metabolism, cell polarity, survival, proliferation, and vesicle trafficking (Engelman et al., 2006). Collectively, the majority of PI3K-dependent functions are mediated by the production of phosphoinositides, the low-abundance phosphorylated forms of phosphatidylinositol (PtdIns; Balla, 2013). All PI3Ks phosphorylate the 3′-position hydroxyl group of inositol membrane lipids to generate phosphoinositides that coordinate the localization and function of multiple effector proteins, which bind these lipids through specific binding domains (Vanhaesebroeck et al., 2012). Although the basic framework of PI3K-dependent signalling has been extensively characterized, the contributions of PI3Ks can be best understood by examining the structural features and substrate preferences of individual isoforms (Vanhaesebroeck et al., 2010). In general, there are eight isoforms of PI3K family lipid kinases in mammals that are grouped into three functionally-discrete classes: class I, class II, and class III (Engelman et al., 2006; Figure 1.9). Importantly, homologs of all PI3K subunits are found throughout vertebrates (Engelman et al., 2006; Brown and Auger, 2011).

1.7.1 Classes of PI3K

Three classes of PI3Ks have been identified based upon molecular structure and substrate specificity (Engelman et al., 2006). Among these, class I PI3Ks are the best characterized and are further subdivided into two subclasses, IA and IB. The class IA subclass comprises three 110 kDa catalytic isoforms (p110α, PIK3CA; p110β, PIK3CB; and p110δ, PIK3CD) that utilize p85-type regulatory subunits encoded by either PIK3R1

(p85α, p55α, p50α), PIK3R2 (p85β), or PIK3R3 (p55γ) and directly interact with intracellular adaptors or receptors possessing intrinsic or associated tyrosine kinase activity (Vanhaesebroeck et al., 2010). The class IB catalytic subunit, p110γ (PIK3CG), binds to either the p101 (PIK3R5) or p84 (PIK3R6) regulatory subunits and is activated by GPCRs through direct interactions with the Gβγ subunits of heterotrimeric Gαβγ complexes (Vanhaesebroeck et al., 2010). Interestingly, the class IA p110β/p85 isoform is also directly activated by Gβγ heterodimers, making it the only class I PI3K that can be directly or synergistically activated by both tyrosine-phosphorylated proteins and GPCRs (Kurosu et al., 1997). Irrespective of the mode of activation, all class I PI3Ks phosphorylate the 3′-hydroxyl group of PtdIns(4,5)P_2 to produce the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3; Vanhaesebroeck et al., 2001; Burke and Williams, 2015). The presence of PtdIns(3,4,5)P_3-sensitive binding domains in a variety of signaling proteins mediates the complexity of signal transduction downstream of class I PI3Ks in cellular, as well as whole-organism, physiology (Vanhaesebroeck et al., 2005, 2010; Burke and Williams, 2015; Figure 1.10).

Unlike the class I PI3Ks, relatively little is known about the specific intracellular functions of either the class II or III PI3Ks (Vanhaesebroeck et al., 2010). Three isoforms of class II PI3Ks have been identified and consist of only a p110-like catalytic subunit: PI3K-C2α (PIK3C2A), PI3K-C2β (PIK3C2B), and PI3K-C2γ (PIK3C2G; Falasca and Maffucci, 2012). Evidence for modest activation of class II PI3Ks by ligands signalling through both receptor tyrosine kinases (RTKs) and GPCRs has been presented, although the molecular mechanisms contributing to the activation of class II PI3Ks by these agonists is not understood (Vanhaesebroeck et al., 2010). *In vitro*, class II PI3Ks

preferentially phosphorylate PtdIns and, to a lesser extent, PtdIns 4-phosphate (PtdIns(4)P) to generate PtdIns 3-phosphate (PtdIns(3)P) and PtdIns 3,4-bisphosphate (PtdIns(3,4)P_2), respectively (Engelman et al., 2006; Falasca and Maffucci, 2012). The vacuolar protein-sorting defective 34 (Vps34; *PIK3C3*) is the only member of the class III PI3Ks and has a highly conserved function in regulating autophagy and vesicle trafficking across eukaryotes (Backer, 2008). Vps34 forms a constitutive heterodimer with the Vps15 (PIK3R4) regulatory subunit and exhibits lipid substrate specificity limited to PtdIns, generating PtdIns(3)P (Backer, 2008). Class II and III PI3Ks could therefore, in principal, share downstream effectors that bind to PtdIns(3)P; however, it is not clear whether, or to what extent, the functions of the class II and III PI3Ks overlap (Vanhaesebroeck et al., 2010). Similar to the class II PI3Ks, it is not completely clear whether Vps34 is directly regulated by extracellular stimuli; however, there is evidence for regulation of Vps34 activity by nutrients including amino acids and glucose, as well as by GPCRs (Vanhaesebroeck et al., 2010). In particular, studies using yeast have demonstrated that activation of Vps34 occurs through a Gα-dependent mechanism at the endosome (Windmiller and Backer, 2003; Slessareva et al., 2006).

Overall, the superfamily of PI3Ks represents a unique complement of signalling effectors that contribute to the regulation of diverse cellular responses. However, it is obvious that there is a significant gap in our understanding of the functions of the class II and III PI3Ks when compared to the class I isoforms. Additionally, among the PI3Ks, the class I PI3Ks are also the only isoforms that are reliably activated downstream of cell-surface receptors, including GPCRs. As a result, the following sections will focus on the canonical signalling downstream of the class I PI3Ks.

1.7.2 Canonical signalling elements downstream of class I PI3Ks

Although growing evidence suggests that different class I PI3K isoforms control distinct intracellular functions, all four isoforms of class I PI3K produce the lipid second-messenger PtdIns(3,4,5)P_3 (Vanhaesebroeck et al., 2010). Binding of PtdIns(3,4,5)P_3 to pleckstrin homology (PH) lipid-binding domains facilitates membrane translocation and/or activation of numerous intracellular effectors, including: serine/threonine protein kinases, non-receptor tyrosine kinases, adaptor proteins, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), as well as direct interactions with plasma membrane transporters and ion channels (Hawkins et al., 2006; Vadas et al., 2011; Burke and Williams, 2015). The best studied PtdIns(3,4,5)P_3-sensitive effectors include members of the AGC family of serine/threonine protein kinases and, to a lesser extent, the Tec family of tyrosine kinases (Wymann and Marone, 2005; Hawkins et al., 2006). In particular, studies in excitable cell types have identified phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK) as canonical intracellular effectors that contribute to the control of PtdIns(3,4,5)P_3-dependent signalling (Figure 1.11).

1.7.2.1 Pleckstrin homology domains

PH domains are found in approximately 250 different human proteins and typically consist of 100-120 amino acids (Lemmon et al., 2008). PH domains vary greatly with their ability to bind phosphoinositides and only a small fraction (~10%) of PH domains actually exhibit high affinity and selectivity for a specific phosphoinositide(s)

(Lemmon et al., 2008; Kutateladze, 2010). As outlined above, PtdIns(3,4,5)P_3 is the major lipid product of agonist-stimulated class I PI3Ks and a small subclass of PH domains, including those of PDK1, Akt, and BTK, recognize PtdIns(3,4,5)P_3 with remarkable specificity and affinity (Lemmon, 2003; Lemmon et al., 2008). In general, PtdIns(3,4,5)P_3 interactions with PH domains coordinates the catalytic activity of PtdIns(3,4,5)P_3-sensitive signalling effectors and also functions as a membrane scaffold for the recruitment of signal transduction intermediates (Lemmon et al., 2008). Interestingly, comprehensive analysis of PtdIns(3,4,5)P_3-regulated proteins across species has shown that the number of PtdIns(3,4,5)P_3-sensitive PH domain-containing proteins has increased from approximately two in *Caenorhabditis elegans* and four in *Drosophila melanogaster*, to roughly 40 in vertebrates including *Danio rerio* (zebrafish; Park et al., 2008). This coincides with the metazoan expansion of the class I PI3K catalytic subunits from a single ancestral PIK3CA-like ortholog within invertebrates, to the modern complement of PIK3CA, PIK3CB, PIK3CD, and PIK3CG homologs found in true vertebrate lineages (Brown and Auger, 2011). Thus it appears that the full complexity of class I PI3K-dependent signalling was first established in basal vertebrates and that PtdIns(3,4,5)P_3-sensitive PH domains are evolutionarily conserved regulators of class I PI3K signalling functions.

1.7.2.2 Phosphoinositide-dependent kinase 1

PDK1 is ubiquitously expressed in eukaryotic cells and functions as an important intermediate during PtdIns(3,4,5)P_3-dependent signalling by phosphorylating members of the AGC family of serine/threonine kinases (Mora et al., 2004; Pearce et al., 2010). In

particular, PDK1 catalytic activity regulates downstream signalling by isozymes of Akt, PKC, protein kinase A (PKA), S6 kinase (S6K), ribosomal S6 kinase (RSK), and serum- and glucocorticoid-induced protein kinase (SGK) by phosphorylating a sequence known as the T-loop that is critical for kinase activation (Mora et al., 2004). PDK1 is also constitutively active in eukaryotic cells following autophosphorylation of Ser241 and, due to its vital role in PDK1 activation, Ser241 is conserved in all known homologs of PDK1, including those identified in *Caenorhabditis elegans*, *Drosophila melanogaster*, and zebrafish (Alessi et al., 1997a; Casamayor et al., 1999). Overall, the coordinate control of AGC kinases is complex and additional signals that selectively prime some targets for phosphorylation by PDK1 may be required (Mora et al., 2004; Pearce et al., 2010). Despite being constitutively active, evidence for the acute activation of PDK1 has emerged (Calleja et al., 2014), although binding of PtdIns(3,4,5)P_3 to PDK1 is not required to regulate its catalytic activity (Alessi et al., 1997a; Stephens et al., 1998; Currie et al., 1999). However, the interaction of PtdIns(3,4,5)P_3 with PDK1 allows PDK1 to act on other PH domain-containing substrates that can also be recruited by PtdIns(3,4,5)P_3. Although PDK1 is able to control the activity of a diverse complement of AGC kinases that regulate many different physiological processes, PDK1-dependent regulation of Akt activity is thought to play a central role in the signal transduction responses downstream of PtdIns(3,4,5)P_3 production by class I PI3Ks (Figure 1.11).

1.7.2.3 Protein kinase B

Akt serves as a central signalling node within all cells of higher eukaryotes and contributes to the complex control of cell survival, growth, proliferation, and metabolism

(Manning and Cantley, 2007). Three Akt isoforms, termed Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) have been identified and each isoform shares a high degree of homology in primary protein sequence as well as in domain structure (Bozulic and Hemmings, 2009). The interaction of Akt isoforms with PtdIns(3,4,5)P_3 leads to their recruitment to the plasma membrane and promotes intermolecular interactions between PDK1 and Akt on two-dimensional membrane surfaces (Stokoe et al., 1997; Milburn et al., 2003). PtdIns(3,4,5)P_3-binding also induces a conformational change in Akt that facilitates phosphorylation of the activation loop at residue Thr308 by PDK1 (Alessi et al., 1996; Calleja et al., 2007). As a result of PtdIns(3,4,5)P_3-binding, it is thought that only a small fraction of endogenous PDK1 is required for efficient and robust activation of Akt. This is supported by *in vitro* biochemical studies demonstrating that activation of Akt by PDK1 in the presence of PtdIns(3,4,5)P_3-containing lipid vesicles requires 100- to 1000-fold lower levels of PDK1 relative to PDK1 substrates that do not have PtdIns(3,4,5)P_3-binding PH domains (Alessi et al., 1997b and 1998). Similar findings using RNA interference show that 90% knockdown of PDK1 abundance is insufficient to block PI3K-dependent activation of Akt in several tissues *in vivo* (Ellwood-Yen et al., 2011).

In addition to phosphorylation of the activation loop by PDK1, activation of Akt also requires phosphorylation of Ser473 within the carboxyl-terminal hydrophobic motif for maximal activity (Sarbassov et al., 2005). Interestingly, similar to Thr308, Akt binding to PtdIns(3,4,5)P_3 facilitates phosphorylation of Ser473 by the target of rapamycin (TOR) complex 2 (TORC2), which consists of the kinase TOR bound to the core components rapamycin-insensitive companion of TOR (RICTOR), SAPK-

interacting protein 1 (SIN1), and mammalian lethal with SEC thirteen 8 (mLST8; Sarbassov et al., 2005; Shimobayashi and Hall, 2014). The relative importance and dynamics of the phosphorylation events at Thr308 and Ser473 are still controversial; however, Akt phosphorylation at Ser473 has been suggested to be necessary for the recognition and activation of Akt by PDK1 (Sarbassov et al., 2005; Scheid and Woodgett, 2001). This is supported by studies demonstrating that phosphorylation of the hydrophobic motif by TORC2 can regulate Akt activation independently of PDK1 interactions with PtdIns(3,4,5)P_3 through a unique mechanism relying on the association of phosphorylated Ser473 with the PIF (PDK1-interacting fragment) pocket of Akt (Najafov et al., 2012). Taken together, class I PI3Ks can promote interactions between PDK1 and Akt through two alternative mechanisms that can independently, as well as synergistically, facilitate efficient activation of Akt even at low plasma membrane concentrations of PtdIns(3,4,5)P_3 (Najafov et al., 2012). Furthermore, in coordination with PDK1, TORC2 catalytic activity may also be required for hydrophobic motif phosphorylation and activation of other AGC family kinases, including conventional isoforms of PKC (Sarbassov et al., 2004; Guertin et al., 2006).

Over 50 mammalian proteins have been identified as putative Akt substrates and signalling downstream of Akt involves a number of important intracellular effectors (Manning and Cantley, 2007). One of the conserved effectors of Akt-dependent signal transduction is the rapamycin-sensitive TOR complex 1 (TORC1) consisting of the core components TOR kinase, regulatory-associated protein of TOR (RAPTOR), and mLST8 (Shimobayashi and Hall, 2014). Akt activates TORC1 through multisite phosphorylation of tuberous sclerosis complex (TSC) 2 (TSC2) which negatively regulates the ability of

the TSC to act as a GAP for the small GTPase RAS homolog enriched in brain (Rheb; Manning et al., 2002; Inoki et al., 2002; Zhang et al., 2003; Long et al., 2005). Inhibition of TSC2 leads to accumulation of GTP-bound Rheb, which activates TORC1 (Manning and Cantley, 2007). Akt can also activate TORC1 by phosphorylating proline-rich Akt substrate of 40 kDa (PRAS40), thereby relieving PRAS40-mediated inhibition of TORC1 (Kovacina et al., 2003; Manning and Cantley, 2007). Activated TORC1 phosphorylates downstream targets to regulate basic cellular processes including translation initiation and ribosome biogenesis (Laplante and Sabatini, 2012). Overall, the coordinate regulation of PDK1-, Akt-, and TOR-dependent signalling is vital for the control cellular metabolism and protein homeostasis (Figure 1.11).

1.7.2.4 Bruton's tyrosine kinase

BTK is a non-receptor tyrosine kinase belonging to the Tec family and possesses modular organization similar to the Src family kinases (SFKs); including Src homology 3 (SH3) and Src homology 2 (SH2) protein interaction domains, and a tyrosine kinase catalytic domain (SH1; Mohamed et al., 2009). This complex domain structure allows BTKs to interact with numerous intracellular effectors, and the functional association of BTK with signalling partners is crucial for the selective control of cellular functions (Takesono et al., 2002). In particular, the interaction of the N-terminal PH domain with PtdIns(3,4,5)P_3 has been shown to target BTK to specific membrane microdomains (Guo et al., 2000). Similar to Akt, PtdIns(3,4,5)P_3 interactions with the PH domain increases BTK catalytic activity *in vitro* (Saito et al., 2001). However, in addition to membrane recruitment through its PH domain, BTK activation requires phosphorylation at Tyr551

in the activation loop by SFKs, and subsequent auto-phosphorylation of Tyr223 in the SH3 domain (Takesono et al., 2002). Downstream of its membrane translocation and activation, BTK is important for the phosphorylation and activation of PLCγ, thereby contributing to Ins(1,4,5)P_3 production and Ca^{2+} mobilization (Takata and Kurosaki, 1996). BTK-dependent signalling is also associated with the regulation of MAPKs and the control of actin cytoskeletal reorganization (Wan et al., 1997; Aimin Jiang et al., 1998; Takesono et al., 2002; Figure 1.11).

1.8 GnRHRs and PI3K-dependent signal transduction

Activation of canonical PtdIns(3,4,5)P_3-dependent signalling elements including, PDK1 and Akt in particular, are also important points of intracellular cross-talk between class I PI3Ks and other important signal transduction pathways; including those involved in the control of GnRH actions in goldfish gonadotropes and somatotropes (Figure 1.12). Briefly, as outlined above, PDK1 can regulate the enzymatic maturation of PKCs (Mora et al., 2004; Pearce et al., 2010) and PKC-dependent phosphorylation of the serine/threonine-specific kinase Raf (Kolch et al., 1993; Marais et al., 1998) or Ras guanyl nucleotide releasing proteins (RasGRP; Ebinu et al., 1998; Zheng et al., 2005) can in turn lead to activation of the MEK1/2 family of MAPKKs that signal through ERK1/2 (Lavoie and Therrien, 2015). On the other hand, Akt has been reported to attenuate Raf-dependent activation of MEK-ERK signalling (Zimmerman and Moelling, 1999; Rommel et al., 1999; Guan et al., 2000). Furthermore, Akt can also activate NO synthase (NOS) leading to the activation of NO-dependent signalling (Fulton et al., 1999; Dimmeler et al., 1999; Michell et al., 1999); whereas BTK-mediated activation of PLCγ can contribute to

Ins(1,4,5)P_3-sensitive Ca^{2+} mobilization, as well as stimulate DAG-sensitive isozymes of PKC (Takata and Kurosaki, 1996; Takesono et al., 2002). As a result, it is possible that GnRHR-mediated activation of class I PI3Ks could contribute to GnRH2 and GnRH3 actions in goldfish gonadotropes and somatotropes through canonical PtdIns(3,4,5)P_3-dependent signalling.

In mammalian models, the recruitment of PI3K-dependent signalling in GnRH-stimulated signal transduction has been reported; however controversies exist. In addition, the specific PI3K isoforms involved GnRH actions have never been investigated and few studies have been carried out in primary pituitary cell cultures. Briefly, studies using immortalized LβT2 and αT3-1 gonadotrope cell lines indicate GnRH analog-induced downregulation of gonadotropin (GTH) subunit promoter activity is mediated by PI3K-dependent signalling (Mutiara et al., 2008; Kanasaki et al., 2008); although an earlier study with LβT2 gonadotropes suggests that GnRH actions on GTH mRNA expression are in fact independent of PI3K catalytic activity (Liu et al., 2002). Outside of gonadotrope cell lines, GnRH activation of downstream transduction targets is PI3K-dependent in GnRHR-transfected COS7 cells (Kraus et al., 2003) and immortalized hypothalamic GT1-7 cell lines (Shah et al., 2006); whereas GnRH actions are independent of PI3K-dependent signalling in bullfrog sympathetic ganglion neurons (Ford et al., 2008) and isolated *Necturus* olfactory sensory neurons (Zhang et al., 2007). On the other hand PI3Ks are consistently involved in the downstream signalling activated by GnRHRs in tumor cells. For example, (1) GnRH effects on uterine leiomyomas are PI3K-dependent (Bifulco et al., 2004), (2) GnRH induces phosphorylation of Akt (Ser473) in ovarian cancer cells (Ling Poon et al., 2011), and (3) a GnRH antagonist

suppresses Akt phosphorylation (Ser473) in prostate cancer cells (Kim et al., 2009). Consequently, it is plausible that Akt could mediate PI3K-dependent actions on GnRH-stimulated hormone release, however there are no reports examining the direct involvement of Akt, or any other PtdIns(3,4,5)P_3-dependent effectors for that matter, in the control of agonist-stimulated pituitary hormone secretion.

Few studies have examined the involvement of PI3K-dependent signalling in the control of pituitary hormone release from untransformed cells by neuroendocrine factors, in general. In terms of GnRH actions, PI3Ks are not thought to be involved in GnRH-stimulated LH secretion in mammals. However, this conclusion is based primarily on the results from one study that identified a wortmannin-sensitive step in sustained GnRH-induced LH exocytosis in cultured rat pituitary cells (Rao et al., 1997). Oddly, the inhibitory actions of micromolar concentrations of wortmannin on GnRH-stimulated LH release were attributed to actions on myosin light-chain kinase (MLCK) and not PI3Ks; even though wortmannin is an irreversible inhibitor of all three classes of PI3K at low nanomolar doses (Vlahos et al., 1994; Walker et al., 2000). Although the authors acknowledged the possible involvement of PI3Ks, they ultimately concluded that it was unlikely that PI3K-dependent signalling would be relevant to the control of calcium-dependent exocytosis in gonadotropes (Rao et al., 1997). Subsequent studies have outlined a prominent role for PI3Ks downstream of GPCR activation as well as, specifically, in the control of vesicular trafficking and membrane remodeling (Czech, 2003; Lindmo and Stenmark, 2006; Di Paolo and De Camilli, 2006). Consequently, in addition to the control of hormone mRNA expression, it is plausible that PI3Ks directly contribute to GnRH actions on hormone secretion. That said, the extent to which

GnRHRs are coupled to PI3K-dependent signalling cascades is likely to be agonist-, cell type-, and function-specific.

1.9 Central hypothesis, general goals, and experimental approaches

As discussed above, growing evidence has identified PI3Ks as important components of GPCR-activated signalling networks, and although very little is known regarding the biased regulation of PI3K-dependent signalling, class I PI3Ks may participate in the differential downstream effects of Gα and Gβγ subunits. On the other hand, PI3Ks are known to participate in the regulation of endocrine cell functions, as well as in some of the actions of GnRHRs in mammalian tumor cells. PI3K-dependent signalling is also known to act upstream of, and/or to interact with, several known signal transduction mechanisms mediating GnRH actions in goldfish pituitary gonadotropes and somatotropes such as PKCs, Ca^{2+}, and MAPKs (Section 1.6 and 1.7). Furthermore, GnRH stimulation of LH and GH release in goldfish pituitary cells is mediated by differential intracellular signalling in a GnRH-selective and cell type-specific manner (Section 1.6). Based on these observations, I hypothesize that PI3Ks, and the class I PI3K isoforms in particular, mediate the differential actions of GnRH2 and GnRH3 on goldfish pituitary gonadotrope and somatotrope functions.

In testing this hypothesis, this thesis not only explores the intracellular mechanisms that contribute to the biased GnRHR-mediated control of pituitary cell functions but also characterizes the involvement of the PI3K superfamily of lipid kinases in the regulation of signal transduction responses downstream of agonist-stabilized GPCRs. In addition, whether the biased actions of the endogenous GnRH isoforms lead

to differences in their ability to control acute hormone release responses, as well as during prolonged effects on the maintenance of cellular hormone availability, will be elucidated. Lastly, the role of PI3K catalytic activity and PtdIns(3,4,5)P_3-sensitive signalling effectors in the regulation of basal hormone release and production will also be evaluated.

1.9.1 Advantages of the goldfish primary dispersed pituitary cells culture study system

The majority of the experiments in this thesis have been performed *in vitro* with dispersed goldfish pituitary cells. Overall, the use of primary goldfish pituitary cell cultures provides several advantages that make it ideal for these studies. First, as outlined above, the goldfish pituitary cell model is one of the few physiological systems in which cell- and peptide-specificity in signal transduction has been characterized, especially with regards to the actions of endogenous GnRHs. Second, goldfish pituitary cells *in vivo* are exposed to multiple GnRH isoforms that both regulate LH and GH secretion (Figure 1.6). Thus, results from *in vitro* studies on cell-specific differences in receptor-signal transduction coupling activated by hypothalamic GnRH peptide isoforms are likely to be physiologically relevant. Third, unlike in mammals, one can identify live goldfish gonadotrope and somatotrope cell types in mixed populations of primary dispersed pituitary cell cultures on the basis of their morphology (Van Goor et al., 1994; Figure 1.6c). Pituitary cell identification provides a considerable advantage since single-cell studies can be performed without prior manipulations that may affect cellular functions. Fourth, in goldfish and other teleost fishes, hypothalamic neurons containing hormone-

releasing and release-inhibitory factors directly innervate the pituitary, terminating in the vicinity of their target cells (Figure 1.5). Importantly, unlike pituitary fragments, primary dispersed goldfish pituitary cell cultures have been shown to be devoid of nerve terminals (Chang et al., 1990). Thus, the use of dispersed goldfish pituitary cells eliminates the possible compounding influence of indirect treatment effects through modulation of the release of neuroendocrine factors from nerve terminals and allow for studies on the direct actions of neuroendocrine effectors on pituitary cell functions. Fifth, studies on mixed cultures of goldfish pituitary cells allow for the simultaneous measurement of hormone secretion from both somatotropes and gonadotropes. Thus, novel details regarding the integrated control of GnRHR-mediated hormone release, including potential signalling bias across two cell types within the same tissue, can be provided.

The expansion of the PI3K subunit repertoire is also exclusive to true vertebrate lineages and coincides with the emergence of a more diverse complement of GPCR-interacting effectors (Brown and Auger, 2011; Khan et al., 2013; also discussed above in Section 1.7). Studying biased coupling of PI3K-dependent signalling, and the activation of unique class I PI3K isoforms in particular, within basal vertebrates such as the goldfish can potentially provide novel insight into how functional selectivity in GPCR-mediated signalling has evolved. Furthermore, the goldfish GnRHR system utilizes only $G\alpha_{q/11}$ family subunits. Therefore, by using a GPCR model that couples to a single family of Gα subunits to examine ligand-selective activation of signal transduction, we can also examine the possibility of Gβγ-directed signalling bias through known Gβγ-dependent effectors such as the class I PI3Ks. As a result, goldfish pituitary cells are an ideal basal vertebrate model system for the study of biased signal transduction by endogenous ligand

isoforms at the intracellular level. Just as importantly, due to the central importance of serum LH and GH levels in regulating reproduction and growth, results from studies using goldfish pituitary cells contribute to the understanding of how whole animal neuroendocrine control is manifested.

1.9.2 General approaches

The [His^5,Trp^7,Tyr^8]GnRH variant of GnRH2 and the [Trp^7,Leu^8]GnRH variant of GnRH3 are the endogenous hypothalamic GnRH forms in goldfish (Yu et al., 1988). Therefore these GnRH2 and GnRH3 isoforms will be used in all experiments in this thesis in order to more closely mimic the natural situations. Acute hormone release responses will be monitored using a well-established cell column perifusion system; this also has the advantage of minimizing the paracrine and autocrine influences of local factors released by goldfish pituitary cells (Chang et al., 1990). Alternatively, long-term hormone release will be examined in static culture systems (Pemberton et al., 2013) and, in all instances hormone concentrations will be quantified by established radioimmunoassays (LH, Peter et al., 1984; GH, Marchant et al., 1987). Across model systems, most contemporary studies on GnRH actions focus almost exclusively on the control of gene expression. However, it is well-established that the relationship between mRNA levels and protein is not linear, but very few models have investigated the integrated control of GnRH actions on pituitary hormone release and production. To address these short-comings, both hormone release and cellular hormonal contents will be monitored as physiological endpoints in long-term studies; with the sum of the amount

released and that remaining within the cells (total) also monitored as an index of hormone protein availabilty, which in turn is a proxy of hormone protein synthesis/replenishment.

In general, I chose to use a pharmacological approach to the study of signal transduction because chemical tools offer the ability to dynamically modulate intracellular signalling effectors in non-transformed cells. The presence of the full complement of PI3K isoforms and PtdIns(3,4,5)P_3-sensitive effectors in basal vertebrates, such as the goldfish, highlights the importance of examining PI3K-dependent signalling at multiple levels of organization. Consequently, research in this thesis utilizes a layered approach that involves pharmacological mapping of both proximal and distal effectors of PI3Ks in order to thoroughly characterized both PI3K-dependent and PI3K-independent aspects of the basal- and GnRH-selective intracellular signalling mechanisms that are activated in both gonadotropes and somatotropes.

As an effort to ensure that pharmacological inhibitors that are mainly designed for studies in mammalian systems are also specific and suitable for use in comparative studies using goldfish pituitary cells, the ability of these drugs to interfere with the activation of downstream signalling elements are examined using immunoblotting of total and phosphorylated target proteins in goldfish pituitary tissue or cell extracts. Where possible, *in silico* analysis using protein homology modeling and sequence alignments generated from the available databases of basal vertebrate genomic sequence data, and those from zebrafish in particular, were performed. These bioinformatics approaches can be used to ensure that the drug-coordinating residues, as well as the three-dimensional shape of the drug-binding pockets, are conserved within the teleost homologs of proteins

targeted by commonly used pharmacological modulators. For clarity, detailed descriptions of the experimental methods used are reported in Chapter 2.

1.10 Specific research aims

Overall, the central hypothesis of my thesis research is that biased activation of PI3K-dependent signal transduction mediates the selective actions of GnRH2 and GnRH3 on goldfish pituitary cells leading to the differential control of hormone secretion and availability. To test this hypothesis, my research is divided into 6 major research aims that each constitutes an individual chapter within this thesis.

(1) Chapter 3 examines whether PI3K-dependent signal transduction, in general, participates in acute GnRH-induced LH and GH release, as well as accompanying increases in gonadotrope and somatotrope $[Ca^{2+}]_i$. In addition, the relationship between PI3K-dependent signalling and PKCs, known mediators of GnRH-induced hormone release, is also addressed.

(2) Experiments in Chapter 4 investigate the involvement of class I PI3Ks in the differential control of GnRH2- and GnRH3-stimulated acute hormone release responses. In particular, the differential roles of Gβγ-sensitive class I PI3K isoforms provide novel insights regarding the molecular events coupling GnRHR activation to class I PI3K-dependent signalling cascades.

(3) Studies in Chapter 5 characterize the multiplicity of signal transduction responses downstream of class I PI3Ks by examining the involvement of canonical PtdIns(3,4,5)P_3-sensitive effectors in GnRHR-mediated signal transduction. In particular,

the requirement for PDK1-, Akt-, and BTK-dependent signalling in mediating GnRH-stimulated acute hormone release is monitored.

(4) In Chapter 6, I begin to examine the long-term involvement of PI3Ks by evaluating the role of PI3K-dependent signal transduction in the coordinate control of long-term GnRH actions on pituitary hormone release and total availability.

(5) GnRH2 and GnHR3 also utilize MEK1/2-dependent signalling in the control of goldfish gonadaotrope and somatotrope functions (Section 1.6) and PI3K-dependent signalling is also known to affect Raf-MEK-ERK activation (Section 1.8). Accordingly, Chapter 7 examines the involvement of MEK1/2-dependent signalling during the acute and prolonged actions of GnRH, as well as its relationship with PI3K-dependent signalling in the control of hormone secretion.

(6) Chapter 8 examines whether differences in the involvement of the canonical PI3K-PDK1-Akt-TOR signalling cascade exist during the long-term control of basal hormone release and cellular content.

Lastly, the findings of this thesis are synthesized in Chapter 9 and used to illustrate that PI3K-dependent signalling represents a unique platform for the integration of basal and agonist-stimulated cellular responses. Based on findings in this thesis, I also propose a novel hypothesis for the selective control of cellular functions as a result of the bifurcation of signalling responses by activated heterotrimeric Gαβγ protein complexes downstream of agonist-stabilized GPCRs.

Figure 1.1 Functional versatility of the conserved GPCR fold is controlled by structural dynamics. The seven-transmembrane (7TM) helix fold of G protein-coupled receptors (GPCRs) controls a wide variety of intracellular signalling responses by providing conformational heterogeneity through linear peptide motifs and embedded posttranslational modification sites. The relative arrangement of the transmembrane helices is conserved across GPCR classes, although there is marked diversity in the ligand-binding positions within and around the 7TM helical bundle. Overall, structured regions permit the receptor to bind to a diversity of ligands, whereas disordered regions in the cytoplasmic side modulate downstream signalling responses. Adapted from Venkatakrishnan et al., *Curr. Opin. Struc. Biol.*, 2014. (Central image is a homology model of the goldfish GfA GnRHR; a member of the *rhodopsin-like* class A GPCRs; figure prepared using PyMol).

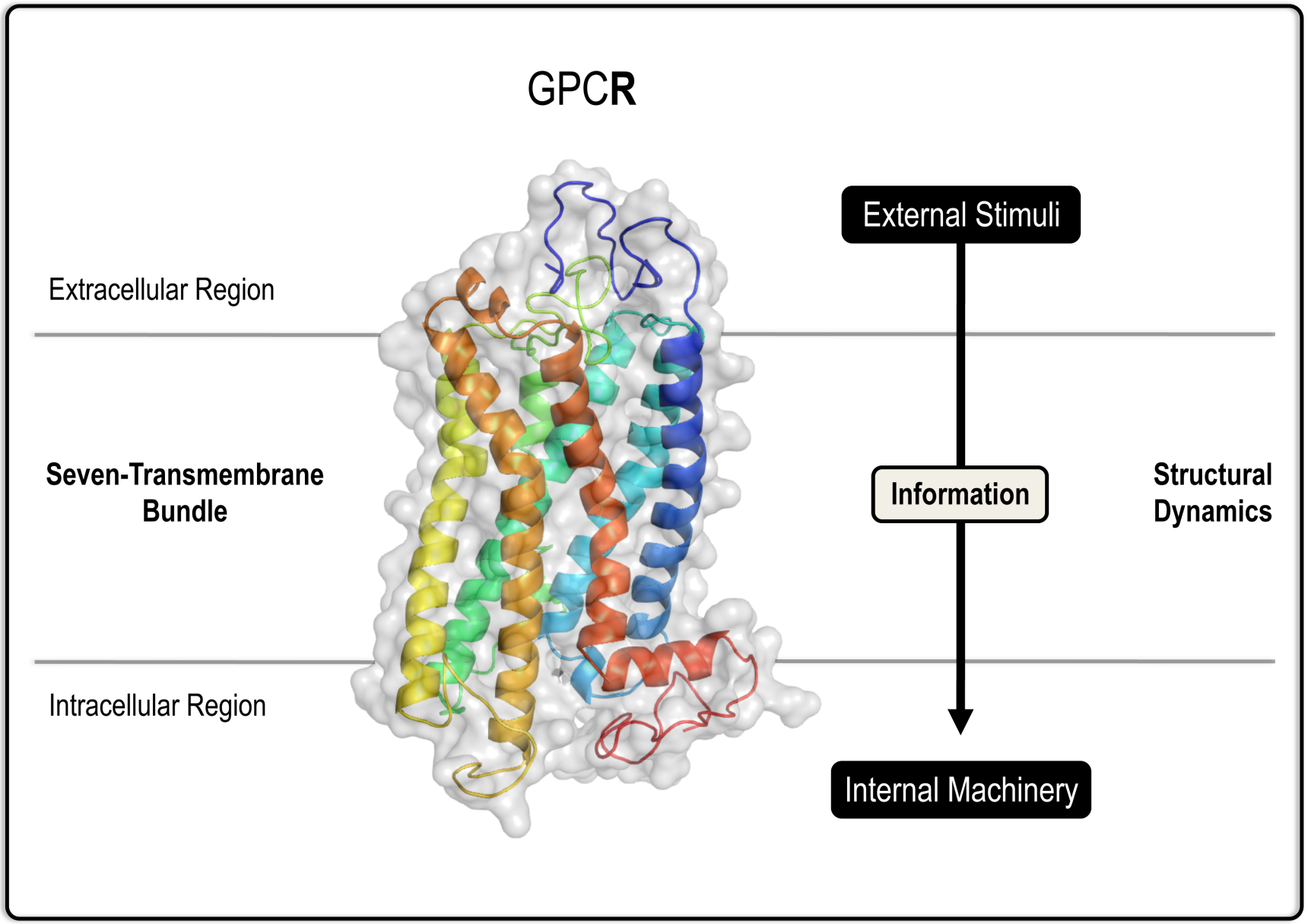

GPCR
Extracellular Region
Seven-Transmembrane Bundle
Intracellular Region
External Stimuli
Information
Internal Machinery
Structural Dynamics

Figure 1.2 Signal transduction outcomes controlled by heterotrimeric Gαβγ complexes. G protein-coupled receptors (GPCRs) operate as a transduction unit containing a transmembrane-spanning receptor, a single heterotrimeric G protein (Gαβγ) complex, and signal-specific intracellular signalling effectors. In general, agonist binding (green circle) stimulates nucleotide exchange for the associated Gα subunit leading to a conformational change that allows for the signalling complex to dissociate into free Gα and Gβγ subunits. Distinct classes of Gα subunits have been identified that consistently couple to conserved intracellular signalling effectors, including: adenylyl cyclases (ACs; $G\alpha_s$ and $G\alpha_{i/o}$) and phosphodiesterases (PDEs; $G\alpha_{i/o}$), phospholipase Cβ (PLC; $G\alpha_{q/11}$), protein kinase Cs (PKCs; $G\alpha_{q/11}$), calcium (Ca^{2+}; $G\alpha_{q/11}$), as well as members of the Rho-family of small GTPases ($G\alpha_{12/13}$). Independent of signalling initiated downstream of the Gα subunits, Gβγ heterodimers also directly associate with downstream effectors and can regulate distinct aspects of cellular physiology. Taken together, heterotrimeric Gαβγ proteins are versatile signalling complexes that can coordinately control many canonical signal transduction cascades and therefore comprise the fundamental unit of GPCR-controlled signalling networks. Additionally, within the Gαβγ complex, agonist-selective control of Gα- and/or Gβγ-dependent signalling responses may allow for the differential control of cellular responses. Adapted from Marinissen and Gutkind, *Trends Pharmacol. Sci.*, 2001 and Khan et al., *Pharmacol. Rev.*, 2013.

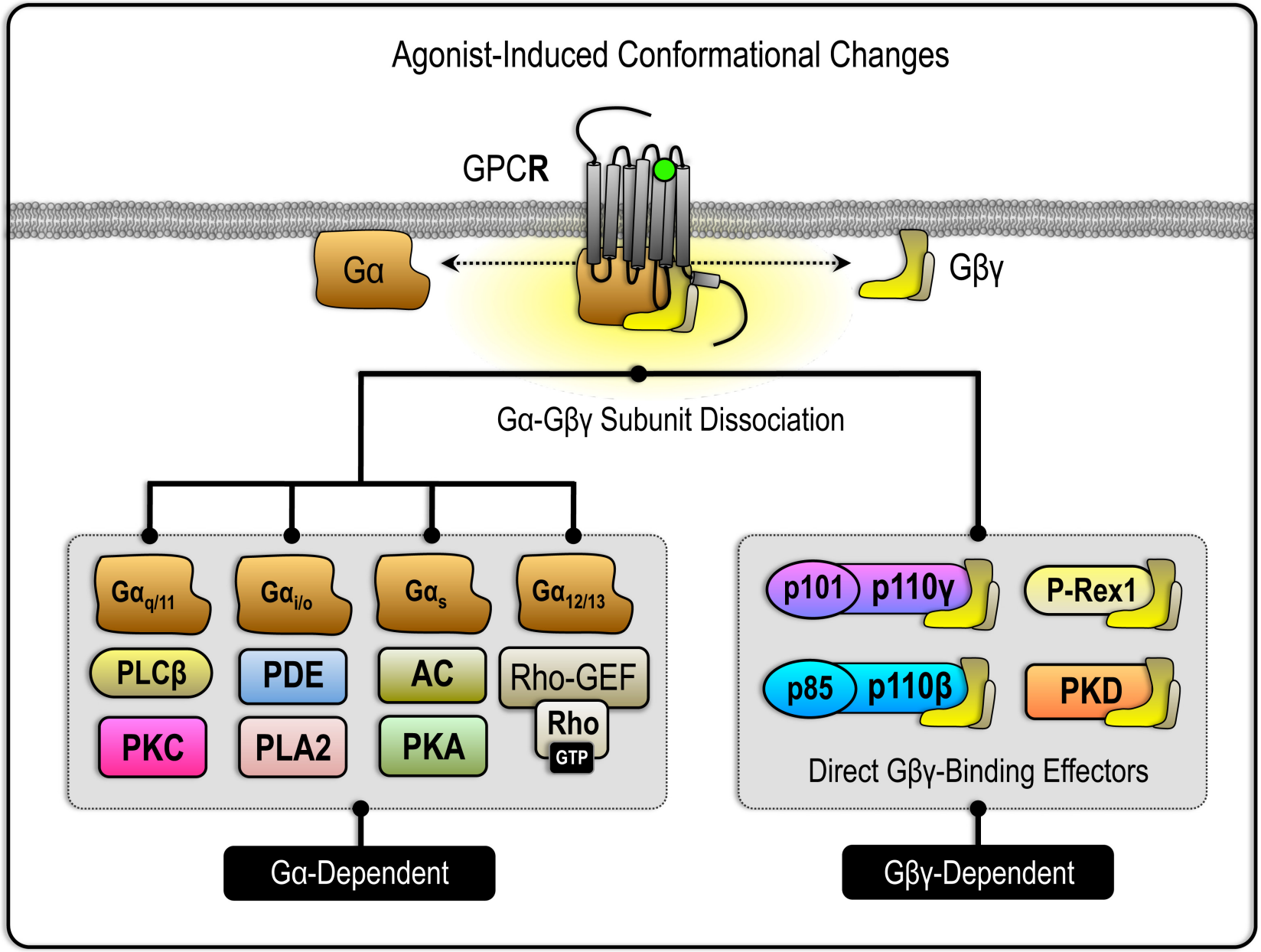

Agonist-Induced Conformational Changes
GPCR
Gα
Gβγ
Gα-Gβγ Subunit Dissociation
Gα$_{q/11}$
Gα$_{i/o}$
Gα$_{s}$
Gα$_{12/13}$
PLCβ
PDE
AC
Rho-GEF
Rho
GTP
PKC
PLA2
PKA
p101
p110γ
P-Rex1
p85
p110β
PKD
Direct Gβγ-Binding Effectors
Gα-Dependent
Gβγ-Dependent

Figure 1.3 Biased signal transduction. G protein-coupled receptors (GPCRs) can be thought of as oscillating between a series of conformational intermediates and the stability of the energy landscape associated with the receptor is influenced by both extracellular ligands and intracellular effector proteins that bind to the receptor. Consequently, structurally-related agonists (blue, agonist variant 1; red, agonist variant 2) have been demonstrated to take advantage of the heterogeneity within the conformational landscape of the receptor by stabilizing agonist-specific GPCR conformations that can selectively activate a subset of signal transduction effectors without activating others. In particular, the current model of GPCR activation has demonstrated that a single GPCR can signal through both heterotrimeric G protein (Gαβγ)-dependent (left; G protein-bound GPCR; blue) and Gαβγ-independent (right; arrestin-bound GPCR; red) transduction cascades. Gαβγ-independent signalling has distinct biochemical and functional consequences from those mediated by Gαβγ proteins; although redundant and/or convergent regulation of intracellular effectors can occur (green). Overall, unique agonist-specific functional profiles promote the differential regulation of intracellular signalling networks and ultimately to divergent physiological responses. Adapted from Wisler et al., *Curr. Opin. Cell. Biol.*, 2014.

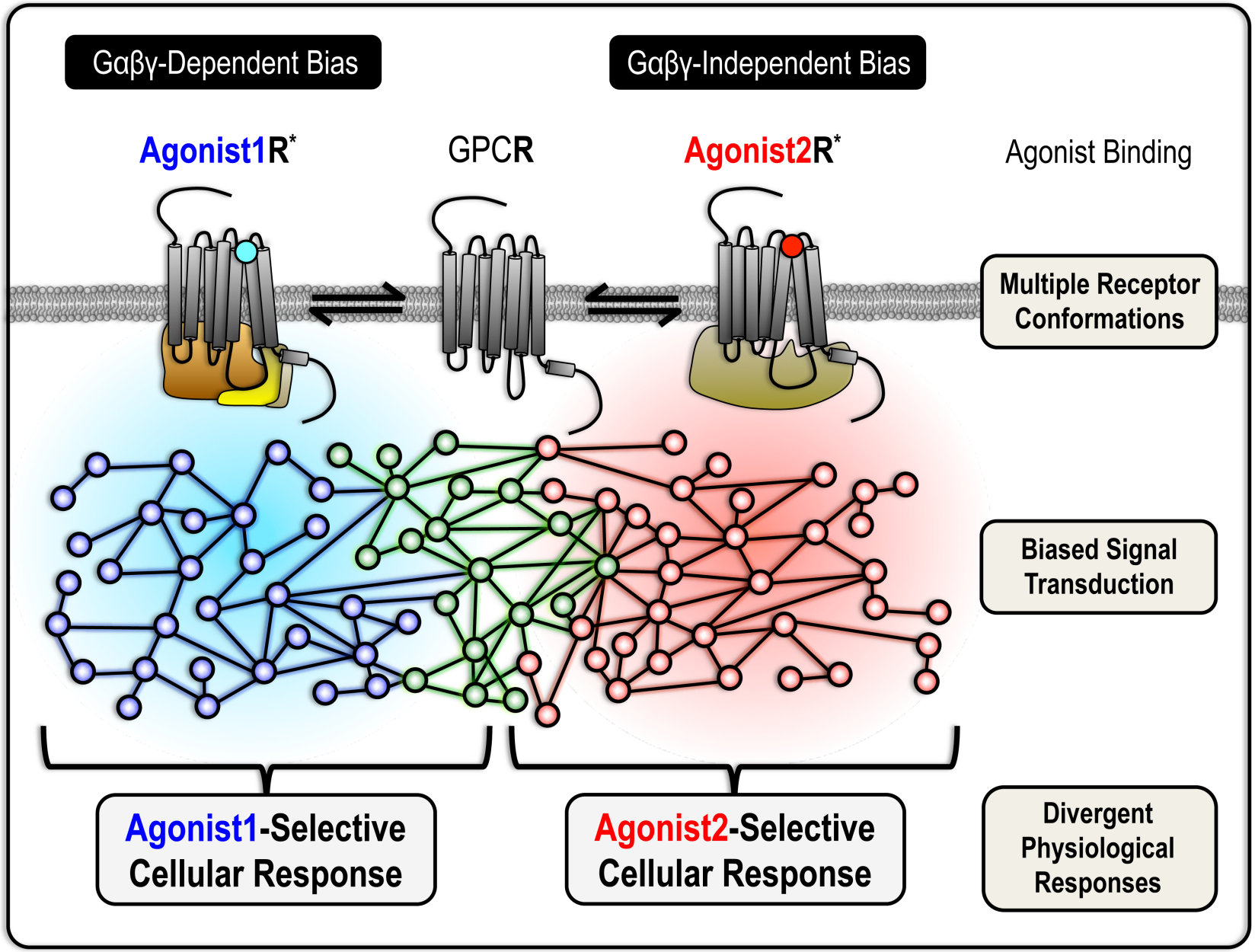
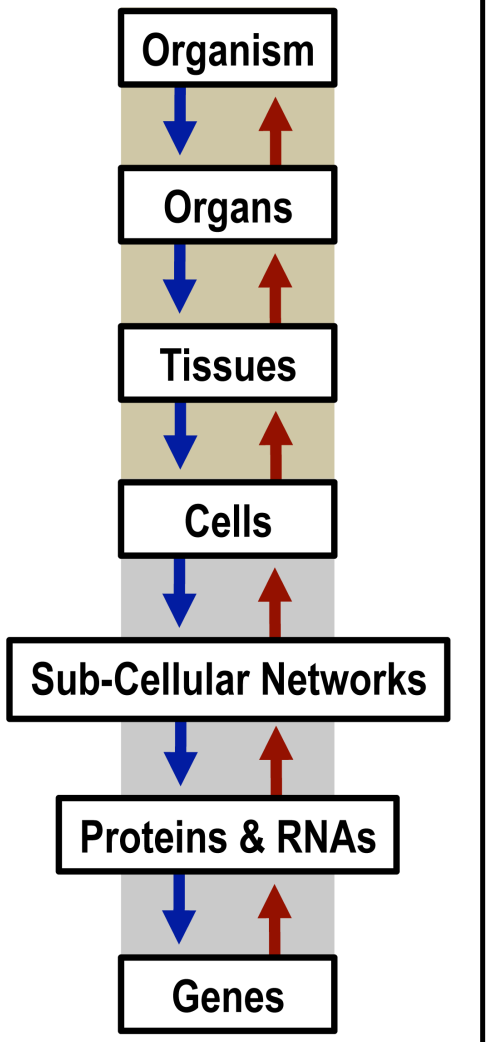

Gαβγ-Dependent Bias
Gαβγ-Independent Bias
Agonist1R*
GPCR
Agonist2R*
Agonist Binding
Multiple Receptor Conformations
Biased Signal Transduction
Agonist1-Selective Cellular Response
Agonist2-Selective Cellular Response
Divergent Physiological Responses

Figure 1.4 Potential for biased signalling during the multifactorial neuroendocrine control of pituitary cell functions in basal vertebrates. Across the levels of biological organization (right), cells represent an important regulatory center that integrates molecular and extracellular information. In order to understand the physiological role for natural biased ligands, we will utilize the goldfish (*Carassius auratus*) pituitary cell model system. Listed are known endogenous variants of several important vertebrate neuropeptide families that are found in goldfish (left). In addition, the majority of these peptides have been shown to elicit functional responses at the level of pituitary cells, and all of them activate G protein-coupled receptors. Taken together, these factors make goldfish pituitary cells an ideal model for understanding the integrated control of cell functions by endogenous hormone variants. GnRH, gonadotropin-releasing hormone; PACAP, pituitary adenylate cyclase activating peptide; NPY, neuropeptide Y; SST, somatostatin; GnIH, gonadotropin-inhibitory hormone; GRL, ghrelin; Kiss, kisspeptin. Adapted, in part, from Noble, *Prog. Biophys. Mol. Biol.*, 2013.

Multifactorial Control of Pituitary Cell Functions



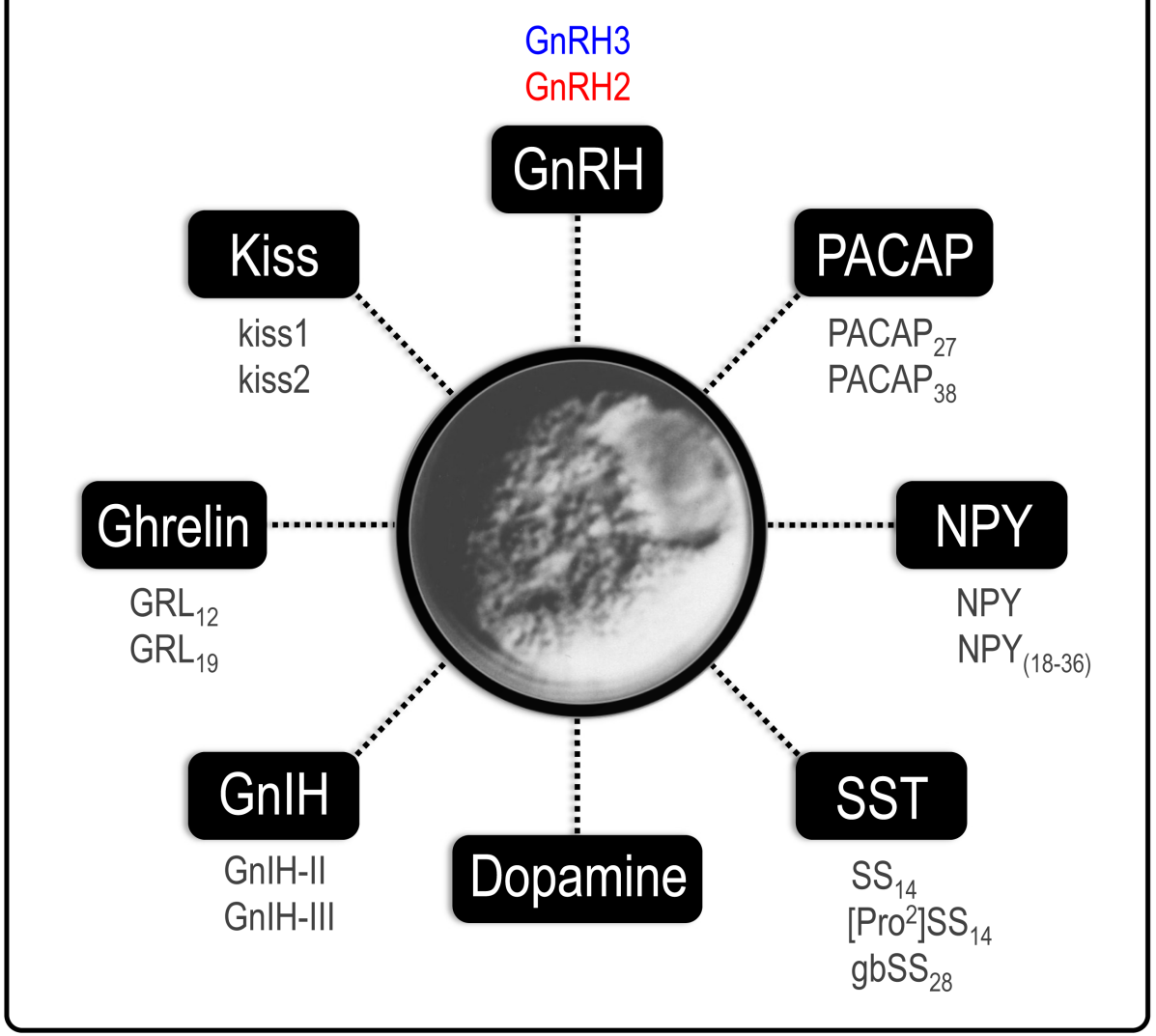


Figure 1.5 Comparative organization of the hypothalamo-pituitary connections in mammals and teleost fishes. (a) In mammals, hypothalamic releasing factors are secreted into the hypothalamohypophysial portal blood system, at the level of the median eminence, and delivered to cells in the adenohypophysis (AH; anterior pituitary; shaded grey; left). (b) In fishes, there is no portal vasculature and the hypophysiotropic neurons send their axonal projections directly into the anterior lobe of the pituitary and either directly innervate secretory cells of the anterior lobe (depicted) or end at a basal membrane that separates the neurohypophysis (NH; shaded beige; bottom) from the adenohypophysis (AH; shaded grey; top). Adapted from Kah & Dufour, *Horm. Reproduct. Vert. – Vol. 1 – Fishes*, 2011 and Zohar et al., *Gen. Comp. Endocrinol.*, 2010.

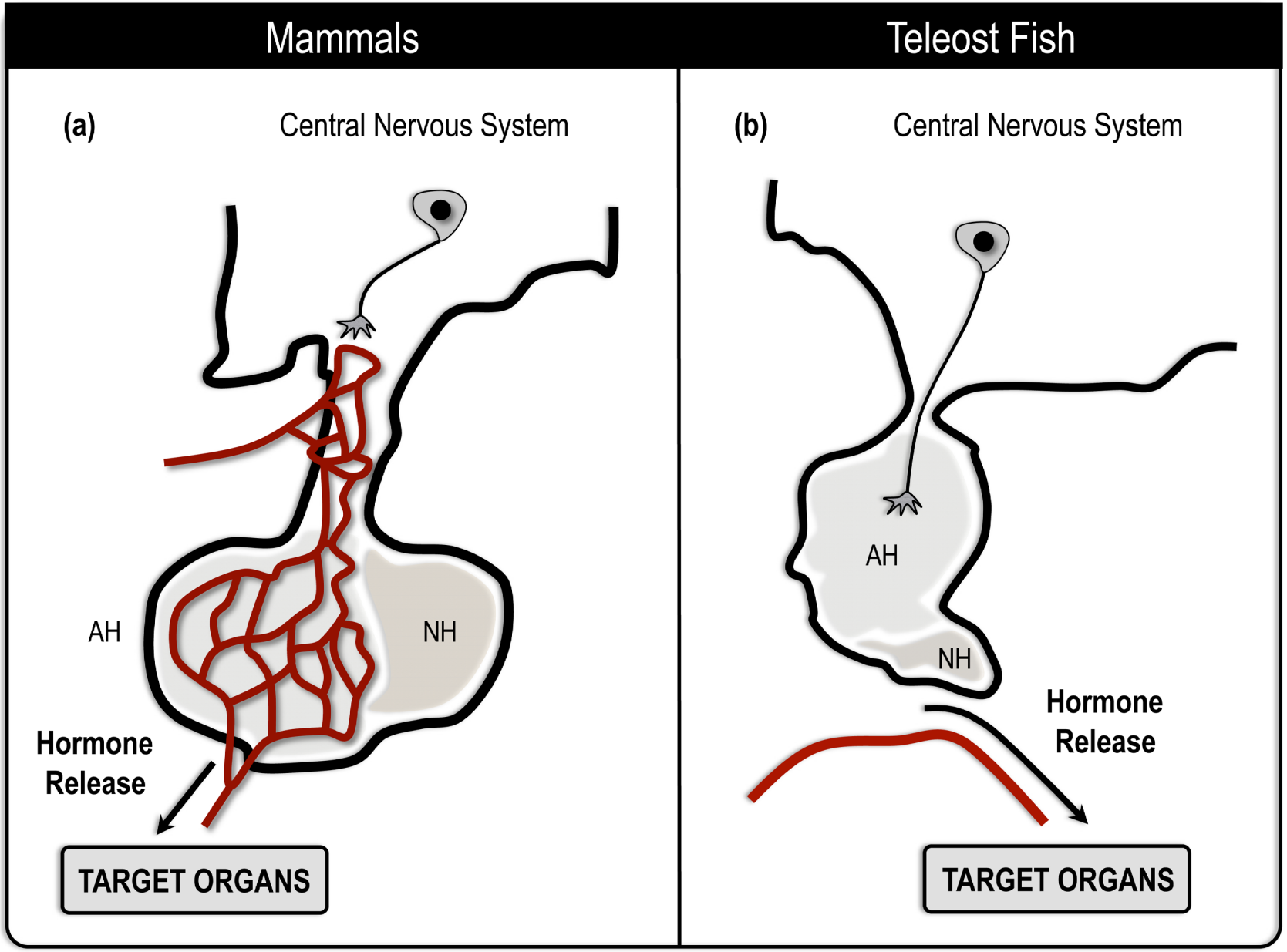
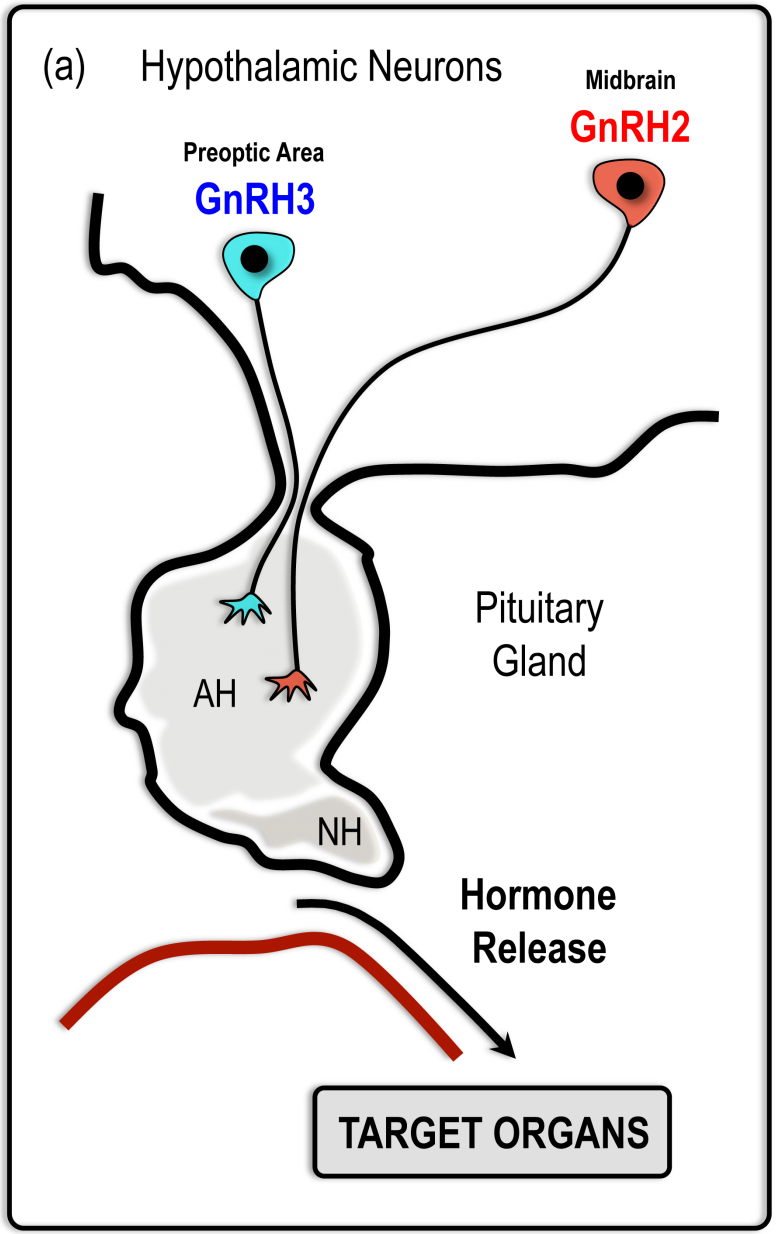

Mammals
Teleost Fish
(a)
Central Nervous System
AH
NH
Hormone Release
TARGET ORGANS
(b)
Central Nervous System
AH
NH
Hormone Release
TARGET ORGANS

Figure 1.6 Organization of the GnRH system within goldfish (*Carassius auratus*). (a) In goldfish, both GnRH2 and GnRH3 are expressed in the brain and secreted from neuronal terminals that directly innervate the adenohypophysis (AH; anterior pituitary; shaded grey). (b) GnRH3-positive perikarya (neuronal cell bodies) are found throughout the preoptic area, whereas GnRH2-positive perikarya reside primarily in the midbrain tegmentum (boxed area is highlighted in (a); hypothalamo-pituitary connection). (c) GnRH2 and GnRH3 both bind to the same population of cell-surface GnRHRs and directly stimulate the release of LH from gonadotropes and GH from somatotropes. Interestingly, goldfish gonadotrope and somatotrope cell types can be identified by their morphology in primary culture (characteristic cells shown). Adapted from Lethimonier et al., *Gen. Comp. Endocrinol.*, 2004 and Van Goor et al., *Cell Tissue Res.*, 1994 (for detailed descriptions of the morphology of each cell type, see Chapter 2 - Section 2.8).


(a) Hypothalamic Neurons
Midbrain
GnRH2
Preoptic Area
GnRH3
AH
NH
Pituitary Gland
Hormone Release
TARGET ORGANS

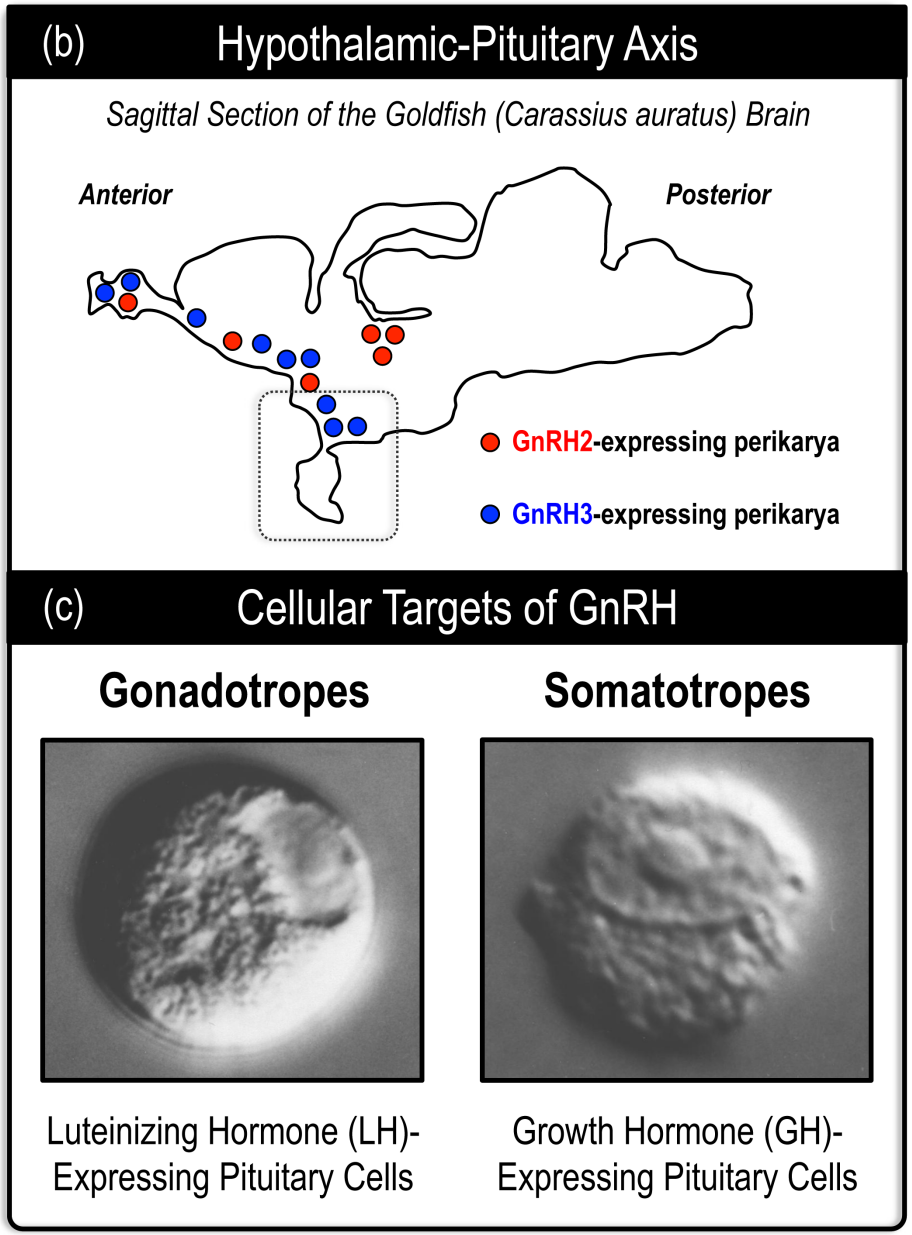

(b) Hypothalamic-Pituitary Axis
Sagittal Section of the Goldfish (Carassius auratus) Brain
Anterior
Posterior
GnRH2-expressing perikarya
GnRH3-expressing perikarya
(c) Cellular Targets of GnRH
Gonadotropes
Somatotropes
Luteinizing Hormone (LH)-Expressing Pituitary Cells
Growth Hormone (GH)-Expressing Pituitary Cells

Figure 1.7 Key intermediates in the G protein-coupled receptor (GPCR) activation mechanism as characterized using crystallography. R represents the inactive state(s) of receptors, which can be stabilized by antagonists or inverse agonists. R′ represents inactive low-affinity agonist (A)-bound state(s), which differ from R states by only small local changes in the orthosteric binding pocket. R′′ represents activated state(s), characterized by global rearrangements of helices and side-chain microswitches on the intracellular side that exposes the G protein-binding surface. $R^{*G\alpha}$ represents activated substate(s) with the initial insertion of the G protein into the intracellular crevice. Finally, $R^{*G\alpha\beta\gamma}$ is a distinct G protein signalling conformation of a receptor, which can achieved upon full engagement and activation of the GPCR-Gαβγ signalling complex. Other distinct GPCR conformational states include complexes of the receptor with Gαβγ-independent effectors such as G protein-coupled receptor kinases and arrestins. Transition from the $R^{*G\alpha}$ state to the full signalling state $R^{*G\alpha\beta\gamma}$ is accompanied by the release of GDP and therefore proceeds unidirectionally. Return to the pre-signalling states requires dissociation of the Gαβγ complex. Adapted from Katritch et al., *Annu. Rev. Pharmicol. Toxicol.*, 2013.

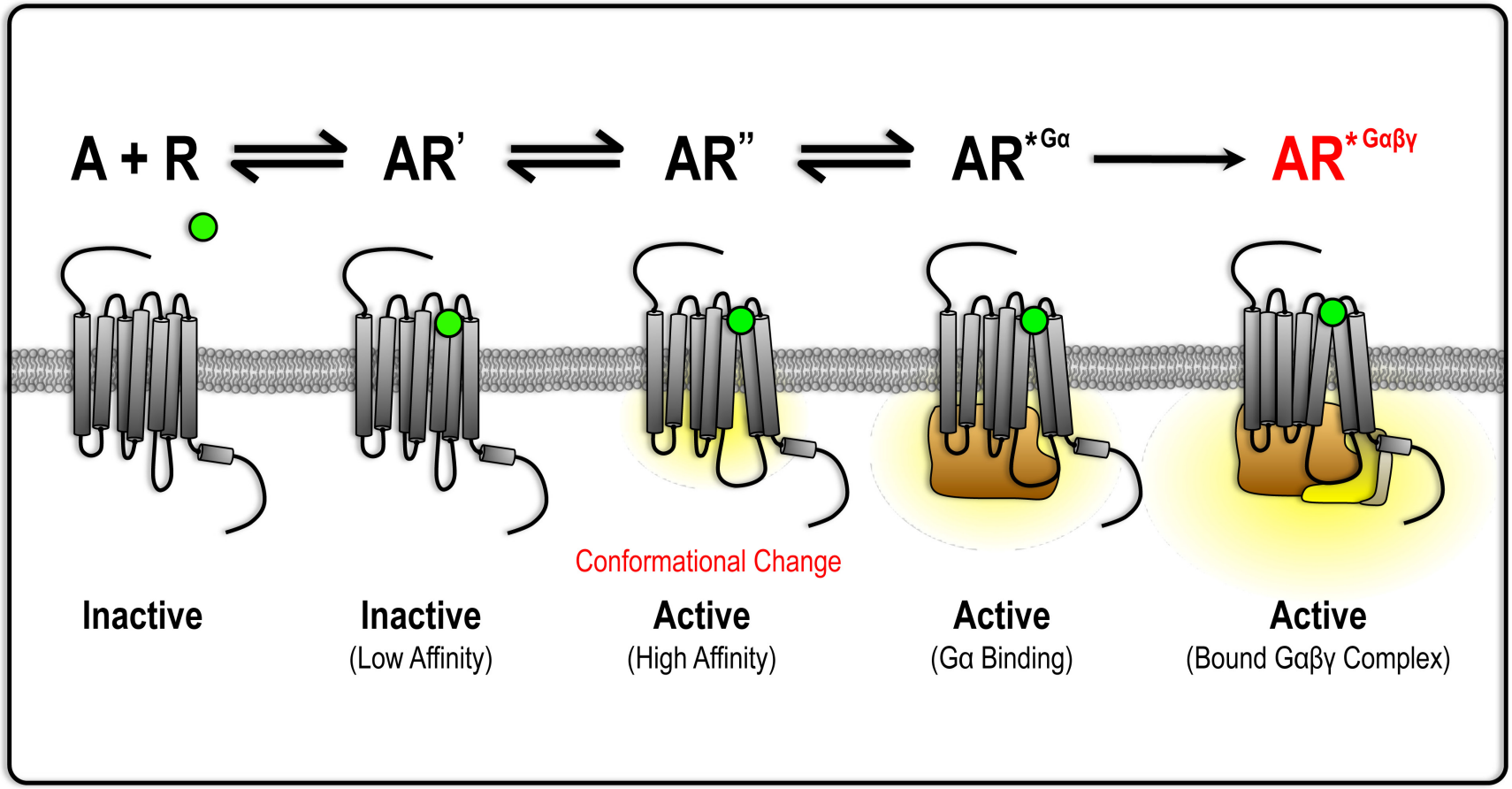

A + R
AR'
AR''
AR*Gα
AR*Gαβγ
Conformational Change
Inactive
Inactive
(Low Affinity)
Active
(High Affinity)
Active
(Gα Binding)
Active
(Bound Gαβγ Complex)

Figure 1.8 Schematic of the signal transduction mechanisms known to be involved in mediating GnRH actions on hormone release in both goldfish gonadotropes and somatotropes. In goldfish (*Carassius auratus*), results to date have demonstrated that the canonical $G\alpha_{q/11}$-coupled effectors phospholipase Cβ (PLCβ), protein kinase C (PKC), and Ca^{2+}-dependent signalling are consistently involved in mediating gonadotropin-releasing hormone (GnRH)-stimulated LH and GH release responses. However, GnRH-specific differences in mechanisms used to control increases in the intracellular Ca^{2+} concentration exist (see main text; Section 1.6). Furthermore, recent studies suggest that signalling initiated by nitric oxide synthases (NOS) and mitogen-activated protein kinases (MAPKs) might be differentially involved in GnRH2- and/or GnRH3-stimulated signalling, and that these effectors might be differentially integrated with known elements of GnRH-dependent signalling, such as PKC or Ca^{2+}, in a cell type-selective manner. Whether and how differences in the intracellular transduction networks activated by either endogenous GnRH isoform leads to alterations in hormone release and production are not fully understood. The goals of this thesis will be to better understand how the biased regulation of transduction responses alters the integrated control of pituitary cell functions stimulated by GnRH2 and GnRH3 (sequence differences in the GnRH2 and GnRH3 decapeptides are provided for comparison; Top).

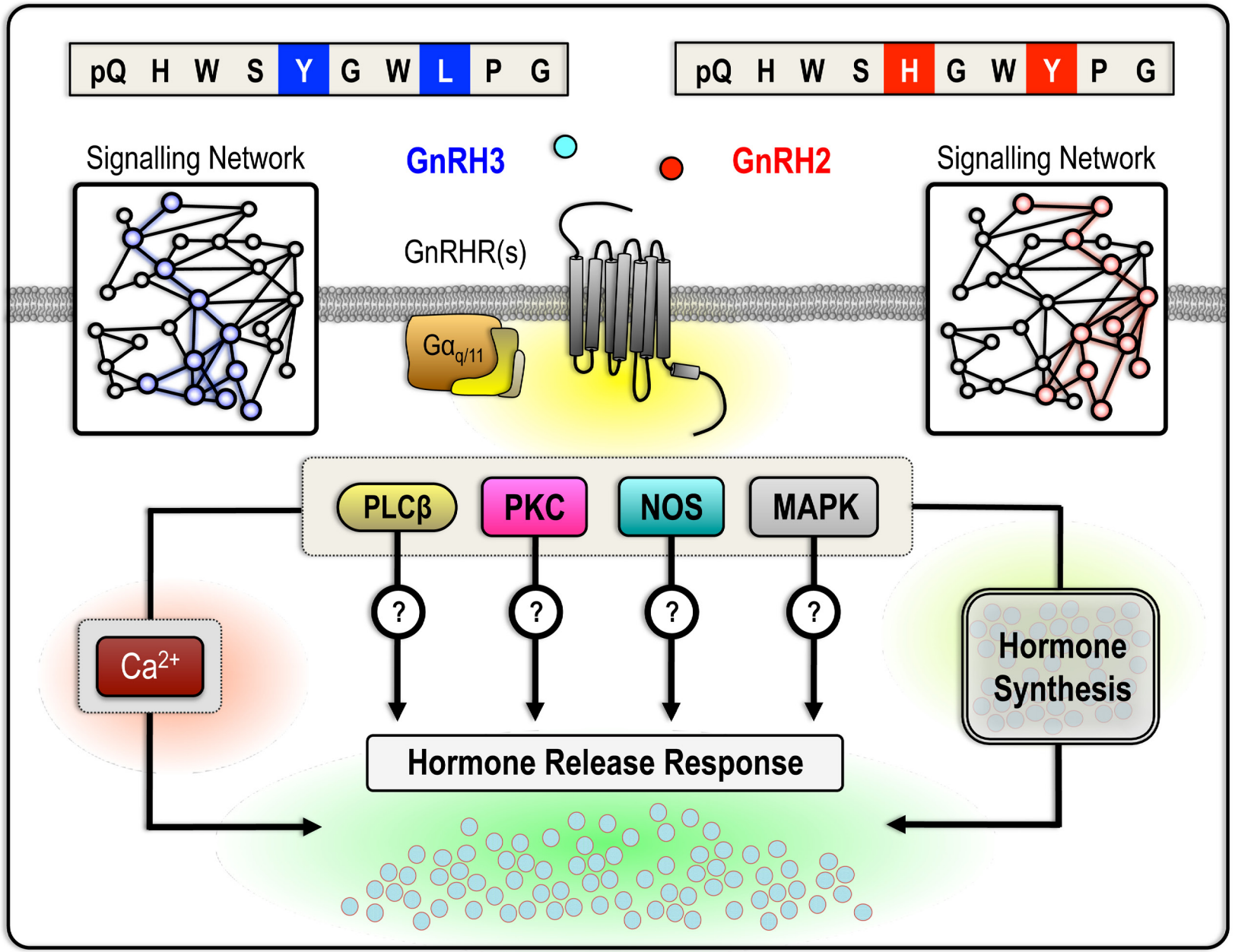

pQ H W S Y G W L P G
pQ H W S H G W Y P G
Signalling Network
GnRH3
GnRH2
Signalling Network
GnRHR(s)
Gαq/11
PLCβ
PKC
NOS
MAPK
?
?
?
?
Ca2+
Hormone Synthesis
Hormone Release Response

Figure 1.9 Classification and domain structure of the PI3K superfamily. Phosphoinositide 3-kinases (PI3Ks) are classified based on their substrate specificities and domain structure. In general, all PI3K catalytic subunits have a PI3K core structure consisting of a C2 phospholipid-binding domain, a helical domain, and a catalytic domain. (a) Class I PI3Ks phosphorylate phosphatidylinositol (PtdIns) 4,5-bisphosphate (PtdIns(4,5)P_2) to produce PtdIns 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). Class IA PI3Ks are heterodimers of a p110 catalytic subunit and a p85-type regulatory subunit. Class IA catalytic isoforms (p110α, p110β, and p110δ) have a p85 binding domain (p85-BD), a Ras-binding domain (RBD), and the PI3K catalytic core. Class IA regulatory subunits (p85α, p85β, p55γ, p55α, and p50α; differential promoter usage encodes p85α, p55α, and p50α from the PIK3R1 gene) have an inter-SH (Src homology)2 (iSH2) domain that binds to the p110 catalytic subunits, that is flanked by SH2 domains that interact with phosphorylated tyrosine motifs. The p85α and p85β subunits additionally have amino-terminal SH3 and breakpoint cluster homology (BH) domains. Alternatively, the class IB PI3Ks are heterodimers of a p110γ catalytic subunit and either a p101 or p84 regulatory subunit. The catalytic p110γ subunit has an RBD in addition to the PI3K catalytic core. The domain structures of the p101 and p84 regulatory subunits are not fully known, but a carboxy-terminal region of p101 directly binds to G protein βγ subunits. (b) Class II PI3Ks use PtdIns as a substrate to produce PtdIns 3-phosphate (PtdIns(3)P), but might also use PtdIns 4-phosphate (PtdIns(4)P) and produce PtdIns 3,4-bisphosphate (PtdIns(3,4)P_2) under certain conditions. The class II PI3Ks are monomeric and consist of only a p110-like catalytic subunit (PI3K-C2α, PI3K-C2β, and PI3K-C2γ) that each have an RBD and the PI3K catalytic core. In addition, class II PI3Ks have an extended amino-terminus containing proline-rich (P) domains, as well as additional C2 and Phox homology (PH) domains at the carboxy-terminus. (c) The only class III PI3K catalytic subunit Vps34 (vacuolar protein-sorting defective 34) forms a constitutive heterodimer with the myristoylated regulatory subunit Vps15. Vps34 consists of the PI3K catalytic core (C2, helical, and catalytic domains) and uses PtdIns as a substrate to produce PtdIns (3)P. Vps15 consists of a catalytic domain (which is thought to be inactive), HEAT domains (involved in protein-protein interactions), and WD40 repeats, which are essential for functional interactions with numerous effectors. Adapted from Vanhaesebroeck et al., *Nat. Rev. Mol. Cell Biol*., 2010 and Thorpe et al., *Nat. Rev. Cancer*, 2015.

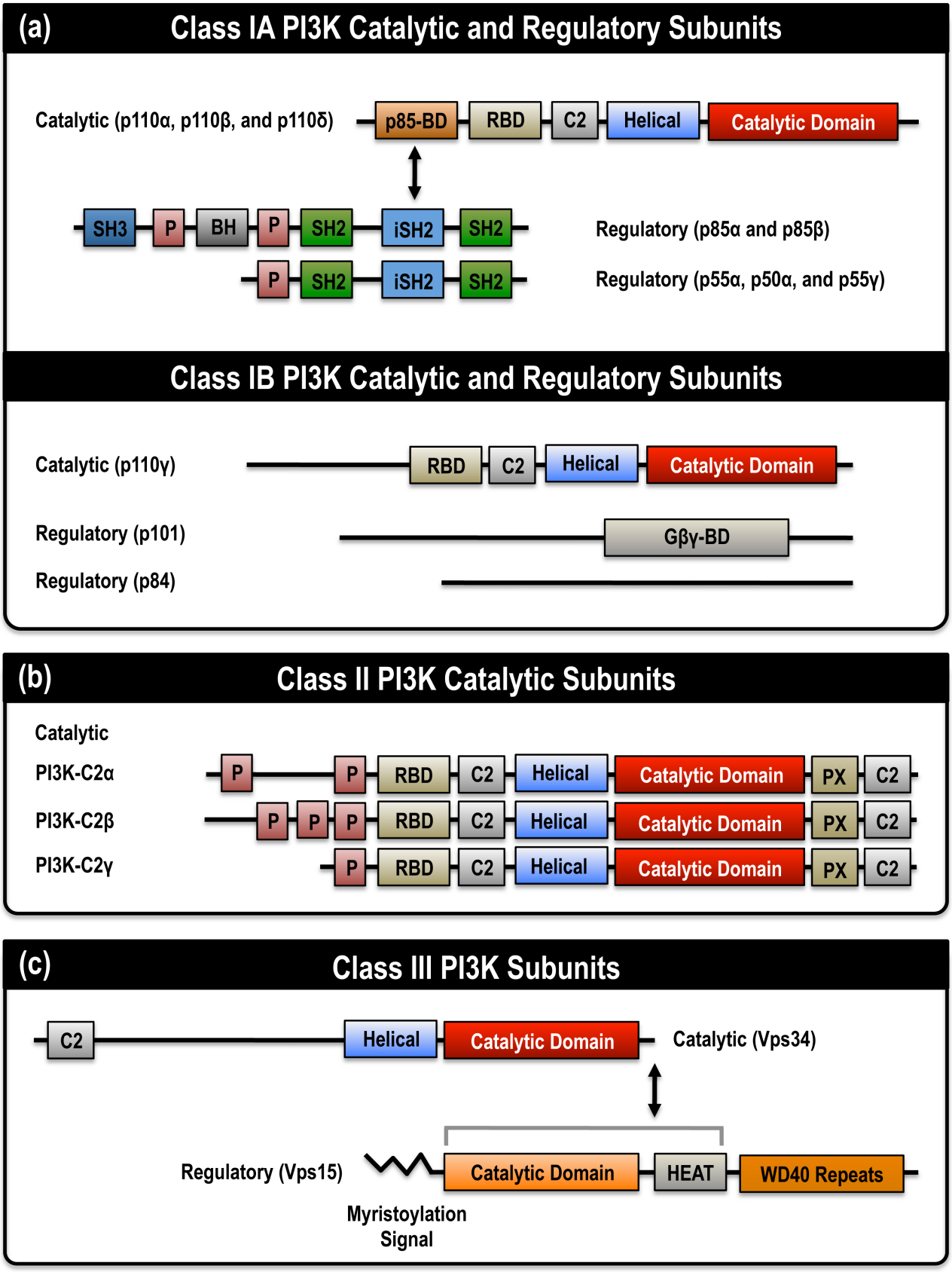

(a)
Class IA PI3K Catalytic and Regulatory Subunits
Catalytic (p110α, p110β, and p110δ)
p85-BD
RBD
C2
Helical
Catalytic Domain
SH3
P
BH
P
SH2
iSH2
SH2
Regulatory (p85α and p85β)
P
SH2
iSH2
SH2
Regulatory (p55α, p50α, and p55γ)
Class IB PI3K Catalytic and Regulatory Subunits
Catalytic (p110γ)
RBD
C2
Helical
Catalytic Domain
Regulatory (p101)
Gβγ-BD
Regulatory (p84)
(b)
Class II PI3K Catalytic Subunits
Catalytic
PI3K-C2α
PI3K-C2β
PI3K-C2γ
P
RBD
C2
Helical
Catalytic Domain
PX
C2
(c)
Class III PI3K Subunits
C2
Helical
Catalytic Domain
Catalytic (Vps34)
Regulatory (Vps15)
Catalytic Domain
HEAT
WD40 Repeats
Myristoylation
Signal

Figure 1.10 General schematic of class I PI3K-dependent signalling. The class IA subclass of PI3Ks comprises three catalytic isoforms (p110α, p110β, and p110δ) that utilize p85-type regulatory subunits that bind phosphorylated tyrosine motifs. As a result, the class IA PI3Ks have been associated with signalling downstream of receptor tyrosine kinases (RTKs). Alternatively, the class IB catalytic subunit p110γ forms a heterodimer with either the p101 or p84 regulatory subunits and is activated by direct interactions with the Gβγ subunits of heterotrimeric Gαβγ complexes; although recent data suggests that p110γ/p101 complexes are preferentially activated by G protein-coupled receptors (GPCRs) due to an additional Gβγ interaction surface present within p101. Interestingly, the class IA p110β/p85 complex is also directly activated by Gβγ heterodimers (*; activated by interactions with either phosphorylated tyrosines or Gβγ). Irrespective of the mode of activation, class IA and IB PI3Ks both phosphorylate the 3′-hydroxyl group of phosphatidylinositol 4,5-bisphosphate to produce the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). The presence of PtdIns(3,4,5)P_3-sensitive binding domains in a variety of signaling proteins mediates the complexity of signal transduction downstream of class I PI3Ks. In particular, canonical intracellular effectors activated downstream of PtdIns(3,4,5)P_3 production include: phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK). The coordinate or selective activity of these effectors results in the regulation of class I PI3K-stimulated cellular responses. Adapted from Engelman et al., *Nat. Rev. Genet*., 2006.

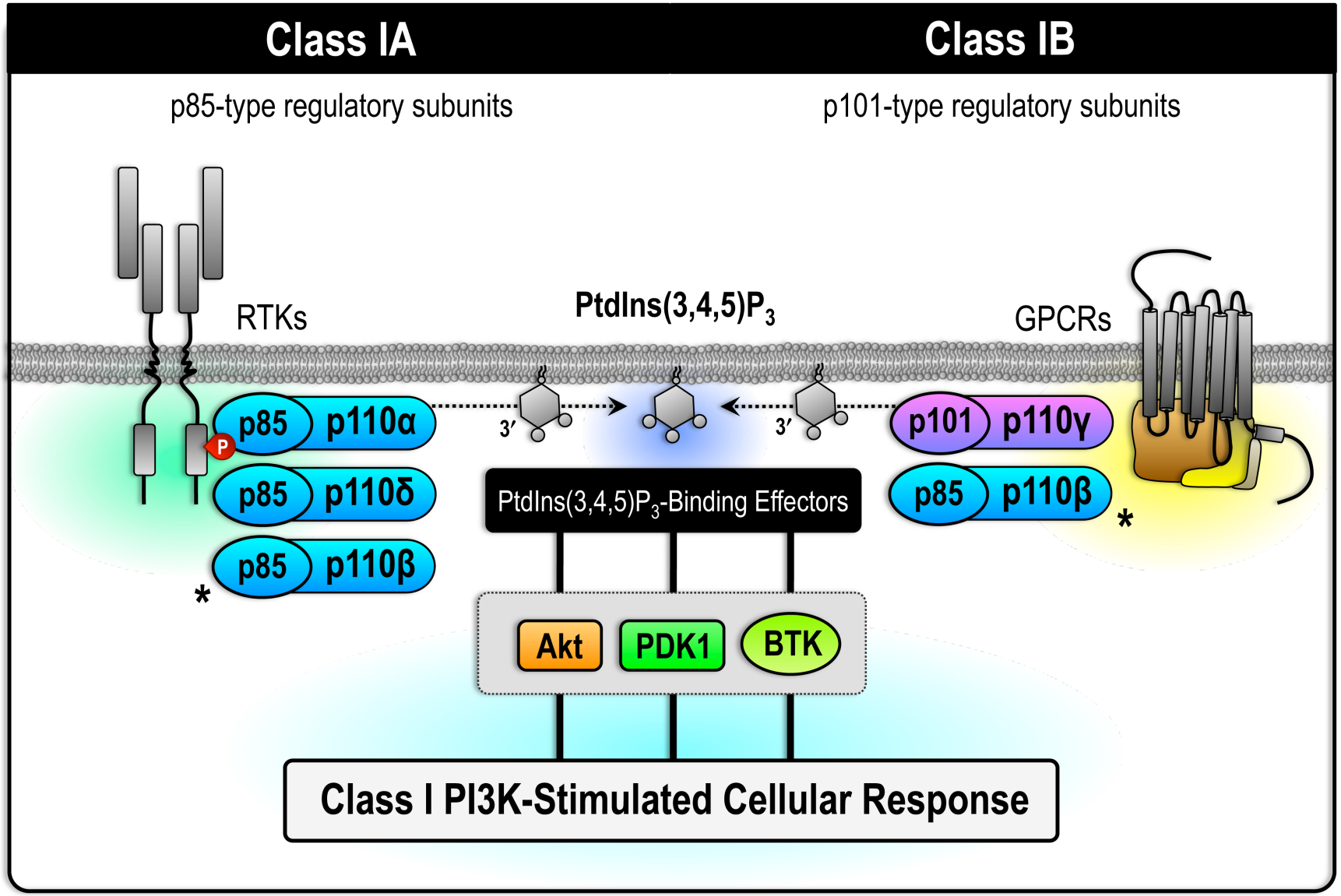

Class IA
Class IB
p85-type regulatory subunits
p101-type regulatory subunits
RTKs
PtdIns(3,4,5)P3
GPCRs
p85 p110α
p85 p110δ
p85 p110β
p101 p110γ
p85 p110β
P
3′
3′
PtdIns(3,4,5)P3-Binding Effectors
Akt
PDK1
BTK
Class I PI3K-Stimulated Cellular Response

Figure 1.11 Canonical signalling downstream of class I PI3K activation and PtdIns(3,4,5)P_3 production. Class I PI3Ks (p110α, p110β, p110δ, and p110γ) selectively phosphorylate the 3′-hydroxyl group of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to produce the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). Phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK) are canonical intracellular effectors that are recruited to the plasma membrane through PtdIns(3,4,5)P_3-selective pleckstrin homology lipid-binding domains. PDK1 functions as an important intermediate during PtdIns (3,4,5)P_3-dependent signalling by phosphorylating the T-loop activation segment of numerous AGC family serine/threonine kinases, including isozymes of: Akt, protein kinase C, protein kinase A, S6 kinase, ribosomal S6 kinase, and serum- and glucocorticoid-induced protein kinase. In addition to phosphorylation of the activation loop by PDK1, activation of Akt as well as conventional isozymes of PKC (cPKC) also requires phosphorylation within the hydrophobic motif by the target of rapamycin (TOR) complex 2 (which consists of TOR kinase bound to the core components rapamycin-insensitive companion of TOR (RICTOR), SAPK-interacting protein 1 (SIN1), and mammalian lethal with SEC thirteen 8 (mLST8); TORC2). Phosphorylation of Akt results in activation of downstream effectors, including the rapamycin-sensitive TOR complex 1 (consisting of the core components TOR kinase, regulatory-associated protein of TOR (RAPTOR), and mLST8; TORC1). Akt activation of TORC1 is indirect and requires the phosphorylation and inactivation of two major negative regulators: tuberous sclerosis complex 2 (TSC2) and phosphorylating proline-rich Akt substrate of 40 kDa (PRAS40; see text for additional details; Section 1.7.2.4). Alternatively, BTK primarily functions by increasing intracellular Ca^{2+} concentrations through the phosphorylation and activation of phospholipase Cγ (PLCγ). Activated PLCγ cleaves the polar head group of PtdIns(4,5)P_2 to produce two important intracellular second messengers: inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 stimulates the mobilization of Ca^{2+} from intracellular stores by binding to specific Ins(1,4,5)P_3 receptors (IP_3 Rs), while DAG binds to and activates several families of effector proteins including cPKC and novel PKC (nPKC) isoforms. Convergence of PDK1-, TORC2-, and BTK-dependent signalling is likely required for the spatiotemporal regulation of cPKCs and nPKCs. Lastly, BTK activity has also been implicated in the regulation of mitogen-activated protein kinases (MAPKs). Overall, the integrated actions of PDK1-, Akt-, TOR-, and BTK-dependent signalling control cellular responses downstream of PtdIns(3,4,5)P_3 production. Adapted from Takesono et al., J. Cell Sci., 2002, Manning and Cantley, *Cell*, 2007, Pearce et al., *Nat. Rev. Mol. Cell Biol*., 2010, Rosse et al., *Nat. Rev. Mol. Cell Biol*., 2010, and Zoncu et al., *Nat. Rev. Mol. Cell Biol*., 2011.

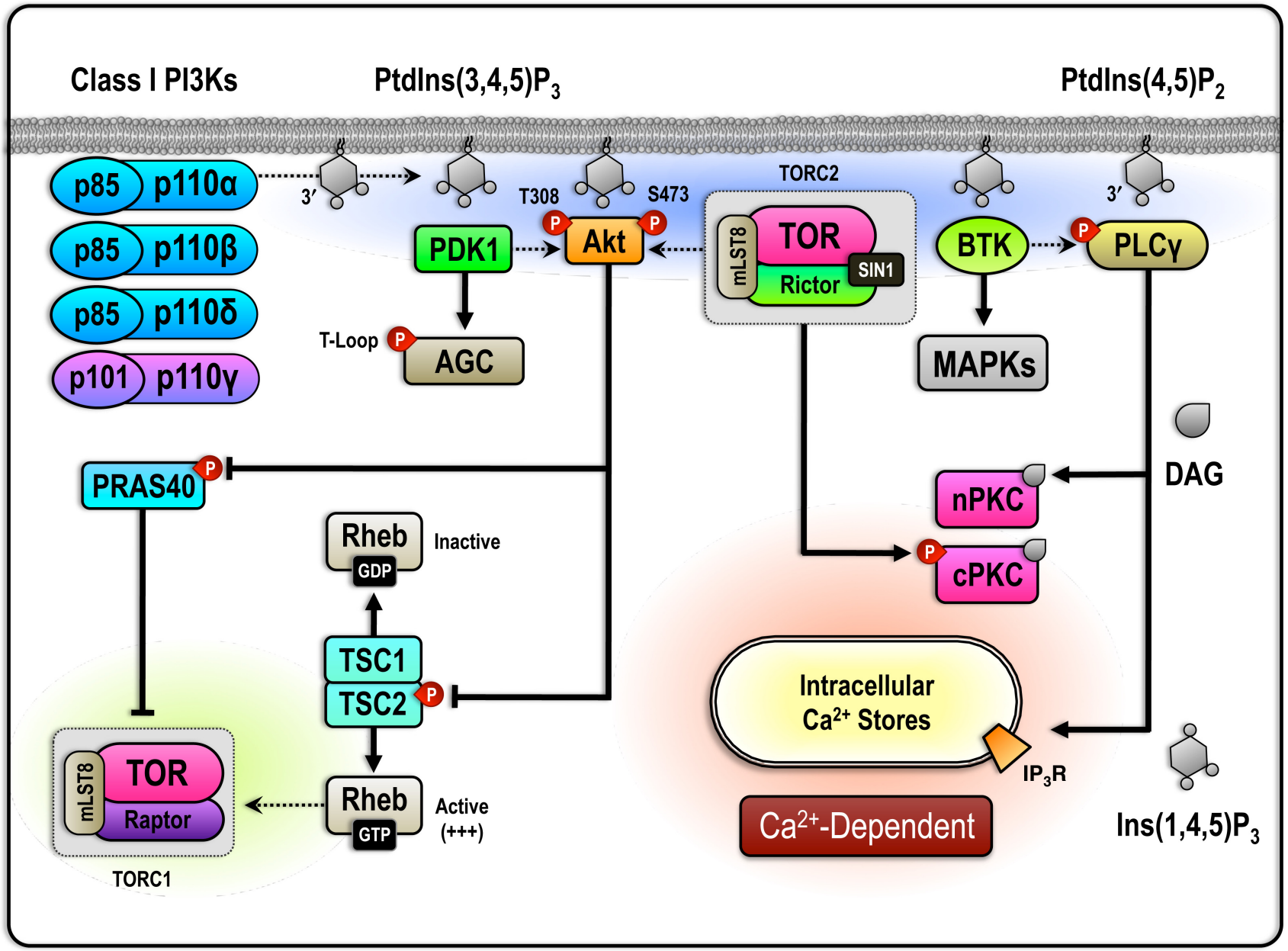
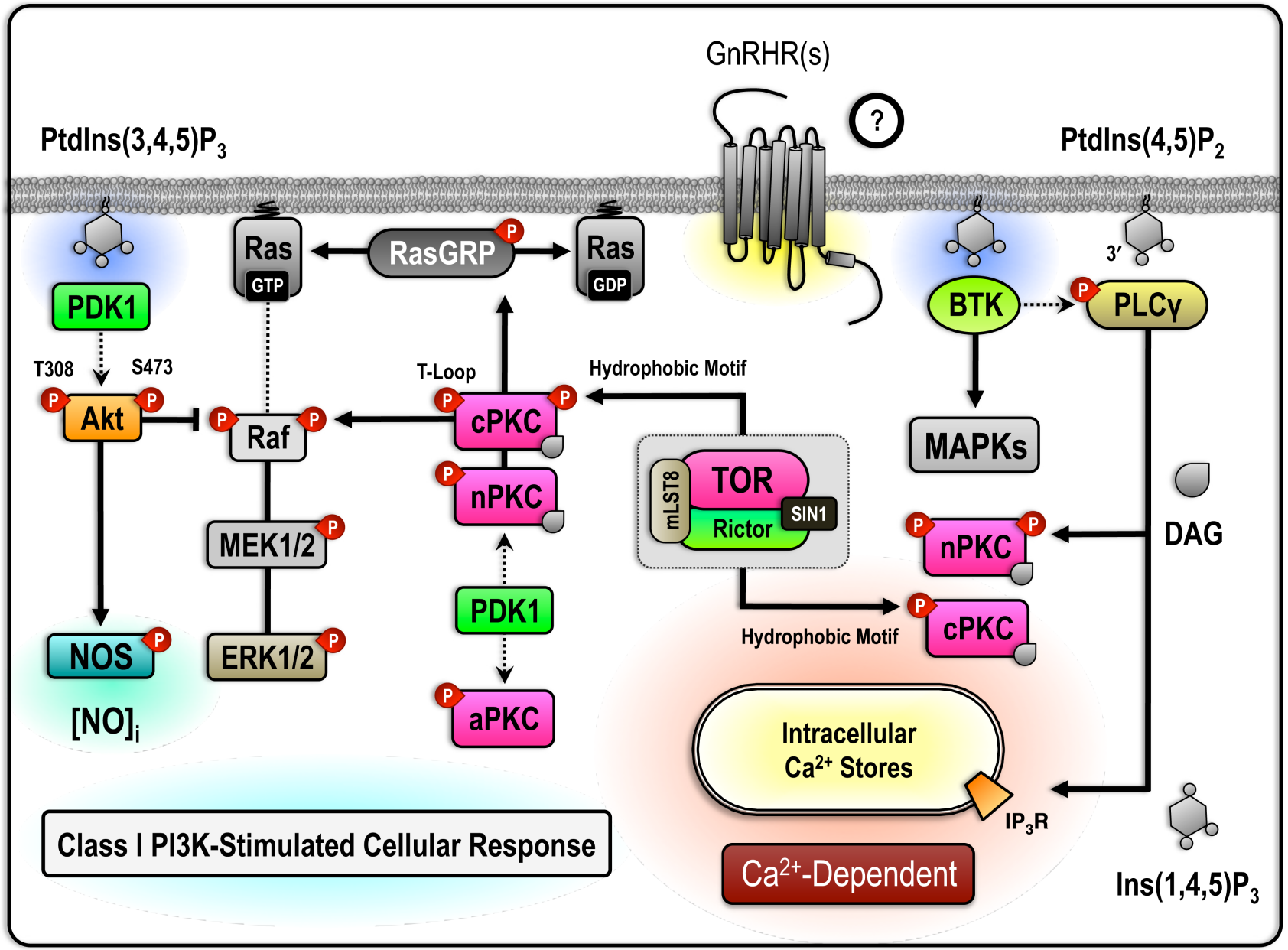
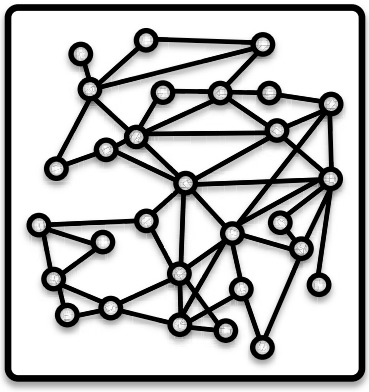

Class I PI3Ks
PtdIns(3,4,5)P3
PtdIns(4,5)P2
p85 p110α
p85 p110β
p85 p110δ
p101 p110γ
3′
PDK1
T308
S473
Akt
P
T-Loop
AGC
TORC2
mLST8
TOR
SIN1
Rictor
BTK
PLCγ
MAPKs
DAG
PRAS40
Rheb
GDP
Inactive
TSC1
TSC2
nPKC
cPKC
Intracellular
Ca2+ Stores
IP3R
Ins(1,4,5)P3
Ca2+-Dependent
mLST8
TOR
Raptor
Rheb
GTP
Active
(+++)
TORC1

Figure 1.12 Integration of canonical class I PI3K signalling with transduction effectors known to be involved in GnRH actions in goldfish gonadotropes and somatotropes. In goldfish (*Carassius auratus*), gonadotropin-releasing hormone (GnRH)-stimulated LH and GH release responses involve the activation of phospholipase C (PLC), protein kinase C (PKC), and Raf-MEK-ERK cascades as well as nitric oxide (NO)- and Ca^{2+}-dependent signalling mechanisms. In the majority of excitable cell models tested, production of the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) by activated class I PI3Ks results in the recruitment of the canonical effectors phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK). PDK1 directly phosphorylates the T-loop activation segment of conventional (cPKC), novel (nPKC), and atypical (aPKC) isozymes of PKC. PKC activation is known to activate the Raf-MEK-ERK cascade through either direct phosphorylation of Raf, or indirectly through the activation of the Ras superfamily guanine nucleotide exchange factor Ras guanyl nucleotide-releasing protein (RasGRP). Alternatively, phosphorylation and activation of Akt by the coordinate activity of PDK1 and the target of rapamycin (TOR) complex 2 (TORC2; which consists of TOR kinase bound to the core components rapamycin-insensitive companion of TOR (RICTOR), SAPK-interacting protein 1 (SIN1), and mammalian lethal with SEC thirteen 8 (mLST8)) directly inhibits the activation of Raf-MEK-ERK. Akt activation also phosphorylates and activates NO synthase (NOS) to stimulate the intracellular production of NO. Alternatively, recruitment of BTK primarily functions to increase intracellular Ca^{2+} concentrations through the phosphorylation and activation of phospholipase Cγ (PLCγ) and subsequent mobilization of Ca^{2+} from intracellular stores with specific Ins(1,4,5)P_3 receptors (IP_3Rs). In addition, PLCγ activity also produces diacylglycerol (DAG), which binds to and activates several families of effector proteins including cPKC and novel PKC (nPKC) isozymes. TORC2 phosphorylation is also thought to contribute to the maximal activation of cPKCs. BTK activity also regulates the signalling activity of mitogen-activated protein kinases (MAPKs), including Raf-MEK-ERK. Overall, it is obvious that class I PI3K signalling has the potential to interact with known transduction elements that are activated downstream of GnRHRs in gonadotropes and somatotropes.


GnRHR(s)
?
PtdIns(3,4,5)P3
PtdIns(4,5)P2
PDK1
Ras
GTP
RasGRP
P
Ras
GDP
BTK
3′
PLCγ
T308
S473
T-Loop
Hydrophobic Motif
Akt
Raf
cPKC
nPKC
MAPKs
mLST8
TOR
Rictor
SIN1
DAG
MEK1/2
nPKC
PDK1
Hydrophobic Motif
cPKC
NOS
ERK1/2
[NO]i
aPKC
Intracellular
Ca2+ Stores
IP3R
Class I PI3K-Stimulated Cellular Response
Ca2+-Dependent
Ins(1,4,5)P3

Chapter Two:

Materials & Methods



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

2.1 Animals

Animal maintenance and handling protocols adhered to the Canadian Council for Animal Care guidelines and were approved by the Biosciences Animal Care and Use Committee of the Research Ethics Office at the University of Alberta. Common goldfish (*Carassius auratus*; post-pubertal, 10-13 cm body length, 25-30 g body weight) were purchased from Aquatic Imports (Calgary, AB, Canada) and maintained at 18°C in flow-through aquaria (1800L capacity; de-chlorinated and charcoal filtered municipal water). Within each aquarium, male and female goldfish were held together. Goldfish were cared for and fed to satiation with a commercial fish food during a scheduled morning feeding period by staffs of the Faculty of Science Animal Support Services. All aquaria were housed under a simulated Edmonton, AB, Canada photoperiod (coordinates 53°32'N and 113°30'W) controlled by graded lighting and an astronomical clock set to the local sunrise and sunset. Goldfish were acclimated to the above conditions for a minimum of seven days prior to experimentation and were used within one month of purchase. Experiments were performed using mixed pituitary cell populations isolated from both male and female goldfish.

In temperate climates, goldfish are seasonal spawners with a tightly regulated reproduction cycle. Generally, spawning occurs between April and early June after which the gonads undergo regression through the summer and fall. Gonadal recrudescence commences in mid-fall (around October/November) and a sexually-matured (pre-spawning) stage is achieved by late March or April. Circulating LH levels are highest at the pre-spawning and spawning stages and lowest during late fall just prior to gonadal recrudescence (Peter, 1979, 1981). On the other hand, serum GH levels are high in the

early spring, coinciding with gonadal maturation, and remain elevated during the summer months when somatic growth is at its greatest. GH levels then drop to low levels over the fall and early winter when body growth slows before increasing again late in the winter months as the fish approach mid- to late-recrudescence (Marchant and Peter, 1986). During this period, GH plays an important role in potentiating the gonadal steroidogenic response to LH (Van der Kraak et al., 1990). However, despite these complex seasonal changes, GnRH stimulation of LH and GH release can be demonstrated at all times of the seasonal reproductive cycle; although, the magnitude of the response may vary.

In order to minimize any variation due to seasonal reproductive changes, replicate experiments in most hormone release studies were performed within a short time period (8-14 days). Different experiments were, however, performed throughout the entire reproductive cycle and testing of the involvement of a signalling pathway was carried out in more than one seasonal reproductive stage. In this case, the results from replicate experiments covering more than one gonadal marurational stage are generally consistent and were pooled for data presentation and analysis. In all instances, to facilitate future comparisons, the time of year and gonadal stage of the goldfish when individual experiments were performed are provided in the figure legends.

2.2 Preparation of dispersed pituitary cell cultures

Male and female goldfish were sacrificed by cervical transection following deep anesthesia with 0.05% tricane methanesulfonate (AquaLife, Syndel Laboratories, Vancouver, BC, Canada). Pituitaries were excised and collected in dispersion medium (Medium M199 with Hank's salts (Gibco, Life Technologies, Grand Island, NY, USA)

supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/l streptomycin, 100,000 units/l penicillin and 0.3% bovine serum albumin (BSA); pH adjusted to 7.2 with NaOH and sterilized by filtration through 0.22 μm Sartorius filter units). For preparing dispersed cells, pituitary fragments were processed using an established trypsin/DNAse dispersion protocol (Chang et al., 1990). Prior to plating, cell yield and viability were determined using trypan blue exclusion. Viability was routinely >98%. Dispersed cells were re-suspended and cultured overnight in plating medium (Medium M199 with Earles' salts (Gibco, Life Technologies) containing 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/l streptomycin, 100,000 units/l penicillin and 1% horse serum; pH adjusted to 7.2 with NaOH and sterilized by filtration through 0.22 μm Sartorius filter units) at 28°C, under 5% CO_2 and saturated humidity. Dispersed pituitary cell cultures prepared in this manner lack residual hypothalamic neuronal terminals that have been shown to be present in pituitary fragments and thus allow for studies on the direct actions of neuroendocrine effectors on pituitary cell functions (Chang et al., 1990). However, where indicated, excised pituitary fragments were also prepared (Yu et al., 1991) and used for static incubation and immunoblotting studies.

2.3 Mammalian cell culture

RBL-2H3 cells (rat basophilic leukemia cell line; obtained from American Type Culture Collection, Manassas, VA, USA) were grown at 37°C and 5% CO_2 in 1X Eagles' Minimum Essential Medium (MEM; Gibco, Life Technologies) supplemented with 2 mM L-glutamine, 100,000 Units/l penicillin, 100 mg/l streptomycin, and 10% heat-inactivated fetal bovine serum (characterized; Hyclone, Logan, UT, USA). Prior to use,

culture media was filter sterilized using 0.22 μm filter units (Corning Incorporated, Corning, NY, USA).

2.4 Drugs and reagents

2.4.1 GnRH isoforms

Synthetic GnRH2 (cGnRH-II; [His^5, Trp^7, Tyr^8]GnRH) and GnRH3 (sGnRH; [Trp^7, Leu^8]GnRH) were purchased from Bachem (San Carlos, CA, USA) and stock solutions were prepared in distilled, deionized water. Aliquots of stock solutions were kept frozen at –20°C until used. For all experiments, equimolar and maximally stimulatory concentrations of GnRH2 and GnRH3 (100 nM; Chang et al., 1990) were used. Final concentrations used for experiments were achieved by diluting stock solutions in testing medium (Medium 199 with Hank's salts (Gibco, Life Technologies) containing 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/l streptomycin, 100,000 units/l penicillin and 0.1% BSA; pH adjusted to 7.2 with NaOH and sterilized filtered through 0.22 μm Sartorius filter units).

2.4.2 Small molecule inhibitors and activators

In general, aliquots of each pharmacological modulator were prepared in the appropriate solvent and stored at –20°C. Final drug concentrations used for experiments were achieved by diluting stock solutions (typically 1000 fold) in testing medium. When required, the concentrations of the organic solvents dimethyl sulfoxide (DMSO) or ethanol were always less than 0.1% and do not effect basal LH or GH release (Van Goor et al., 1997; Wong et al., 1992).

Pan-PI3K inhibitors wortmannin ((1*S*,6b*R*,9a*S*,11*R*,11b*R*) 11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3*H*-furo[4,3,2-*de*]indeno[4,5,-*h*]-2-*h*]-2-benzopyran-3,6,9-trione) and LY294002 (2-(4-morpholinyl)-8-phenyl-4H-l-benzopyran-4) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tocris Biosciences (Bristol, UK). BYL719 (p110α-selective inhibitor; (2S)-1-N-[4-methyl-5-[2-(1,1,1-trifluoro-2-methylpropan-2-yl)pyridin-4-yl]-1,3-thiazol-2-yl]pyrrolidine-1,2-dicarboxamide) and rapamycin (allosteric TORC1 inhibitor; (3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone) were purchased from Selleck Chemicals (Houston, TX, USA). TGX221 (p110β-selective inhibitor; (±)-7-methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one), IC87114 (p110δ-selective inhibitor; 2-((6-amino-9H-purin-9-yl)methyl)-5-methyl-3-o-tolylquinazolin-4(3H)-one), and Akt$_i$VIII (Akt inhibitor; 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one) were purchased from Calbiochem (Billerica, MA, USA). CZC24832 (p110γ-selective inhibitor; 5-(2-amino-8-fluoro[1,2,4]triazolo[1,5-a]pyridin-6-yl)-N-(1,1-dimethylethyl)-3-pyridinesulfonamide), NSC8668 (allosteric Gβγ inhibitor; Gallein; 3',4',5',6'-tetrahydroxyspiro[isobenzofuran-1(3*H*),9'-(9*H*)xanthen]-3-one), and GSK2334470 (PDK1 inhibitor; (3*S*,6*R*)-1-[6-(3-amino-1*H*-indazol-6-yl)-2-(methylamino)-4-pyrimidinyl]-*N*-cyclohexyl-6-methyl-3-piperidinecarboxamide) were

purchased from Tocris Biosciences. PIT-1 (PtdIns(3,4,5)P_3-sensitive PH domain inhibitor; N-[[(3- chloro- 2- hydroxy- 5- nitrophenyl)amino]thioxomethyl]- benzamide), GDC0941 (pan-class I PI3K inhibitor; 2- (1H- indazol- 4- yl)- 6- [[4- (methylsulfonyl)- 1- piperazinyl]methyl]- 4- (4- morpholinyl)- thieno[3, 2- d]pyrimidine), and INK128 (TOR kinase inhibitor; 3- (2- amino- 5- benzoxazolyl)- 1- (1- methylethyl)- 1H- pyrazolo[3, 4- d]pyrimidin- 4- amine) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). CGI1746 (BTK inhibitor; 4-tert-butyl-N-(2-methyl-3-(4-methyl-6-(4-(morpholine-4-carbonyl)phenylamino)-5-oxo-4,5-dihydropyrazin-2-yl)phenyl)benzamide) was purchashed from Axon Med Chem (Reston, VA, USA). U0126 (MEK1/2 inhibitor; 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) and PD098059 (MEK1/2 inhibitor; 2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one) were purchased from EMD Chemicals (Gibbstown, NJ, USA) and dissolved in DMSO.

The Ca^{2+} ionophore ionomycin, as well as the PKC activators TPA (4β-tetradecanoyl-phorbol-13-acetate) and DiC8 (1,2-dioctanoyl-sn-glycerol), were purchased from EMD Chemicals (Gibbstown, NJ, USA). Working concentrations of these pharmacological agents that have been shown to be maximally stimulatory in the goldfish pituitary cell system were used (ionomycin, 10 μM; Chang et al., 1990; TPA, 100 nM, and DiC8, 100 μM; Chang et al., 1991) .

2.4.3 Antibodies and antigenic peptides

Polyclonal rabbit anti-human p110α (sc-7174), p110β (sc-602), and p110δ (sc-7176) IgG antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX,

USA). Monoclonal rabbit anti-human Akt (pan; C67E7), Akt1 (C73H10), Akt2 (D6G4), Akt3 (62A8), phospho-Akt (Ser473; D9E XP™), phospho-Akt (Thr308; C31E5E), PDK1 (D37A7), phospho-PDK1 (Ser241; C49H2), ERK1/2 (p44/p42 MAPK; 137F5), phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™), p110α (C73F8), p110γ (D55D5), p101 (D32A5), p55 (D2B3), and p85 (pan; 19H8) IgG antibodies, as well as polyclonal rabbit anti-human phospho-p85 (Tyr458) IgG, were all purchased from Cell Signaling Technologies (Danvers, MA, USA). Recombinant full-length human PI3K p85α peptide (N terminal His tag) was purchased from Abcam Inc., Cambridge, MA, USA. Antibody specificity was examined by pre-incubation of the primary antibody with the corresponding antigenic peptide using Akt (pan; C67E7), phospho-Akt (Ser473), ERK1/2, and phospho-ERK1/2 (Thr202/Tyr204) blocking peptides (Cell Signaling Technologies).

2.5 Cell column perifusion for analysis of acute hormone release responses

Perfusion studies not only allow for quantification of the acute hormone release responses but also reveal the kinetics of the hormone release profile. This allows for the comparison of basal- and agonist-induced effects on hormone release across treatments, as well as in duplicate columns. In addition, with the continuous removal of perifusate from the column, potential autocrine and/or paracrine effects of pituitary secretions can be minimized. Column perifusion experiments were carried out using an Accusyst system as previously described (Chang et al., 1990). Dispersed pituitary cells were cultured overnight with pre-swollen Cytodex beads (Cytodex I; Sigma-Aldrich, St. Louis, MO, USA) in plating medium at 28°C, under 5% CO_2 and saturated humidity. Cells attached

to Cytodex beads were loaded into temperature controlled columns (1.5 x 10^6 cells/column, 500 µl chamber volume, 18°C) and perifused with testing medium at a flow rate of 15 ml/hr (time to clear total dead-space is approximately 5-6 min) for a minimum of 4 hr prior to the onset of experiments in order to stabilize basal hormone secretion. In general, individual perifusion experiments consisted of stimulator alone, inhibitor alone, and combinatorial treatments repeated in duplicate columns. Perifusion experiments always began with the collection of five, 5 min fractions of basal hormone secretion to determine the average pre-treatment hormone release levels (application of testing medium without pharmacological agents). For combinatorial treatments, a 20 min application of inhibitor alone, followed by a 5 min pulse of stimulator in the presence of the inhibitor and a further 40 min treatment with inhibitor were applied. Control treatments with stimulator alone or inhibitor alone followed a timeline parallel to that established for the combination treatment. Fractions of perifusate were stored at –20°C until being assayed for LH and GH content by validated radioimmunoassays (RIAs; Cook et al., 1983; Marchant et al., 1987; Peter et al., 1984). When required, the concentrations of the organic solvents DMSO or ethanol were always less than 0.1% and do not effect basal LH or GH release (Van Goor et al., 1997; Wong et al., 1992).

For perifusion studies, LH and GH release from individual columns were expressed as a percentage of the corresponding pretreatment value (% pretreatment), which is calculated as the average hormone release (ng/ml) of the first five fractions of perifusate collected at the beginning of the experimental period prior to pharmacological manipulations. This conversion allowed for pooling of perifusion data from different columns without distorting the shape of the hormone release kinetic. The magnitude of

hormone release responses to treatments with agonists were quantified from individual columns by calculating the net response (sum of the net change in hormone release in each of the collected fractions over the quantification period) of the LH or GH levels as the area under the response curve (baseline value subtracted; with baseline defined as the average % pretreatment value of the three fractions prior to the administration of agonist treatment). Termination of hormone release response to agonist application in a column was deemed to have occurred when hormone release values returned to within one standard error of the mean (SEM) from the average baseline value. The net response for treatment columns exposed to small molecule inhibitors alone or combination treatments were quantified over the equivalent time frame as that used for agonist alone treatments. Additionally, where indicated, the effect of selective inhibitors on basal release was evaluated by comparing the average of the % pretreatment hormone release values obtained over the duration of the inhibitor alone treatment (normally 30 to 95 min of experiment; pooled across experiments) to the corresponding average values from 0 to 20 min of the treatment protocol in the same columns. All treatments were performed in duplicate columns in each experiment and all perifusion experiments were repeated a minimum of three times using different cell preparations.

2.6 Analysis of hormone release and cellular hormone content in static pituitary cell cultures

A static incubation protocol was used to examine the signal transduction mechanisms involved in controlling basal and GnRH-stimulated LH and GH release, as well as cellular hormone content, over a longer time period. Dispersed pituitary cells

were plated in centrifuge-safe tissue culture tubes (Techno Plastic Products (TPP) T-10; Midwest Scientific; Valley Park, MO, USA) and cultured overnight. On the day of experiments, cells were harvested by centrifugation at 200 x g for 10 min at 5°C, re-suspended in testing medium, and then allowed to recover for 30 min prior to treatment. Where applicable, this 30 min period was used to apply a pretreatment with selective small molecule inhibitors. Cell cultures were left either untreated or challenged with endogenous GnRHs, pharmacological agents, or combination treatments for 2, 6, 12, or 24 hr at 28°C, under 5% CO_2 and saturated humidity. After the appropriate incubation period, cells were harvested by centrifugation and the testing medium was collected for analysis of hormone release. Pelleted cells were subsequently washed twice with ice-cold phosphate-buffered saline and lysed by sonication in cell lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% NP-40 (nonylphenoxypolyethoxylethanol; Thermo Scientific, Waltham, MA, USA)) for evaluation of cellular LH and GH contents. Pituitary cell culture supernatants and total cell content lysates were stored at –20°C prior to RIA. All experiments were performed a minimum of three times with different cell preparations. Prolonged 24 hr exposure to GnRHs does not significantly reduce the cell number or viability of the goldfish pituitary cell in static cultures as assessed by trypan blue exclusion (Johnson et al., 2003).

Data from static incubation experiments were presented as total μg of LH or GH within the cell culture supernatant (released) or cellular protein extracts (cell content), or as a percentage of the time-matched untreated control. The total hormonal protein present (total) was calculated as the sum of cell content and released for each individual treatment tubes and used as an index of hormonal protein availability and production

during the incubation time period. Measurements of cell hormone release, cell hormone contents, and total hormone within cell cultures were analyzed independently.

2.7 Radioimmunoassays

Competative RIA was performed using a double antibody method. Throughout the protocol, all dilutions were made with 0.08 M sodium barbital buffer, pH 8.6, containing 0.5% BSA. Values obtained were corrected for non-specific binding of the labelled hormone (determined in assay tubes where primary antibody was omitted). Serial dilutions of perifusate or medium collected from static incubations resulted in displacement curves that parallel those generated for the purified LH or GH standard curves, indicating that these RIA are suitable for measuring hormone levels from these in vitro experiments. RIA results were analyzed using a weighted regression of a log-logit plot as described previously (Cook et al., 1983). Samples and standards were always assayed in duplicate and radioactivity measurements were made using a high-throughput gamma counter (Packard Cobra II 10-channel Auto-Gamma Model D5005).

2.7.1 Measurement of maturational LH

Radioactive LH tracer was prepared from 5 μg of purified carp pituitary LH (GTH-II (Van der Kraak et al., 1992), gift from the late Dr. R. E. Peter, University of Alberta) labeled with ^{125}I using a modified chloramine-T method (Peter et al., 1984) and purified by chromatography with a 1.1 × 20 cm glass column of Sephadex G-50 fine beads (Sigma-Aldrich). The iodination mixture was eluted with 0.08 M barbital buffer and the saved ^{125}I-LH fractions (stored in 0.08 M barbital buffer containing 0.5% BSA)

are generally free of unreacted iodide and radio-damaged proteins. Radio-iodinated LH tracer was prepared at between 15,000 to 17,000 cpm per 200 µl. Perifusion or static incubation samples were diluted as required (final volume of 50 µl) and pipetted with 200 µl of primary antibody (rabbit anti carp cGTH-378; produced at the University of Alberta by the late Dr. R.E. Peter) at a dilution of 1:220,000 (in 0.08 M barbital buffer containing 0.5% BSA and 1% normal rabbit serum; anti-serum preparations were titred to establish dilutions which bound approximately 25-30% of the added hormone). Importantly, the anti-LH primary antibodies used detect only single protein bands in Western blot analysis with carp pituitary cells with molecular size appropriate for mature holomeric LH protein (Yang et al., 2010). These observations suggest that our radioimmunoassay does not measure immature LH forms or individual LHβ and GTHα subunits. There is also minimal cross-reactivity (approximately <5%) of the anti-LH primary antibody with purified carp GH or goldfish prolactin (PRL, Peter et al., 1984) or carp GTH-I (FSH-equivalent; Van der Kraak et al., 1992). No reactivity was detected in this system when serum samples from hypophysectomized fish were tested (Peter et al., 1984). Standard curves were generated using purified carp pituitary LH standards (range from 0.16-100 ng/ml) diluted from a 200 ng/ml stock and added in 50 µl aliquots per assay tube. Pipetted samples and standards mixed with primary anti-LH antibody were incubated with 200 µl ^{125}I-LH tracer for 48 hr at 5°C. Precipitation of antibody-bound hormone was induced by addition of 200 µl of a 1:40 initial dilution of goat anti-rabbit IgG secondary antibody (IgG-GARGG, lot# 2801; IgG Corp, Nashville, TN, USA) and incubation overnight at 5°C. RIA tubes were then centrifuged for 25 min at 1,000 x g and 5°C to

pellet the bound fraction. Supernatants were decanted to remove unbound ^{125}I-LH and radioactivity in the pellet was determined.

2.7.2 Measurement of GH

Radioactive GH tracer was prepared from 5 μg of recombinant carp GH protein (GenWay Biotech, Inc., San Diego, CA) labeled with ^{125}I using a modified lactoperoxidase method (Cook et al., 1984). Unreacted iodide and radio-damaged proteins were separated from intact ^{125}I-GH by chromatography with a 1.1 × 20 cm glass column of Sephadex G-50 fine beads (Sigma-Aldrich). The iodination mixture was eluted with 0.08 M barbital buffer containing 0.5% BSA. Radio-iodinated ^{125}I-GH tracer was prepared at approximately 13,000 to 15,000 cpm per 100 μl. Perifusion or static incubation samples were diluted as required (final volume of 25 μl) and pipetted with 100 μl of primary antibody (rabbit anti-carp kcGH R#1; produced at the University of Alberta by the late Dr. R. E. Peter) at a dilution of 1:52,000 (in barbital buffer containing 0.5% BSA and 2% normal rabbit serum). The anti-GH primary antibody used detected a single immunoreactive band at an appropriate molecular size for mature GH protein (Zhou et al., 2004). Purified carp LH and PRL do not show any significant cross-reactivity (<0.1%) with the anti-GH primary antibody, nor was reactivity detected when serum from hypophysectomized goldfish were assayed (Marchant et al., 1989). GH standard curves (range from 0.32-200 ng/ml) were prepared from a 200 ng/ml stock of purified carp pituitary GH standards (kcGH #3; gift from Dr. H. Kawauachi, Kitasato University, Sanriku, Japan) and added in 25 μl aliquots per assay tube. Pipetted samples and standards mixed with primary anti-GH antibody were incubated for 24 hr at 5°C prior to

the addition of 100 µl ^{125}I-GH tracer. RIA tubes were then incubated for an additional 24 hr at 5°C and precipitation of the bound fraction was induced by the addition of 200 µl of a 1:40 initial dilution of goat anti-rabbit IgG secondary antibody (IgG-GARGG, lot# 2801; IgG Corp, Nashville, TN, USA) and further incubation overnight at 5°C. RIA tubes were then centrifuged for 25 min at 1,000 x g and 5°C to pellet the bound fraction. Supernatants were decanted to remove unbound ^{125}I-GH and radioactivity in the pellet was determined.

2.8 Morphological identification of pituitary cell types

In all vertebrates, the pituitary gland is made up of several different hormone-secreting cell types. Goldfish pituitary gonadotropes, somatotropes, and lactotropes can be reliably distinguished using distinct morphological features under Nomarski differential interference contrast (DIC) optics, as previously described (Van Goor et al., 1994). This established identification methodology has been subject to repeated double-blind verification and has an accuracy of >98% (as determined by immunostaining with LH, GH, or PRL anti-serum). Importantly, identification of pituitary cell types is effective in primary pituitary cell cultures prepared from male and female goldfish during all stages of the seasonal reproductive cycle (Van Goor et al., 1994).

2.8.1 Gonadotropes

Gonadotropes are ovoid in shape and are consistently the largest (12-19 µm in diameter; Van Goor et al., 1994) of the three identifiable cell types in culture. The defining feature of gonadotropes is that they possess a small and peripherally-situated

reniform nucleus that occupies less than a third of the cross-sectional area of the cell. A single, large, irregularly-shaped nucleolus can usually be detected. Additionally, the cytoplasm of live gonadotropes possesses numerous small granules and large spherical globules with only a few large spherical or irregularly-shaped membrane inclusions (Van Goor et al., 1994).

2.8.2 Somatotropes

Somatotropes are spherical and are, in general, smaller than gonadotropes (9-11 μm in diameter; Van Goor et al., 1994). The most distinctive feature of somatotropes is a large ovoid nucleus containing a centrally located nucleolus. The nucleus occupies approximately half of the cross-sectional area of the cell. Unlike gonadotropes, somatotropes tend to have several small cytoplasmic extensions and large globules were never observed in the cytoplasm of somatotropes (Van Goor et al., 1994).

2.9 Measurements of $[Ca^{2+}]_i$ in morphologically-identified pituitary cells

Fluctuations in cytosolic free Ca^{2+} concentrations were measured using the ratiometric Fura2-AM imaging protocol previously described (Johnson et al., 1999). Dispersed pituitary cells were cultured overnight on poly-$_L$-lysine-coated (0.01 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) coverslips (1 oz., 22 mm x 22 mm, and 0.13-0.17 mm thickness; 0.4 million cells/coverslip) in plating medium at 28°C, under 5% CO_2 and saturated humidity. The Ca^{2+}-sensitive fluorescent dye Fura2-AM (10 mM; Invitrogen, Carlsbad, CA, USA) was prepared in DMSO with 20% Pluronic F-127 and sonicated for 12 min before cell loading. Cells were then loaded with Fura2-AM (final loading

concentration 10 μM) by incubation for 45 min at 28°C under 5% CO_2; this concentration of Fura2-AM has been demonstrated to produce stable and uniform loading of the dye within goldfish gonadotropes (Johnson et al., 1999) and somatotropes (Johnson and Chang, 2000). Once the incubation period had elapsed, cells were washed three times with clear testing medium (Medium 199 with Hank's salts (Gibco, Life Technologies) without phenol red (for imaging purposes) but containing 26 mM $NaHCO_3$, 25 mM HEPES, streptomycin 100 mg/l, 100,000 units/l penicillin and 0.1% BSA; pH 7.2, sterile filtered through a 0.22 μm Sartorius filter unit) and mounted in a closed bath imaging chamber (260 μl chamber volume, series 20 imaging chamber; Warner Instruments, Hayden, CT, USA). Treatment reagents were applied to cells using a gravity driven perifusion system at a flow rate of 1 ml/min at 18-20°C (time to replace bath solution is approximately 15-18 sec). Fura2 was excited with a Hg-Xe arc lamp (Hammamatsu, Japan) at 340 nm and 380 nm using a computer controlled filter wheel (Empix Imaging, Missassauga, ON, Canada). Emission fluorescence at 510 nm was recorded through an oil-immersion objective (1.3 numerical aperture) mounted on a Zeiss Axiovert 135 inverted microscope (Carl Zeiss Inc., Don Mills, ON, Canada) with a Paultek Imaging ICCD camera (Grass Valley, CA, USA) linked to a computer. Exposure time and camera gain were adjusted to optimize the signal-to-noise ratio, while neutral density filters (Omega Optical, Brattleboro, VT, USA) were used to reduce photobleaching. Pairs of images at different excitation wavelengths were captured at 15 sec intervals. F340 and F380 ratios were converted to $[Ca^{2+}]_i$ using constants determined by an *in vitro* Ca^{2+} calibration kit (Molecular Probes, Eugene, OR, USA) according to the formula described in Tsien (1989).

Ca^{2+} traces from individual gonadotropes and somatotropes were normalized as a percentage of basal $[Ca^{2+}]_i$ (defined as the average of the first five recorded values during perifusion with media in the absence of pharmacological manipulations; % pretreatment). $[Ca^{2+}]_i$ profiles from individual cells were pooled for presentation. The net Ca^{2+} response was calculated from individual traces as the area under the normalized $[Ca^{2+}]_i$ response curve with baseline subtracted during pharmacological challenge (baseline defined as the average % pretreatment value of the four fractions immediately prior to drug administration). The termination of a $[Ca^{2+}]_i$ response was defined as when the response return to within 10% of the baseline value (Yu et al., 2008). The response durations were calculated as the amount of time over the response period (Yu et al., 2008). The maximal amplitude of the Ca^{2+} responses was the maximal value (% pretreatment) recorded during the response period (Yu et al.,, 2008). All treatments were replicated in a minimum of 8 individual cells from at least three independent cell preparations. In all instances, final concentrations of DMSO were less than 0.1% and had no effect on basal $[Ca^{2+}]_i$ in identified goldfish somatotropes or gonadotropes (Johnson et al., 1999; Johnson and Chang, 2000). Furthermore, a depolarizing treatment of KCl (30 mM) was applied following a 10 min washout period (imaging chamber perifused with clear testing media alone) at the end of each experimental sequence as a positive control to ensure that the cells remained healthy and responsive following drug treatments. Depolarizing clear testing media for imaging was made by equimolar substitution of NaCl with KCl (Johnson et al., 1999) and was able to routinely cause a robust increase in $[Ca^{2+}]_i$ in >99% of cells within each mixed culture. All cells included as experimental replicates for data analysis responded to this KCl positive-control pulse (data not shown).

2.10 Western blot analysis

2.10.1 Protein extraction and quantification

Protein extracts from cell cultures and pituitary fragments were harvested in cell lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% NP-40 with PhosSTOP phosphatase inhibitor cocktail and complete EDTA-free protease inhibitor cocktail tablets; Roche, Basel, Switzerland) and sonicated on ice. Following sonication, cell lysates were agitated at 4°C for a minimum of 20 min to liberate membrane-associated proteins before being centrifuged at 16,000 x g for 20 min. Supernatants were stored at –80°C until used. Where indicated, the protein concentration within each lysate was quantified using the RC DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) modified from the Lowry protocol (Lowry et al., 1951). The RC DC assay is a colorimetric assay using BSA as a protein standard and can accurately measure protein concentrations in the presence of both reducing agents and detergents.

2.10.2 Gel electrophoresis and immunoblotting

Samples in lysis buffer were diluted in equal volumes of 2x sodium dodecyl sulfate (SDS) reducing buffer (Laemmili sample buffer with 2β-mercaptoethanol; Bio-Rad Laboratories) or 2x Laemmili sample buffer (non-reducing conditions) and boiled for 10 min at 95°C prior to use. Proteins were separated on 8-10% SDS-polyacrylamide gels and transferred to Trans-Blot Pure nitrocellulose membranes (0.45 μm pore size; Bio-Rad Laboratories). Pre-stained molecular weight standards were run on each gel in order to monitor the electrophoretic mobility of samples and to determine the relative size

of immunoreactive bands. Efficiency of the protein transfer was assessed using PonceauS staining (0.1% PonceauS in 5% acetic acid; Sigma-Aldrich, St. Louis, MO, USA). Membranes were washed twice (10 min each) with 1x Tris Buffered Saline (TBS) supplemented with 0.1% Tween-20 (TBST) and then blocked overnight at 4°C or for 60 min at room temperature in 1x TBST with 5% bovine serum albumin (BSA, Fraction V; Roche). Membranes were incubated overnight at 4°C with primary antibodies diluted in 1x TBST and 5% BSA. Positive controls for immunoblotting were performed using whole cell lysates from RBL-2H3 mast cells. In all instances, membranes were washed using two sequential applications of TBST followed by TBS (10 min each at room temperature), and incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in 1x TBST and 5% BSA; Bio-Rad Laboratories) for 120 min at room temperature prior to application of Pierce® ECL Western Blotting Substrates (Thermo Scientific) and exposure to Kodak BioMax XAR film (Rochester, NY, USA). Films were scanned and quantified by densitometry using a computer-based image processing and analysis program (ImageJ Version 1.49; National Institutes of Health, Bethesda, MD, USA). In general, immunoblotting experiments were replicated a minimum of three times using individual dispersed cell or pituitary fragment preparations. Where possible, detailed descriptions of the antigen sequence used for immunization and the experimental conditions tested for each antibody are provided.

2.10.3 Preabsorption using antigenic peptides

When possible, antibody specificity was examined by pre-incubation of the primary antibody with the competing antigenic peptide. Identical protein samples were

loaded on opposite halves of a single gel. Following transfer, membranes were split and blocked as described above. Primary antibodies were diluted (1x TBST and 5% BSA) and, where indicated, pre-incubated with the corresponding antigenic peptide for 60 min at room temperature. Split membranes were then placed within either the standard or pre-absorbed antibody solutions overnight at 4°C. Membranes were washed twice (10 min each) with 1x TBST, twice (10 min each) with 1x TBS, prior to incubation with secondary antibody (diluted in 1x TBST with 5% BSA; Bio-Rad Laboratories) for 2 hr at room temperature.

2.10.4 Phosphorylation-specific immunoblotting

Depending upon the target protein, phosphorylation of specific residues can be used as an index of enzymatic activity. The relative amount of phosphorylated and total protein in each sample was quantified using specific primary antibodies. Pituitary fragments or dispersed primary pituitary cell cultures were either left unstimulated or treated with pharmacological agents for between 5 and 120 min at 28°C, under 5% CO_2 and saturated humidity. Protein samples were prepared and immunoblots were performed as described above. In all instances, membranes were first blotted with phospho-specific primary antibodies (diluted in 1x TBST and 5% BSA). Following processing with secondary antibody and exposure to film as described above, membranes were stripped of the phospho-specific antibodies by incubating in mild stripping buffer (1x TBST with 15% glycine, 1% SDS, 0.01% Tween 20; pH adjusted to 2.2 with HCl; recipe from Abcam Inc., Cambridge, MA, USA) for 10 min at room temperature. Membranes were then washed with 1x TBS (2 x 10 min), 1x TBST (2 x 5 min), and re-blocked prior to

blotting with antibodies against total protein (diluted in 1x TBST and 5% BSA). Positive controls for phosphorylation studies were performed using lysates prepared from RBL-2H3 cells in the presence of hydrogen peroxide (10 mM) and sodium pervanadate (0.1 mM) for 15 min as previously described (data not always shown; Tsuboi et al., 2008). Films from total and phosphorylated protein experiments were quantified by densitometry as described above. Where possible, immunoreactive phosphorylated protein levels were normalized against the corresponding total immunoreactive protein levels and cellular responses were quantified and expressed as a percentage of the corresponding time-matched unstimulated controls (usually minimum replicate of 3 experiments using independent cell preparations).

2.11 Flow cytometric analysis of mixed pituitary cell cultures

Dispersed goldfish pituitary cells were cultured overnight in plating media in centrifuge-safe tissue culture tubes (TPP T-10). On the following day, cells were centrifuged (200 x g, 10 min) and washed with testing or clear testing media. Following treatment with neuroendocrine modulators and/or pharmacological agents, cells were centrifuged, re-suspended, and lightly fixed in 1% isotonic formaldehyde for 20 min on ice. Cell images were then examined using an ImageStream MKII imaging flow cytometer (Amnis, EMD Millipore). Images were analyzed using IDEAS Version 6.0 software. Initial gating was done with unstained cells to identify single cells (based on area and aspect ratio) and degree of focus (gradient RMS), as previously described (Rieger et al., 2010; Chang et al., 2014). Nuclear counter-staining was achieved using Hoechst 33342 (Pierce Biotechnology; excitation 361 nm, emission 486 nm). Using the

collected images, individual goldfish gonadotropes or somatotropes were identified based on their unique morphological characteristics (Van Goor et al., 1994) and their unique size and scatter properties. Cells within the somatotrope gate were confirmed to be positive for GH staining using the rabbit anti-carp kcGH R#1 primary antibody (1:3000; Cook et al., 1991; Chang et al., 2014). Other larger cells were confined to an alternative cytometic gate and were confirmed to stain positively for anti-LH (rabbit anti carp cGTH-378, 1:6000; Cook et al., 1991; Chang et al., 2014) and possessed morphological characteristics of identified gonadotropes (Van Goor et al., 1994). For intracellular staining, pituitary cells were lightly fixed as described above and then permeabilized with saponin buffer (1X PBS, 2% fetal bovine serum, 0.1% saponin) for 10 min on ice. Fixed cells were treated with primary antibodies for 30 min on ice followed by an additional 20 min incubation at room temperature. Primary antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to either fluorescein isothiocyanate (FITC; excitation 495 nm, emission 519 nm; 1:200) or R-phycoerythrin (PE; excitation 496 nm, emission 578 nm; 1:200; both from Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and analyzed using flow cytometry as described above. Alternatively, were indicated, fluorescently conjugated phospho-Akt (Ser473; D9E XP™; Alexa Fluor® 647 Conjugate; excitation 650 nm, emission 665 nm) or phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™; Alexa Fluor® 488 Conjugate; excitation 495 nm, emission 519 nm) primary antibodies from Cell Signaling Technologies were used.

2.12 Bioinformatics

Protein sequences were obtained and aligned using the integrated workflow at the Universal Protein Resource Knowledgebase (UniProtKB; http://www.uniprot.org) and

Clustal Omega Version 1.2.1 (http://www.clustal.org/omega). Searches used human sequences as the initial queries and identified homologs across species with a cut-off of the E-value at $\leq 1.0e^{-10}$. Representative species for homology modeling were selected on the basis of sequence completeness. Advanced protein sequence alignments for structural predictions used the pairwise comparison of hidden Markov models (HMM-HMM comparison; HHpred; http://toolkit.tuebingen.mpg.de/hhpred) and/or profile hidden Markov models (HMMER; http://hmmer.janelia.org). Three-dimensional homology models were obtained by submitting query sequences or sequence alignments to the Protein Homology/AnalogY Recognition Engine Version 2.0 (Phyre2; http://www.sbg.bio.ic.ac.uk/phyre2) web portal. The quality of each computational model generated was evaluated using both the ProQ2 model assessment server (http://bioinfo.ifm.liu.se/ProQ2) and MolProbity (http://molprobity.biochem.duke.edu) to determine the Ramachandran statistics. Figures of structural models were prepared using the PyMOL Molecular Graphics System (Version 1.7, Schrödinger, LLC.). Modular protein domains were identified and conserved structural features were integrated into homology models using the annotated National Center for Biotechnology Information Conserved Domain Database (NCBI CCD; http://www.ncbi.nlm.nih.gov/cdd/).

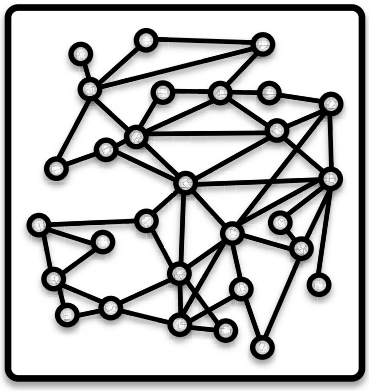
2.13 Statistics

All values are expressed as mean ± SEM. Results were compared using either Student's t-test or analysis of variance (ANOVA). Where ANOVA showed that differences exist between groups, further statistical analyses were done using the protected Fisher's least significant difference (LSD) or Tukey's honest significant

differences (HSD) multiple comparisons. Nonparametric analysis was done as needed using the Kruskal-Wallis test followed by pairwise comparison using the Mann-Whitney U test. Two-way ANOVA tests were performed to examine the interactions of time and drug treatment effects across the different static incubation time points. The level of significance was set at $p < 0.05$.

Chapter Three:

GnRH stimulation of acute hormone release involves PI3K-dependent intracellular signalling in both gonadotropes and somatotropes



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

3.1 Introduction

As reviewed in Chapter 1, in goldfish, GnRH2 and GnRH3 both stimulate the release of LH and GH through PKC- as well as CaMK-dependent signal transduction cascades (Chang et al., 2009, 2012). The actions of both GnRHs also involves activation of Ca^{2+}-dependent signalling pathways, and the increases in $[Ca^{2+}]_i$ are achieved via increased entry of Ca^{2+} from extracellular sources and Ca^{2+} mobilization from intracellular stores (Johnson and Chang, 2000; Chang et al., 2009, 2012). Multiple pharmacologically distinct intracellular Ca^{2+} stores exist and those implicated in GnRH2 and GnRH3 stimulation of LH and GH release are not identical (Chang et al., 2009, 2012). The mechanisms controlling GnRH isoform-selective signal transduction, including how differential GnRH-evoked Ca^{2+} signals are produced, are not fully understood and several canonical intracellular signalling cascades have yet to be thoroughly investigated in pituitary cell models. In particular, recent evidence indicates that the PI3K superfamily serve as central intracellular regulators of hormone actions in numerous endocrine and immune cell types (Balla, 2006; Hirsch et al., 2007; Okkenhaug et al., 2013). However, few studies have examined the role of PI3K-dependent signalling in the control of pituitary hormone release, in general, and it remains extremely unclear whether PI3Ks are transduction targets of GnRHRs.

In this Chapter, I utilized the broad-spectrum PI3K inhibitors wortmannin and LY294002 to investigate the involvement of PI3K-dependent signalling in GnRH-stimulated LH and GH release from mixed cultures of dispersed goldfish pituitary cells in cell column perifusion. I also measured basal and GnRH-evoked changes in the $[Ca^{2+}]_i$ within morphologically identified gonadotropes and somatotropes preloaded with the

ratiometric Ca^{2+}-sensitive dye Fura2-AM. Lastly, to better understand the intracellular signalling network architecture responsible for GnRH actions, the relationship between PI3K- and PKC-dependent signalling in acute LH and GH release, and whether PKC activation is upstream of PI3K in particular, was investigated. Results provide the first insight into the role of PI3K-dependent signalling on pituitary hormone release in any system, and reveals novel cell type-selective interactions with known intracellular mediators of GnRH action.

In general, the involvement of PI3K-dependent signalling in GnRH-stimulated LH and GH release from dispersed goldfish pituitary cells was examined in a column perifusion system using the pan-specific PI3K inhibitors wortmannin (100 nM) and LY294002 (10 μM). The reported specificity and experimental concentrations used for both wortmannin and LY294002 are provided in Table 3.1. Briefly, wortmannin irreversibly inhibits all three classes of PI3K with an IC_{50} in the nanomolar range (4.2 nM; Walker et al., 2000) by covalently associating with the highly conserved catalytic Lys802 residue (p110α numbering) of PI3K catalytic subunits (Wymann et al., 1996). At nanomolar concentrations, wortmannin does not significantly inhibit the catalytic activity of PKC, CaMKII, or protein tyrosine kinase (Powis et al., 1994; Nakanishi et al., 1992). However, wortmannin inhibition of polo-like kinase (PLK) has been demonstrated at nanomolar doses (IC_{50} = 24 nM; Liu et al., 2005) and inhibition of type II phosphatidylinositol 4-kinase (PI4K) isoforms and MLCK (Nakanishi et al., 1992) can occur, but only at elevated micromolar concentrations (i.e., at least 10 fold higher than the dose used in this study; Vanhaesebroeck et al., 2001). In order to validate our results, we utilized an additional selective pan-PI3K inhibitor LY294002 (IC_{50} = 1.4 μM; Walker

et al., 2000). LY294002 inhibits PI3K-superfamily kinases with an IC_{50} in the micomolar range, but does demonstrate some selectivity for class I PI3K isoforms over class II and III PI3Ks (Knight et al., 2004). Additionally, even at concentrations as high as 50 μM, LY294002 does not inhibit other major protein and lipid kinases, including: PKC, PKA, MAPKs, c-Src, and PI4K (Vlahos et al., 1994). In general, pretreatment with low nanomolar doses of wortmannin (≤100 nM) or mid-micromolar concentrations of LY294002 (≤20 μM), the dosages used throughout this Chapter, are reliably selective for PI3Ks in cell culture systems (Knight et al., 2004; Manning and Cantley, 2007).

3.2 Results

3.2.1 PI3K superfamily inhibitors abolish GnRH-stimulated LH and GH release from mixed populations of dispersed goldfish pituitary cells

Treatments with maximally effective concentrations of GnRH2 (100 nM; Chang et al., 1990) or GnRH3 (100 nM; Chang et al., 1990) significantly increased LH (Figures 3.1 and 3.2) and GH (Figures 3.3 and 3.4) secretion. Both the LH (Figures 3.1 and 3.2) and GH (Figures 3.3 and 3.4) release responses to GnRH2 and GnRH3 were significantly reduced in the presence of wortmannin (100 nM) or LY294002 (10 μM). Interestingly, treatment with either of the PI3K inhibitors alone elicited a rapid, transient increase in basal hormone release that decayed by 60-80% by the time of GnRH applications.

To evaluate if the transient increase in basal hormone release elicited by wortmannin or LY294002 alone decreased the releasable hormone pool or the sensitivity of the releasable hormone pool to agonist-stimulation in general, we examined the effects of PI3K inhibition on hormone release responses to the Ca^{2+} ionophore ionomycin.

Ionomycin (10 μM) has been shown to effectively elicit GH and LH release and to increase $[Ca^{2+}]_i$ in goldfish pituitary cells (Chang et al., 1990). Similarly, ionomycin, applied in place of GnRH as in the above experimental protocol, stimulated LH and GH secretion in this study (Figures 3.5 and 3.6). Pretreatment with pan-PI3K inhibitors did not significantly alter the net LH and GH release responses to ionomycin (Figures 3.5 and 3.6).

When the average LH and GH release magnitudes before and during applications of the PI3K inhibitors alone were compared, an overall significant elevation in hormone secretion was observed in the presence of either of the pan-PI3K inhibitors tested (Figures 3.7 and 3.8).

3.2.2 Wortmannin and LY294002 effects on GnRH-stimulated increases in $[Ca^{2+}]_i$ from isolated pituitary gonadotropes and somatotropes

To evaluate the involvement of PI3Ks in GnRH-stimulated Ca^{2+} signalling, we examined the effects of wortmannin and LY294002 on $[Ca^{2+}]_i$ in identified goldfish gonadotropes or somatotropes preloaded with the Ca^{2+}-sensitive dye Fura2-AM under basal and GnRH-stimulated conditions. Application of either wortmannin (100 nM) or LY294002 (10 μM) alone elicited a transient increase in basal $[Ca^{2+}]_i$ of gonadotrope or somatotrope (Figures 3.9, 3.11, 3.13, and 3.15).

Applications of 2 min pulses of GnRH2 (100 nM) or GnRH3 (100 nM) significantly elevated $[Ca^{2+}]_i$ in gonadotropes (Figures 3.9, 3.10 3.11, and 3.12) and somatotropes (Figures 3.13, 3.14, 3.15, and 3.16). In the presence of either wortmannin or LY294002, GnRH2- and GnRH3-evoked net $[Ca^{2+}]_i$ responses in gonadotropes were

significantly reduced (Figures 3.9, 3.10, 3.11, and 3.12). Interestingly in somatotropes, although wortmannin and LY294002 reduced GnRH2-stimulated net increases in $[Ca^{2+}]_i$, both of the PI3K inhibitors used did not significantly alter the corresponding response to GnRH3 (Figures 3.13, 3.14, 3.15, and 3.16). Similar results were obtained when the maximal amplitude of $[Ca^{2+}]_i$ responses were analyzed (Table 3.2). Likewise, the duration of the Ca^{2+} response to GnRH2 and GnRH3 were consistently reduced in the presence of LY294002 in both gonadotropes and somatotropes (Table 3.2).

A depolarizing treatment of KCl (30 mM) was applied at the end of each experimental sequence as a positive control to ensure that the cells remained healthy and responsive following treatments ($[Ca^{2+}]_i$ traces not shown). KCl applications elicited rapid increases in $[Ca^{2+}]_i$ in all cells regardless of the type of prior treatments. In particular, the net $[Ca^{2+}]_i$ responses, the maximal response amplitude, and the response duration to this KCl application were largely similar across all treatment groups (Table 3.3). These observations suggest that changes in responses to stimuli in the presence of PI3K inhibitors are not due to changes in cell viability or general detrimental effects on cell health.

3.2.3 LY294002 does not significantly alter acute LH and GH release responses to PKC activators

To address the possibility that activation of PI3K-dependent signalling occurs downstream of PKC, we examined the effect of LY294002 on LH and GH release responses stimulated by the novel and conventional PKC isoform activators TPA and DiC8 in column perifusion. Wortmannin was not used because of its potential effects on

cytosolic type III PI4Ks (Table 3.1). Briefly, PKC-dependent cellular responses are tightly controlled by phosphoinositide levels, and in particular the turnover of PtdIns(4,5)P_2 at the plasma membrane (Rosse et al., 2010). Sustained formation of hormone-sensitive inositide pools, including those responsible for Ins(1,4,5)P_3 turnover and $[Ca^{2+}]_i$ responses, requires the activity of wortmannin-sensitive type III PI4Ks (Nakanishi et al., 1995). Although the catalytic activity of membrane-bound type II PI4Ks are not significantly inhibited by either wortmannin or LY294002, wortmannin completely abrogates the acitivity of cytosolic type III PI4Ks at nanomolar doses (IC_{50} = ~50 nM; Downing et al., 1996). In contrast, type III PI4Ks are not effected by low micromolar doses of LY294002 (IC_{50} = ~100 μM; Downing et al., 1996). Therefore, a 10 μM dose of LY294002 was used for studies using PKC activators.

As expected, maximally stimulatory concentrations of TPA (100 nM) and DiC8 (100 μM) elicited LH and GH release from perifused goldfish pituitary cells (Figures 3.17 and 3.18; Chang et al., 1991). Treatment with LY294002 (10 μM) did not significantly alter the net LH or GH release responses to either of the PKC activators (Figures 3.17 and 3.18).

3.3 Discussion

There are only a small number of studies examining the involvement of PI3K-dependent signalling in the control of GnRH actions on hormone release. This is the first study to compare and contrast the involvement of PI3Ks in mediating the actions of two endogenous GnRH isoforms on hormone release responses from pituitary cells in primary culture. In each experimental series, replications were performed at different times

throughout the seasonal reproductive cycles of the goldfish and the results obtained are similar regardless of the gonadal stage of the pituitary donor. When taken together, our findings suggest that integration of PI3K- and Ca^{2+}-dependent signalling is both pituitary cell type- and GnRH-specific (Figures 3.19 and 3.20). In gonadotropes, both GnRH2 and GnRH3 actions on $[Ca^{2+}]_i$ involves PI3K-dependent signalling; in contrast, pan-PI3K inhibitors selectively attenuated the $[Ca^{2+}]_i$ response in identified somatotropes to GnRH2, but not GnRH3. These results provided the first evidence for the differential activation of PI3K-dependent signalling in GnRH-induced LH and GH release responses. Furthermore, results from the present study suggest that PI3K-dependent signalling is upstream or independent of acute activation of conventional or novel PKC isoforms.

3.3.1 Involvement of PI3Ks in GnRH-stimulated hormone release, and $[Ca^{2+}]_i$

Although PI3Ks have been implicated in the control of pituitary cell functions in transformed pituitary cell lines (reviewed in Chapter 1), very little is known regarding the involvement of PI3Ks in LH and GH release in primary pituitary cell cultures in any vertebrate species. In this study, the pan-specific PI3K inhibitors wortmannin and LY294002 both inhibited GnRH2 and GnRH3-induced hormone release from goldfish gonadotropes and somatotropes. Wortmannin, but not LY294002, irreversibly blocks PI3K activity through a covalent interaction with the catalytic subunit of PI3Ks (Wymann et al., 1996; Walker et al., 2000). That both inhibitors show a similar effect strongly suggests that PI3Ks are an integral part of the mechanisms mediating GnRH stimulation of LH and GH release in goldfish. Although a wortmannin-sensitive component of mGnRH1 stimulation of LH release from rat pituitary cells has been reported, the

inhibitory actions of wortmannin were largely attributed to a PI3K-independent mechanism involving inhibitory actions on MLCK due to the high concentration of wortmannin used (10 μM; Rao et al., 1997). Another rationale for this interpretation was that RTK-regulated PI3Ks were not likely to be relevant to the control of Ca^{2+}-dependent exocytosis (Rao et al., 1997). However, these results using mammalian gonadotropes are also not at variance with our findings.

At the concentration used in the present study, LY294002 and wortmannin are generally acknowledged to be selective inhibitors of PI3Ks (Table 3.1; also see Section 3.2); however, recent reports suggest that LY294002 may also bind to TOR, DNA protein kinase (DNA-PK), glycogen synthase kinase 3 (GSK3), and casein kinase 2 (CK2; Gharbi et al., 2007); while nanomolar concentrations of wortmannin also inhibit PLK and type III PI4Ks (Downing et al., 1996; Liu et al., 2005). Signalling downstream of TOR, DNA-PK, GSK3, and PLKs are not classically associated with actions on exocytosis (Katso et al., 2001; Vanhaesebroeck et al., 2001). In addition, while a number of studies have implicated CK2 in the control of GPCR-mediated signalling and cytoskeletal remodeling (Canton and Litchfield, 2006), no information is available regarding CK2 potential involvement in pituitary hormone secretion. Thus despite having some limitations as pharmacological tools, results using the ATP-dependent inhibitors wortmannin and LY294002 support the idea that PI3K-dependent signalling participates in GnRH actions on goldfish somatotropes and gonadotropes.

In goldfish, GnRH2 and GnRH3 stimulation of LH and GH release is dependent on $[Ca^{2+}]_i$ increases achieved via enhanced extracellular Ca^{2+} entry and mobilization from intracellular stores (reviewed in Chapter 1). Extracellular Ca^{2+} entry occurs through

VSCCs, whereas intracellular Ca^{2+} involvement is through the use of a number of pharmacologically distinct and agonist-specific Ca^{2+} stores (Johnson and Chang, 2000; Chang et al., 2009, 2012). Application of wortmannin and LY294002 attenuated the Ca^{2+} signal induced by both GnRHs in identified goldfish gonadotropes and by GnRH2 in somatotropes. These results suggest that PI3Ks are involved in the transduction step(s) proximal to increases in $[Ca^{2+}]_i$ in the case of GnRH3 stimulation of gonadotropes and GnRH2 stimulation of gonadatropes and somatotropes. Whether the PI3K-dependent step(s) affect extracellular Ca^{2+} entry and/or release of Ca^{2+} from intracellular stores in these cells is not known. However, the inability of wortmannin and LY294002 to affect ionomycin-induced hormone release suggest that PI3K involvement in receptor-independent secretion is not at steps downstream of $[Ca^{2+}]_i$ increases.

On the other hand, PI3K-dependent downstream signalling has been linked to PLC activation. The lipid second messenger produced by class I PI3Ks, PtdIns(3,4,5)P_3, regulates the membrane translocation and activity of PLCγ (reviewed in Chapter 1). In general, PLCs hydolyze PtdIns(4,5)P_2 to produced Ins(1,4,5)P_3 and DAG to stimulate downstream signalling, including inducing the intracellular release of Ca^{2+} from non-mitochondrial Ins(1,4,5)P_3-sensitive Ca^{2+} stores (reviewed in Chapter 1). Consistent with this activity, low nanomolar concentrations of wortmannin or injections of class I PI3K isoform-specific primary antibodies significantly inhibits antigen-stimulated production of Ins(1,4,5)P_3 and global $[Ca^{2+}]_i$ increases in RBL-2H3 cell lines (Barker et al., 1999; Smith et al., 2001). In addition, extracellular Ca^{2+} entry is also important for GnRH action and PI3Ks are known to affect extracellular Ca^{2+} entry directly and indirectly. Specifically, PI3K-dependent signalling enhances L-type VSCC currents in vascular

myocytes (Viard et al., 1999; Macrez et al., 2001; Quignard et al., 2001; Le Blanc et al., 2004) and cerebellar granule neurons (Blair et al., 1997). PtdIns(3,4,5)P_3 has also been shown to modulate agonist-stimulated Ca^{2+} entry in lymphocytes by directly interacting with the transient receptor potential (TRP) channel isoform TRPC6 (Hsu et al., 2000; Ching et al., 2001; Tseng et al., 2004). PI3K-dependent signalling also affects the plasma membrane insertion and recycling of ion channels to indirectly affect extracellular Ca^{2+} entry; including, rapid plasma membrane insertion of VSCCs is PI3K-dependent in COS-7 cell lines and rat dorsal root ganglion neurons (Viard et al., 2004). PI3Ks also regulate the membrane insertion of TRP ion channels (Bezzerides et al., 2004), which have been implicated in both extracellular Ca^{2+} influx and store-operated Ca^{2+} mobilization (Salido et al., 2009). Thus, it is likely that PI3K-dependent signalling mediates the $[Ca^{2+}]_i$ responses to GnRH3 and GnRH2 stimulation of goldfish gonadotropes, and GnRH2 in somatotropes, at the level of extracellular Ca^{2+} entry and/or Ca^{2+} release from stores. In terms of the involvement of intracellular Ca^{2+} stores, Ryn-sensitive stores mediates GnRH2 action on both gonadotropes and somatotropes whereas Ins(1,4,5)P_3 -sensitive stores participate in GnRH3 action on gonadotropes (Chang et al., 2009, 2012), Whether PI3Ks target these Ca^{2+} signalling components and/or other mechanisms modulating $[Ca^{2+}]_i$ to mediate GnRH effects on hormone release remains to be examined.

Interestingly, both pan-PI3K inhibitors had no significant effects on GnRH3-evoked Ca^{2+} responses in isolated somatotropes, suggesting that PI3K involvement in mediating GnRH3-stimulated GH release is not at step(s) proximal to the GnRH3-generated Ca^{2+} signal in this cell type. This is surprising given that GnRH3 stimulation of LH and GH release involves mobilization of Ca^{2+} from caffeine and Ins(1,4,5)P_3 -

sensitive Ca^{2+} stores, as well as Ca^{2+} influx through VSCCs (Chang et al., 2009, 2012). However, GnRH3 stimulation of GH release also involves unique signalling mechanisms not utilized by GnRH2; these include the use of AA-mediated signalling and Caff-sensitive Ca^{2+} stores, as well as, the participation of mitochondrial Ca^{2+} buffering (Johnson and Chang, 2002, 2005). Whether these known differences in the activation of signal transduction cascades are the reason(s) behind the observed differential effects of PI3K inhibitors on GnRH3- and GnRH2-selective Ca^{2+} signalling is not known.

Recently, NO has also been shown to mediate GnRH3-induced LH and GH release; while GnRH2-stimulated GH secretion also has a NO-sensitive signalling component (Urestsky et al., 2003; Meints et al., 2012). Interestingly, the classical PtdIns(3,4,5)P_3-sensitive effector Akt directly phosphorylates endothelial (e)NOS at Ser1177 to enhance enzymatic activity and NO production (Fulton et al., 1999; Dimmeler et al., 1999; Michell et al., 1999). Akt-dependent phosphorylation also alters the sensitivity of eNOS to Ca^{2+}, allowing for maximal eNOS activity at sub-physiological $[Ca^{2+}]_i$ (Dimmeler et al., 1999; Fulton et al., 2004). Thus, it is possible that PI3K-dependent regulation of NO production through Akt is involved in the Ca^{2+}-independent signalling functions of PI3Ks responsible for controlling hormone release-responses, such as for GnRH3-stimulated LH release or GnRH2-elicited GH secretion. On the other hand, since GnRH3-induced $[Ca^{2+}]_i$ changes in somatotropes are unaffected by the pan-PI3K inhibitors, a linear linkage between PtdIns(3,4,5)P_3-sensitive signalling through Akt, NOS, and Ca^{2+} appears to be absent in GnHR3 actions in somatotropes. Nonetheless, the difference in the effects of pan-PI3K inhibition on GnRH2 and GnRH3 actions on somatotropes, as well as in GnRH3 action between gonadotropes and somatotropes,

support the idea that GnRH-selective signalling exists in goldfish. It also indicates that the interaction of GnRHR-mediated transduction effectors with PI3K-dependent signalling is part of this complexity.

3.3.2 Relationship between PI3K- and PKC-dependent signalling in the acute stimulation of LH and GH release in goldfish pituitary cells

PI3K- and PKC-dependent signal transduction pathways are both involved in mediating GnRH signalling in LH and GH release in goldfish pituitary cells, raising the possibility that these two kinase systems interact in mediating GnRH actions. Interestingly, GPCRs can cause activation of PI3K-dependent signalling downstream of PKC catalytic activity in several cell types (Shah et al., 2006; Lee et al., 2011). Of particular interest for the present study, PKC-dependent signalling is upstream of GnRH-stimulated phosphorylation of Akt in the GnRHR-expressing hypothalamic GT1-7 cell line (Shah et al., 2006). Additionally, new evidence has demonstrated that a conventional PKC isozyme (PKCβ) can directly phosphorylate and activate the class IB PI3K catalytic subunit p110γ; providing a biochemical basis for the activation of PI3Ks by classical Ca^{2+}- and PKC-mediated signalling (Walser et al., 2013). In the present study, pretreatment with LY294002 did not alter TPA- or DiC8-stimulated LH or GH release responses Although the exact PKC isozyme(s) mediating GnRH stimulation of LH and GH release in goldfish has not been investigated, TPA and DiC8 specifically bind to and activate conventional (α, βI, βII, γ) and novel (δ, ε, θ, η) PKC isozymes (Newton, 2001). Taken together, these results indicate that activation of PI3K signalling does not occur downstream of conventional or novel PKCs in mediating acute LH and GH release

responses. These findings are consistent with an earlier study demonstrating that wortmannin, even at high micromolar concentrations, does not affect LH release responses stimulated by TPA in primary cultures of rat pituitary cells (Rao et al., 1997). Additionally, a recent study demonstrated that PKCβ-mediated phosphorylation of p110γ at Ser582 disconnects p110γ from its canonical inputs from GPCRs, and enables p110γ to operate downstream of increases in $[Ca^{2+}]_i$ (Walser et al., 2013). Interestingly, in teleost homologs of p110γ, Ser582 is substituted with a cysteine residue; suggesting that serine/threonine kinases such as PKCs cannot regulate Ser582 phosphorylation of p110γ in basal vertebrates. Furthermore, goldfish pituitary cells are not thought to express PKCβ isozymes (Klausen et al., 2005). Taken together, these data provide a potential mechanism for understanding why PI3K-dependent signalling is not downstream of PKC- and Ca^{2+}-dependent signalling in goldfish pituitary cells.

Evidence presented indicates that PKC activation is not upstream of PI3K-dependent signalling in goldfish gonadotropes and somatotropes during stimulated LH and GH release; however, other relationships between PKC- and PI3K-dependent signalling have yet to be explored. Studies in other cell systems have shown that the class I PI3K signal transduction target PDK1 phosphorylates the activation loop of conventional (α and βII; Dutil et al., 1998; Balendran et al., 2000), novel (δ; Le Good et al., 1998; Balendran et al., 2000) and atypical (ζ; Chou et al., 1998; Le Good et al., 1998; Balendran et al., 2000) PKC isozymes. Furthermore, PDK1 activation of PKCζ is enhanced by increasing intracellular concentrations of PtdIns(3,4,5)P_3 (Chou et al., 1998; Le Good et al., 1998). Since immunoreactivity for conventional (α), novel (θ, δ), and atypical (ζ) PKC isozymes are present in goldfish pituitary extracts (Klausen et al., 2005),

it is plausible that PI3K-dependent signalling cascades activate PKCs in goldfish pituitary cells. Thus, whether activation of the atypical PKCζ isozyme occurs downstream of PI3K signalling and/or that PI3K catalytic activity acts upstream or independent of PKCs to control GnRH-stimulated hormone release are possibilities that require further study. However, long-term treatment with TPA which can deplete cells of conventional and novel, but not atypical, PKCs almost completely abolishes GnRH-stimulated LH and GH release responses (Jobin et al., 1993); suggesting that PKCζ does not play a major role in mediating GnRH action in goldfish pituitary cells.

3.3.3 GnRHR activation of class I PI3K-dependent signalling

As reviewed in Chapter 1, three unique classes of PI3Ks have been identified based upon molecular structure and substrate specificity. Classically, receptor-activated PI3K-dependent signalling leading to hormone release is usually associated with recruitment and activation of the class I PI3Ks (Vanhaesebroeck et al., 2010; Burke and Williams, 2015). In general, class IA PI3Ks are most often linked to receptors with intrinsic or associated tyrosine kinase activity; whereas the class IB catalytic subunit p110γ (characterized by its own unique regulatory proteins, p101 or p84) is activated through a direct interaction with the Gβγ heterodimers of ligand-activated GPCRs (Vadas et al., 2011, 2013). In addition, as outlined in Chapter 1, the class IA p110β/p85 isoform can also be directly activated by Gβγ heterodimers (Guillermet-Guibert et al., 2008; Dbouk et al., 2012). How the activation of the GnRHRs regulates the catalytic activity of class IA and/or IB PI3Ks in goldfish pituitary cells (or any other primary pituitary cell type for that matter) is not known; but, direct coupling of class IA or IB isoforms to

GnRHRs through interactions with Gβγ subunits is a strong possibility. However, alternative mechanisms for GnRHR-mediated activation of class I PI3Ks may also exist. In immortalized hypothalamic neurons, GnRH-mediated activation of PI3K-dependent signalling involves transactivation of epidermal growth factor receptors, (EGFR; Shah et al., 2006). GPCR-RTK transactivation is achieved through the GPCR-dependent activation of matrix metalloproteinases (MMPs; Wetzker and Bohmer, 2003). Activated MMPs cleave membrane-anchored growth factor-like substances (such as heparin-bound EGF) that upon release can bind and activate resident RTKs (Daub et al., 1996; Wetzker and Bohmer, 2003). In general, GPCR agonists can activate MMPs through multiple intracellular mediators, including: SFK family non-receptor tyrosine kinases, Pyk2 protein tyrosine kinase, Ca^{2+}-dependent signalling, and PKCs (Wetzker and Bohmer, 2003; Liebmann, 2011). Our results do not differentiate between GnRHR-RTK transactivation and direct coupling of class I PI3Ks to GPCRs. However, based on our studies using ionomycin and synthetic PKC activators, it is unlikely that GnRHRs transactivate RTK-sensitive class IA PI3Ks downstream of Ca^{2+}- or PKC-dependent signalling.

3.3.4 Possible involvement of Class II and III PI3Ks

Although results from this Chapter strongly implicate a role for the canonical receptor-mediated class I PI3Ks, they do not exclude the involvement of the lesser-studied class II or class III PI3Ks in the control of GnRH actions. In particular, the common catalytic product of both the class II and III PI3Ks, PtdIns(3)P, is emerging as an important component of regulated exocytosis (Falasca and Maffucci, 2012; Backer,

2008). For example, the catalytic activity of the class II PI3K-C2α isoform is regulated by Ca^{2+} and contributes to the production of a dynamic pool of PtdIns(3)P localized on secretory vesicles (Wen et al., 2008). PI3K-C2α also plays an important role in the ATP-dependent priming of neurosecretory granules (Meunier et al., 2005) and insulin secretion (Dominguez et al., 2011). However, PI3K-C2α is insensitive to wortmannin and LY294002 (Knight et al., 2004) but both inhibitors abrogated GnRH-stimulated hormone release, suggesting that PI3K-C2α likely does not contribute to GnRH actions on hormone secretion. On the other hand, both inhibitors at the doses tested can inhibit class II PI3K isoforms C2β and C2γ. Although the cellular activity of PI3K-C2β and PI3K-C2γ is not as well characterized, available evidence indicates that these class II PI3K isoforms also have intracellular signalling functions (Falasca and Maffucci, 2012). Likewise, the class III PI3K isoform Vps34 is known to regulate vesicle trafficking in the endosomal system, as well as signalling to TOR and MAPKs (Backer, 2008). These events play important roles in vesicle reformation and receptor-mediated signalling (Lindmo and Stenmark, 2006; Kononenko and Haucke, 2015). The emergence of new isoform-selective inhibitors of class II (PI3K-C2γ; Freitag et al., 2014) and III (Bago et al., 2014; Ronan et al., 2014; Dowdle et al., 2014) PI3Ks will allow for future studies examining the possible involvement of these PI3K isoforms in goldfish pituitary cell functions.

3.3.5 Broad-spectrum PI3K inhibitors and effects on basal hormone release

PI3Ks are part of the intricate network of lipid-modifying enzymes that are universally important for the maintenance of inositol phospholipid metabolism and membrane dynamics (Di Paolo and De Camilli, 2006). Specifically, class I PI3Ks are

responsible for the formation of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2; whereas class II and III PI3Ks primarily synthesize PtdIns(3)P from PtdIns (Vanhaesebroeck et al., 2010). Application of either pan-specific PI3K inhibitor alone transiently elevated basal LH and GH release in goldfish pituitary cells suggesting that PI3Ks may also be involved in basal hormone secretion. Phosphoinositides alter lipid bilayer properties through a number of potential mechanisms and these changes likely contribute to changes in membrane dynamics, including alterations in exocytosis (Rusinova et al., 2013). In general, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 coordinate signalling responses through specific interactions with unique lipid-binding domains (Lemmon et al., 2008). In addition, they regulate plasma membrane availability of signalling, cytoskeletal, and transport proteins through ionic protein-lipid interactions between the positively charged polybasic motifs of these proteins and the negatively charged PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 headgroups (Do Heo et al., 2006). PtdIns(4,5)P_2 clusters can also act as molecular scaffolds for vesicle docking (Honigmann et al., 2013) and facilitate the formation of membrane microdomains independent of cholesterol or lipid phases (van den Bogaart et al., 2011). Furthermore, maintenance of the PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 ratio by interconverting enzymes may also be important for the regulation of intracellular Ca^{2+} signalling (Fukami et al., 2010). Thus, it is likely that treatment with pan-PI3K inhibitors causes both an overall reduction in PtdIns(3,4,5)P_3 availability, as well as a transient accumulation of the class I PI3K substrate PtdIns(4,5)P_2, that ultimately contributes to changes in basal hormone release. Indeed, LY294002 elevates basal PRL secretion from GH4C1 cells and primary cultures of rat pituitary cells, while it attenuates IGF-1-stimulated PRL secretion (Romano et al., 2006). How inhibition of PI3Ks may lead to

elevated basal goldfish LH and GH release is not known but in addition to the aforementioned influences on vesicle docking and molecular scaffolding, modulation of $[Ca^{2+}]_i$ equilibrium is also a possibility. In particular, as discussed in section 3.3.1, PI3Ks can potentially alter multiple aspects of both extracellular Ca^{2+} entry, as well as internal release of Ca^{2+} in multiple cell types. Negative perturbation of extracellular Ca^{2+} entry in goldfish somatotropes and gonadotropes have been shown to elicit transient increases in hormone release and $[Ca^{2+}]_i$ as release from intracellular Ca^{2+} stores compensates for the decrease in extracellular Ca^{2+} entry (Johnson and Chang, 2000; Johnson et al., 2000). The transient nature of the hormone secretion and $[Ca^{2+}]_i$ responses to wortmannin and LY294002 suggest that such a adjustment and re-establishment of a new equilibrium in terms of basal hormone secretion and $[Ca^{2+}]_i$ occurred in these cells upon exposure to these inhibitors.

3.3.6 Summary

Overall, this study demonstrates the participation of PI3K-dependent signalling in GnRH-induced Ca^{2+} signalling in a cell type- and GnRH isoform-specific manner throughout the seasonal reproductive cycle in goldfish. Specifically, involvement of PI3Ks in the regulation of acute GnRH2-evoked $[Ca^{2+}]_i$ responses leading to LH and GH release, as well as GnRH3-stimulated LH release and $[Ca^{2+}]_i$ responses are demonstrated. PI3Ks similarly mediate GnRH3 action on GH release, but this does not involve the modulation of overall $[Ca^{2+}]_i$ in somatotropes. On the other hand, PI3K-dependent signalling is either upstream or independent of novel or conventional PKC activity in the control of acute LH and GH release. These observations with $[Ca^{2+}]_i$ changes and PKC

activators provide insight into the possible relationships of multiple signal transduction pathways mediating GnRH2 and GnRH3 actions on goldfish pituitary cells. Column perifusion studies also suggest that PI3K-dependent signalling may play a role in the regulation of basal hormone secretion. These results add to our knowledge of the complexity of signal transduction systems responsible for the regulation of GnRH-stimulated hormone release in the goldfish pituitary. In addition, these findings set the stage for novel studies into the selective involvement of specific PI3K isoforms, and their commonly targeted downstream mediators, in the control of LH and GH release.

Table 3.1 Selectivity profiles for the pan-PI3K inhibitors wortmannin and LY294002.

Inhibitor	IC_{50} Values (μM) for pan-PI3K Inhibitors														[Used]	Additional Off-Target Effects
	Class IA PI3K[a]			Class IB PI3K[a]	Class II PI3K[a]			Class III PI3K	PI4Ks		PI3K-Related Protein Kinases					
	p110α	p110β	p110δ	p110γ	PI3K-C2α	PI3K-C2β	PI3K-C2γ	Vps34	Type II	Type III	DNA-PK	ATM	ATR	TOR		
wortmannin	**0.0001**[a]	**0.014**[a]	**0.009**[a]	**0.005**[a]	0.42[b]	**0.016**[d]	**0.032**[e]	**0.0025**[a]	> 100[g]	**0.05**[g]	0.016[h]	0.15[h]	1.8[h]	0.2[i]	**100 nM**	**PLK**[k]
LY294002	**0.72**[a]	**0.31**[a]	**1.33**[a]	**7.26**[a]	~100[c]	**6.9**[d]	**40**[c]	**3.49**[f]	> 100[g]	~ 100[g]	**0.66**[c]	> 100[c]	> 100[c]	**8.9**[c]	**10 μM**	**PLK**[j], **CK2**[c]

Values are drawn from published reports ([a]Hawkins et al., 2006; [b]Domin et al., 1997; [c]Knight et al., 2004; [d]Arcaro et al., 1998; [e]Misawa et al., 1998; [f]Kong et al., 2010; [g]Downing et al., 1996; [h]Sarkaria et al., 1998; [i]Brunn et al., 1996; [j]Bain et al., 2007; [k]Liu et al., 2005). It should be noted that these data were generated from various *in vitro* assays at varying concentrations of ATP. Targets with IC_{50} values written in **bold** are thought to be intracellular targets of either wortmannin (top row) or LY294002 (bottom row) at the doses used in this study. Please note, that proposed selectivities for these compounds are based on multiple cellular assays and may not perfectly match the IC_{50} results presented for *in vitro* kinase assays.

Table 3.2 Effects of the pan-PI3K inhibitors wortmannin (100 nM) and LY294002 (10 μM) on GnRH-stimulated $[Ca^{2+}]_i$ response parameters. Results (mean ± SEM) represent pooled data from three to seven individual cell preparations covering all stages of the seasonal reproductive cycle in goldfish.

	n	Average Response Duration (sec)	Max Amplitude (% Pretreatment)
Gonadotropes			
GnRH2	13	285 ± 47	164 ± 18
GnRH2 + wortmannin	21	217 ± 21	48 ± 12*
GnRH2 + LY294002	14	151 ± 23*	39 ± 11*
GnRH3	13	297 ± 25	237 ± 46
GnRH3 + wortmannin	18	218 ± 18*	71 ± 16*
GnRH3 + LY294002	13	201 ± 32*	73 ± 20*
Somatotropes			
GnRH2	12	293 ± 33	155 ± 15
GnRH2 + wortmannin	9	187 ± 44*	61 ± 14*
GnRH2 + LY294002	12	136 ± 27*	72 ± 16*
GnRH3	8	261 ± 34	113 ± 19
GnRH3 + wortmannin	11	241 ± 26	86 ± 11
GnRH3 + LY294002	14	201 ± 32	73 ± 20

* Significantly different from GnRH control treatment (t-test, $p < 0.05$).

Table 3.3 Parameters of $[Ca^{2+}]_i$ response to a 2 min pulse of depolarizing 30 mM KCl treatment applied between 1245-1365 s in experiments shown in Figures 3.9, 3.11, 3.13, and 3.15. This KCl stimulus served as a positive control to ensure that cells were viable and able to respond with a $[Ca^{2+}]_i$ elevation following inhibitor treatment. The Ca^{2+} response parameters to KCl did not differ between the treatments groups.

	n	Net Response (% Pretreatment)	Response Duration (sec)	Maximum Amplitude (% Pretreatment)
Gonadotropes				
wortmannin	14	2127 ± 345	209 ± 12	346 ± 55
LY294002	10	3227 ± 659	231 ± 6	383 ± 56
GnRH2	14	2333 ± 396	213 ± 10	394 ± 52
GnRH2 + wortmannin	21	2296 ± 402	203 ± 10	354 ± 43
GnRH2 + LY294002	14	3192 ± 549	212 ± 12	422 ± 54
GnRH3	13	2216 ± 333	193 ± 11	419 ± 52
GnRH3 + wortmannin	18	1929 ± 301	209 ± 11	308 ± 45
GnRH3 + LY294002	13	2659 ± 406	185 ± 15	404 ± 48
Somatotropes				
wortmannin	13	2254 ± 355	211 ± 12	357 ± 43
LY294002	8	3411 ± 629	214 ± 11	412 ± 58
GnRH2	13	2361 ± 311	178 ± 16	425 ± 37
GnRH2 + wortmannin	10	2026 ± 518	206 ± 16	296 ± 45
GnRH2 + LY294002	14	3249 ± 372	189 ± 13	505 ± 38
GnRH3	10	2340 ± 475	206 ± 14	384 ± 45
GnRH3 + wortmannin	13	2000 ± 225	200 ± 12	338 ± 40
GnRH3 + LY294002	14	3460 ± 759	196 ± 14	400 ± 51

Figure 3.1 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH2 (100 nM) induced LH release responses. For each panel, LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 5.09 ± 0.23 ng/ml). Pooled responses are shown from experiments using individual cell preparations. Four experiments (n = 8) with wortmannin and five (n = 10) with LY294002 were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

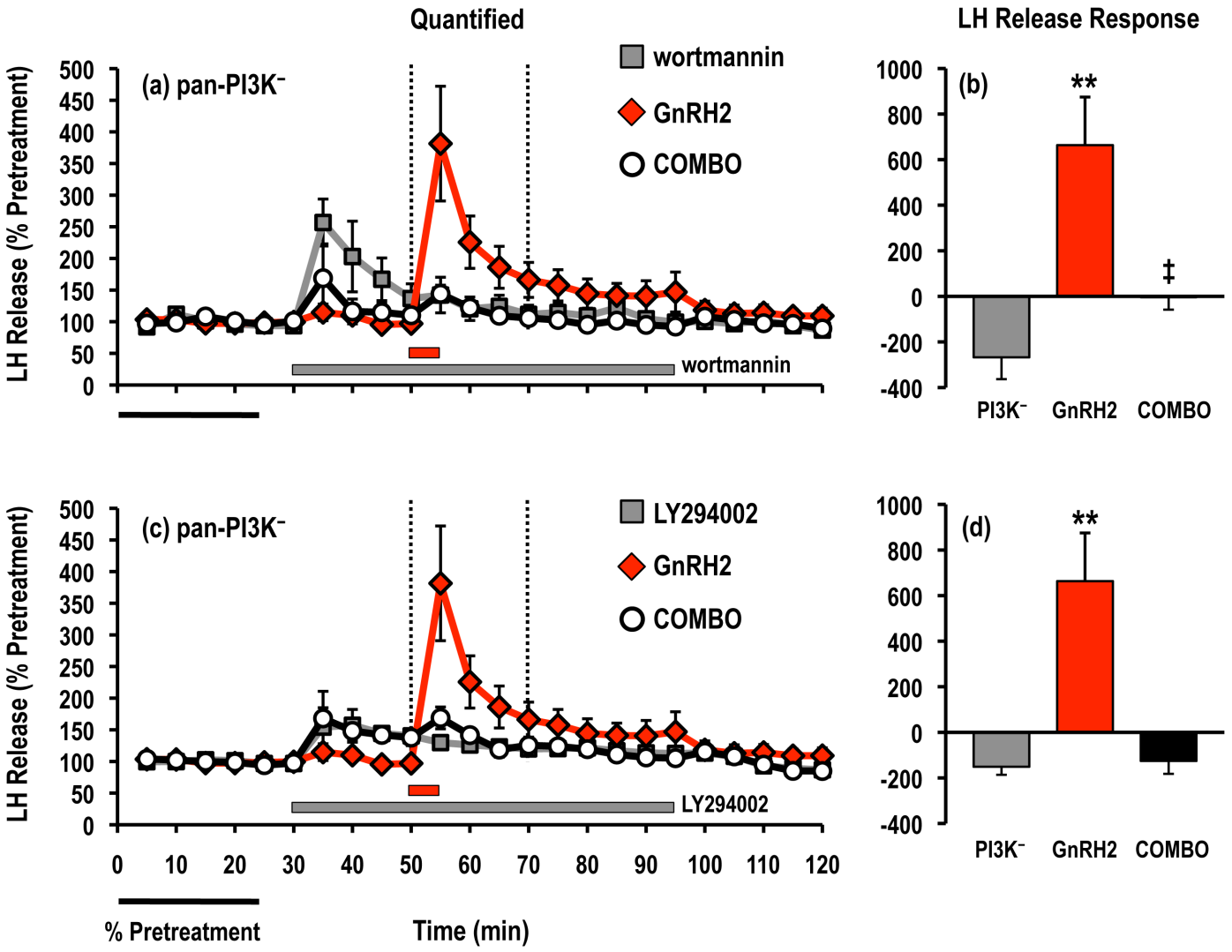

Quantified
LH Release Response
(a) pan-PI3K⁻
wortmannin
GnRH2
COMBO
LH Release (% Pretreatment)
wortmannin
(b)
**
‡
PI3K⁻
GnRH2
COMBO
(c) pan-PI3K⁻
LY294002
GnRH2
COMBO
LY294002
(d)
**
PI3K⁻
GnRH2
COMBO
% Pretreatment
Time (min)

Figure 3.2 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH3 (100 nM) induced LH release responses. For each panel, LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 4.29 ± 0.28 ng/ml). Pooled responses are shown from experiments using individual cell preparations. Four experiments (n = 8) with wortmannin and five (n = 10) with LY294002 were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

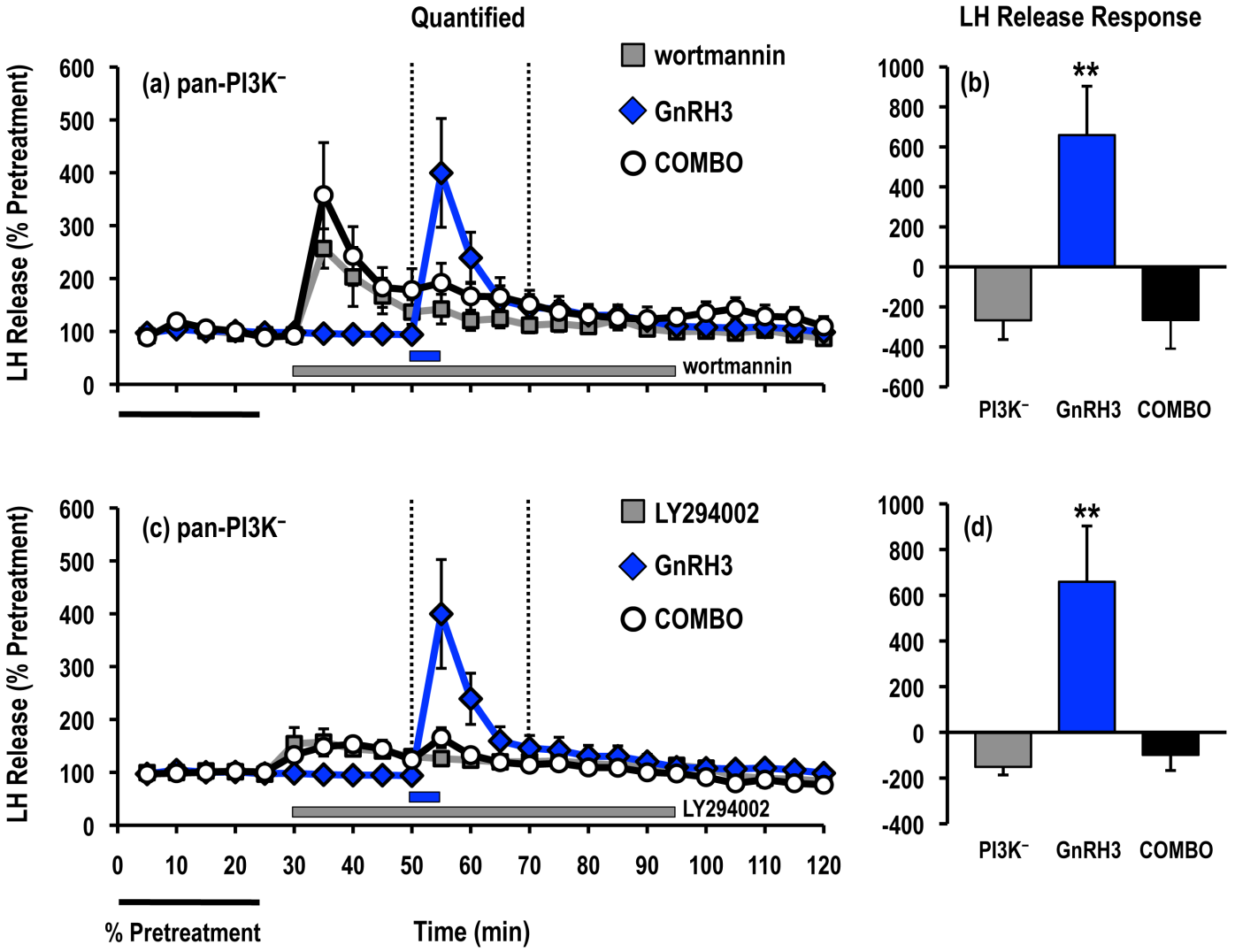

Quantified
LH Release Response
(a) pan-PI3K⁻
wortmannin
GnRH3
COMBO
LH Release (% Pretreatment)
600
500
400
300
200
100
0
wortmannin
(b)
**
1000
800
600
400
200
0
-200
-400
-600
PI3K⁻
GnRH3
COMBO
(c) pan-PI3K⁻
LY294002
GnRH3
COMBO
LY294002
(d)
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)

Figure 3.3 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH2 (100 nM) induced GH release responses. For each panel, GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 7.37 ± 0.81 ng/ml). Pooled responses are shown from experiments using individual cell preparations. Four experiments (n = 8) with wortmannin and five (n = 10) with LY294002 were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

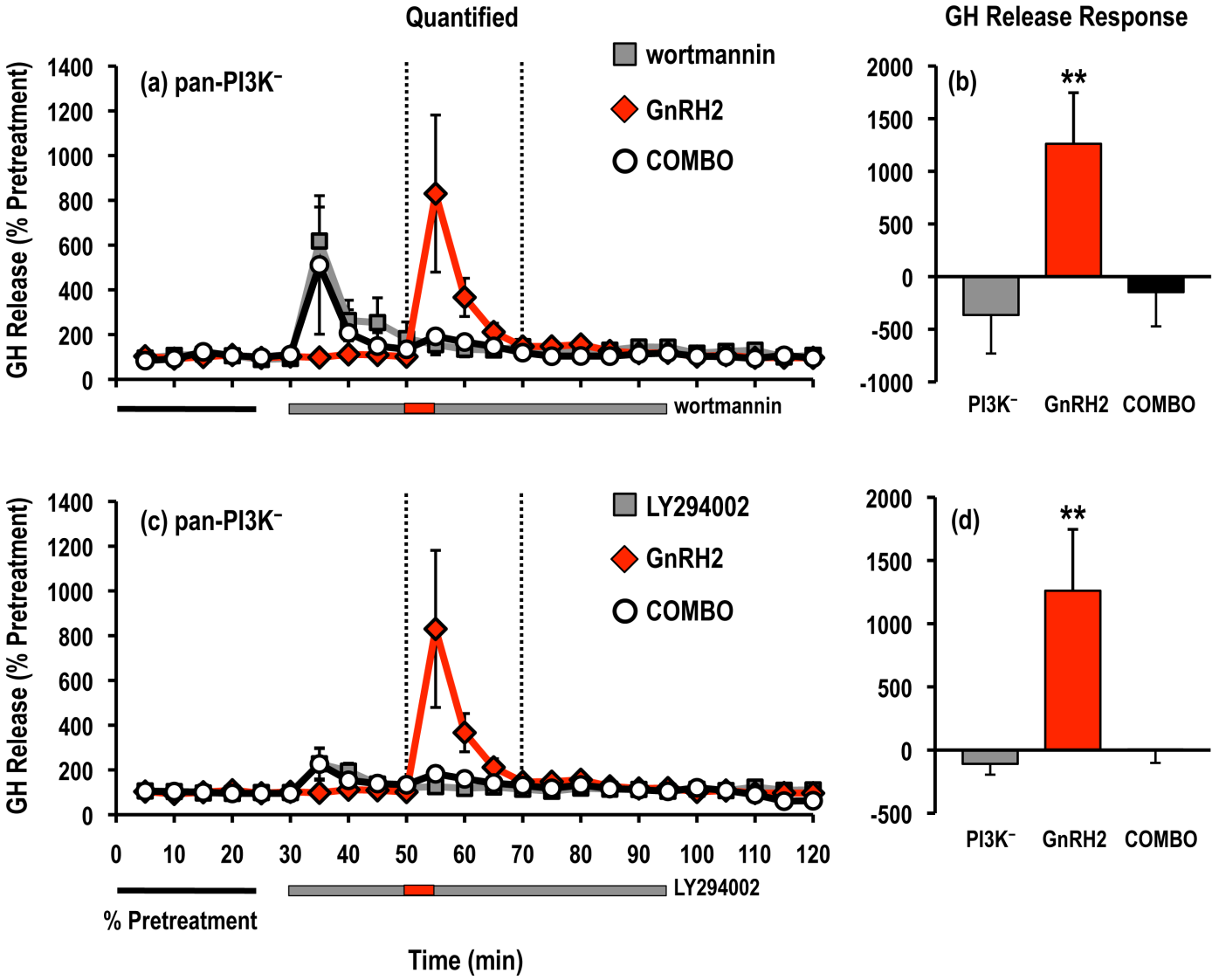

Quantified
GH Release Response
(a) pan-PI3K⁻
wortmannin
GnRH2
COMBO
GH Release (% Pretreatment)
1400
1200
1000
800
600
400
200
0
wortmannin
(b)
**
2000
1500
1000
500
0
-500
-1000
PI3K⁻
GnRH2
COMBO
(c) pan-PI3K⁻
LY294002
GnRH2
COMBO
(d)
0
10
20
30
40
50
60
70
80
90
100
110
120
LY294002
% Pretreatment
Time (min)

Figure 3.4 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH3 (100 nM) induced GH release responses. For each panel, GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 8.91 ± 0.93 ng/ml). Pooled responses are shown from experiments using individual cell preparations. Four experiments (n = 8) with wortmannin and five (n = 10) with LY294002 were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

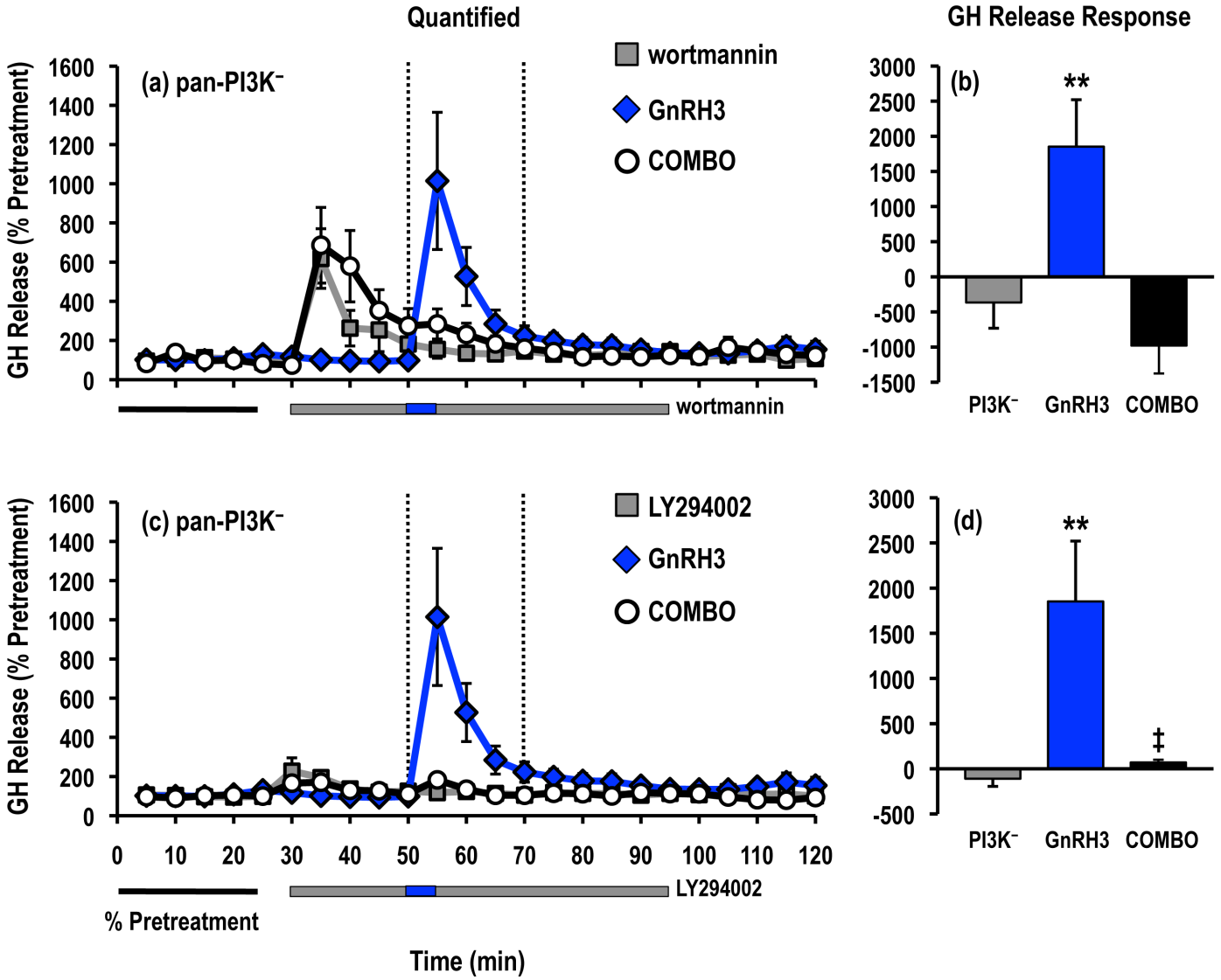

Quantified
GH Release Response
(a) pan-PI3K⁻
wortmannin
GnRH3
COMBO
GH Release (% Pretreatment)
1600
1400
1200
1000
800
600
400
200
0
wortmannin
(b)
**
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
PI3K⁻
GnRH3
COMBO
(c) pan-PI3K⁻
LY294002
GnRH3
COMBO
LY294002
(d)
‡
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)

Figure 3.5 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and ionomycin (10 μM) induced LH release responses. For each panel, LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; green solid diamond, ionomycin alone; open circle, ionomycin + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment; $[Ca^{2+}]^+$ denotes ionomycin treament). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 3.38 ± 0.38 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations using goldfish undergoing sexual recrudescence (February and March). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the green horizontal bar represents the 5-min ionomycin (10 μM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

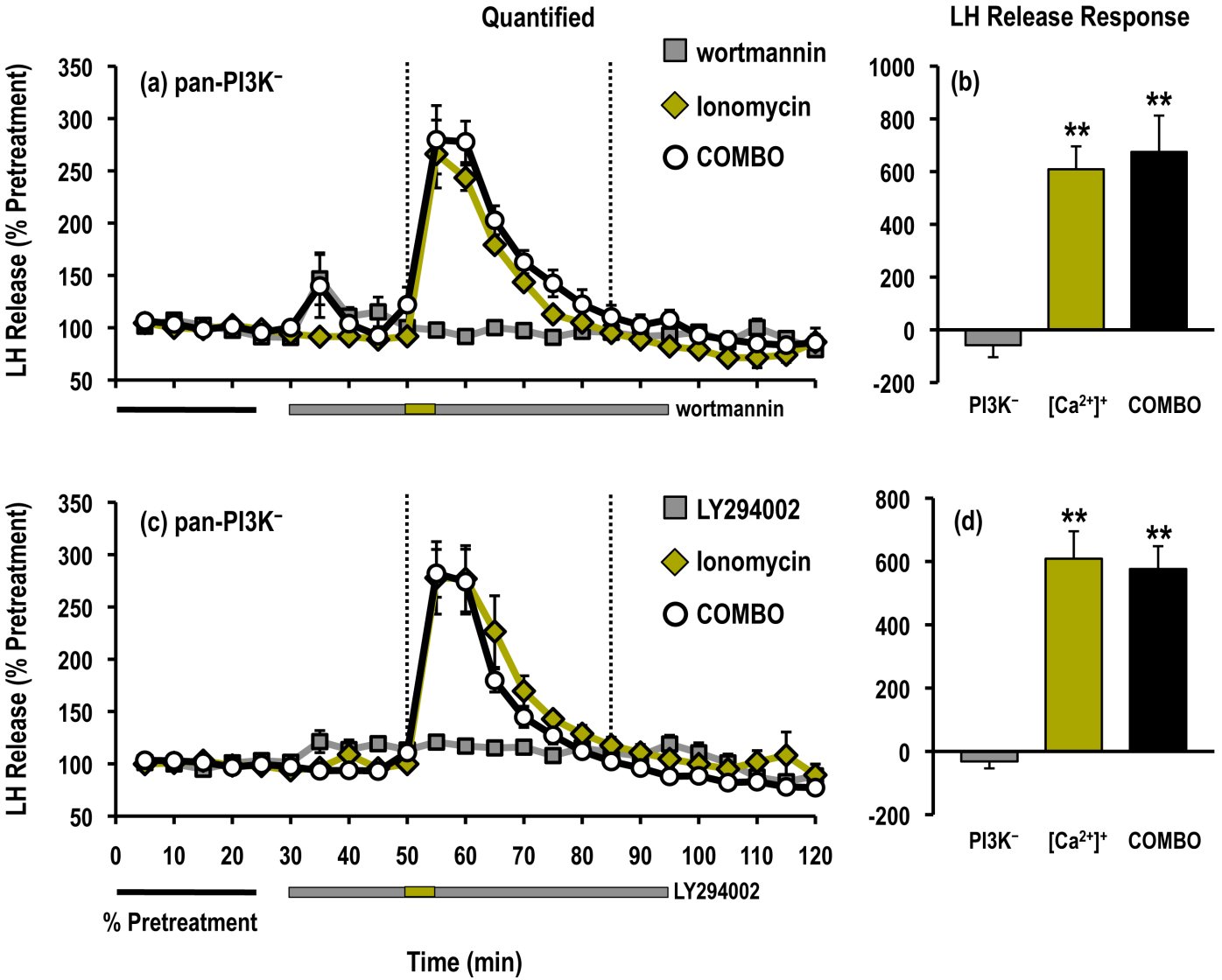

Quantified
LH Release Response
(a) pan-PI3K⁻
wortmannin
Ionomycin
COMBO
LH Release (% Pretreatment)
350
300
250
200
150
100
50
wortmannin
(b)
1000
800
600
400
200
0
-200
**
**
PI3K⁻
[Ca2+]+
COMBO
(c) pan-PI3K⁻
LY294002
Ionomycin
COMBO
LH Release (% Pretreatment)
0
10
20
30
40
50
60
70
80
90
100
110
120
LY294002
% Pretreatment
Time (min)
(d)
800
600
400
200
0
-200
**
**
PI3K⁻
[Ca2+]+
COMBO

Figure 3.6 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and ionomycin (10 μM) induced GH release responses. For each panel, GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; green solid diamond, ionomycin alone; open circle, ionomycin + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; $PI3K^{-}$ denotes inhibitor alone treatment; $[Ca^{2+}]^{+}$ denotes ionomycin treament). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 5.98 ± 0.68 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations using goldfish undergoing sexual recrudescence (February and March). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the green horizontal bar represents the 5-min ionomycin (10 μM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

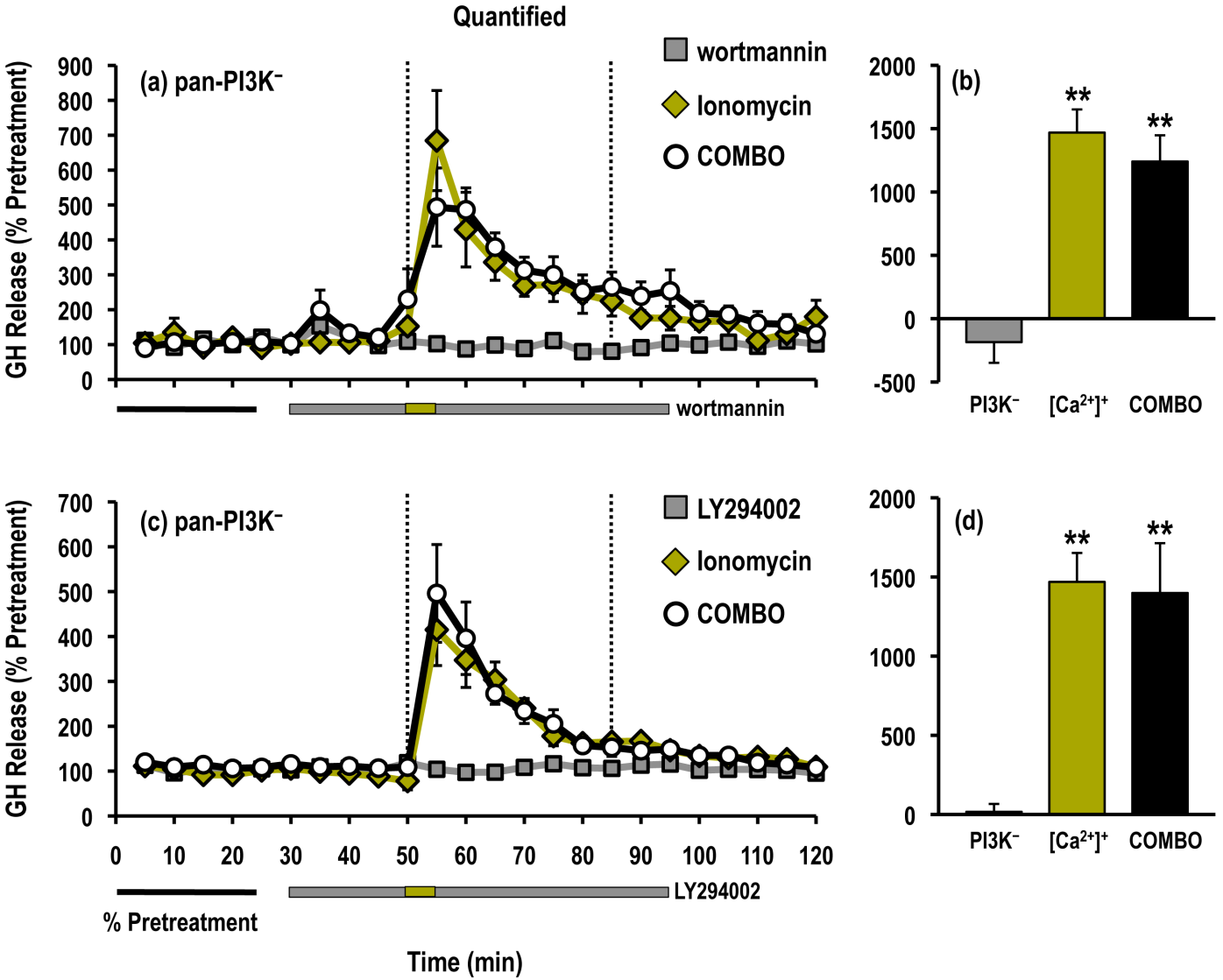

Quantified
(a) pan-PI3K⁻
wortmannin
Ionomycin
COMBO
GH Release (% Pretreatment)
900
800
700
600
500
400
300
200
100
0
wortmannin
(b)
2000
1500
1000
500
0
-500
**
**
PI3K⁻
[Ca2+]+
COMBO
(c) pan-PI3K⁻
LY294002
Ionomycin
COMBO
GH Release (% Pretreatment)
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
LY294002
% Pretreatment
Time (min)
(d)
2000
1500
1000
500
0
**
**
PI3K⁻
[Ca2+]+
COMBO

Figure 3.7 Effects of pan-PI3K inhibitors wortmannin (100 nM; a) and LY294002 (10 μM; b) on basal LH release responses. LH release profiles are shown on the left panel and quantified responses are shown on the right (mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar; 4.69 ± 0.26 ng/ml) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (a, wortmannin, orange bars; b, LY294002, gold bars) was quantified as the average % pretreatment value over the entire duration of the pan-PI3K inhibitor treatment (30-95 min; the grey horizontal bar). Pooled responses are shown from experiments using individual cell preparations. Experiments with wortmannin (n = 15) and with LY294002 (n = 14) were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). Within each trace, the grey horizontal line represents the 100% pre-treatment mark. Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

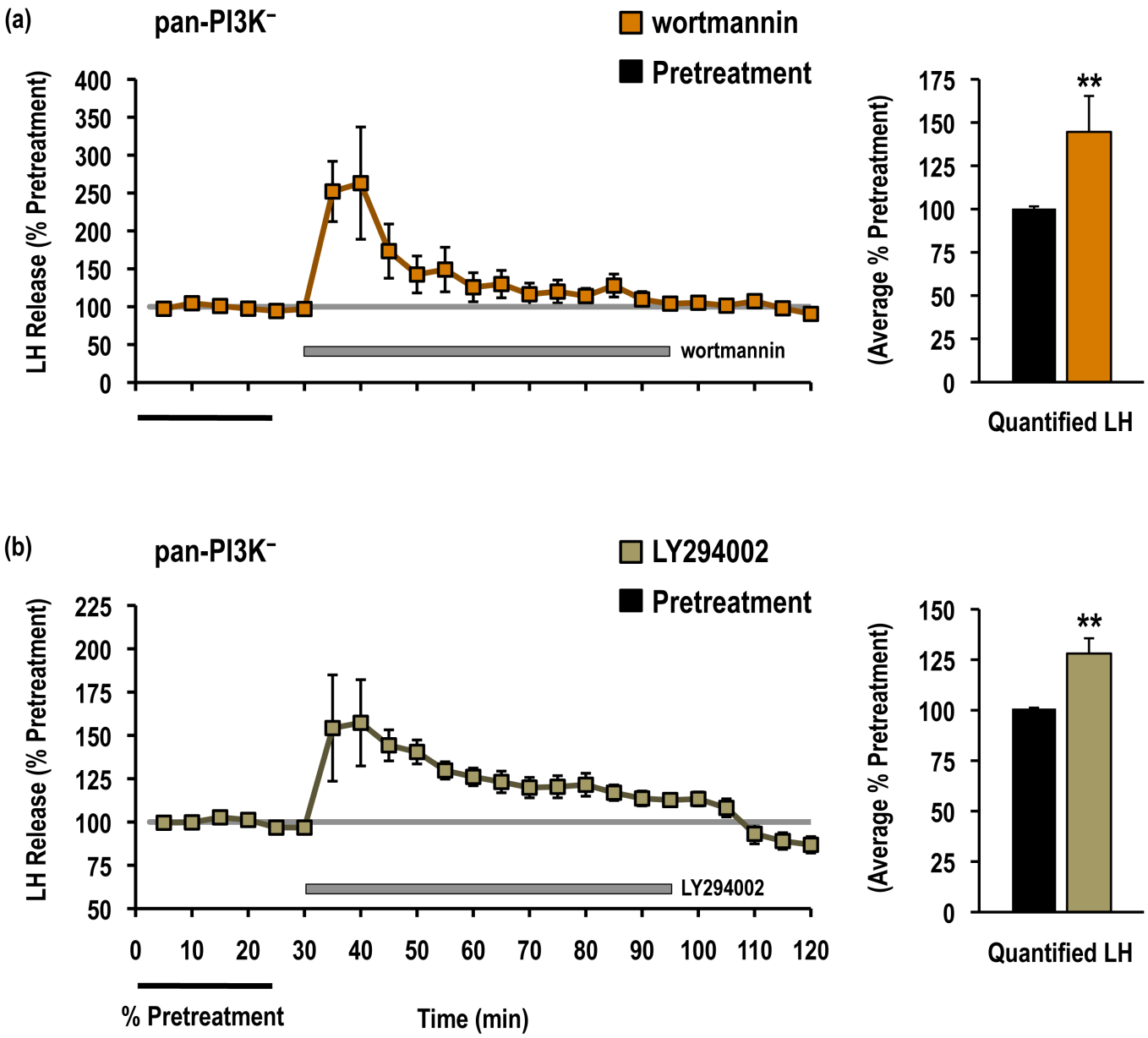

(a)
pan-PI3K⁻
wortmannin
Pretreatment
LH Release (% Pretreatment)
400
350
300
250
200
150
100
50
0
wortmannin
(Average % Pretreatment)
175
150
125
100
75
50
25
0
**
Quantified LH
(b)
pan-PI3K⁻
LY294002
Pretreatment
LH Release (% Pretreatment)
225
200
175
150
125
100
75
50
LY294002
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)
(Average % Pretreatment)
150
125
100
75
50
25
0
**
Quantified LH

Figure 3.8 Effects of pan-PI3K inhibitors wortmannin (100 nM; a) and LY294002 (10 μM; b) on basal GH release responses. GH release profiles are shown on the left panel and quantified responses are shown on the right (mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar; 8.14 ± 0.87 ng/ml) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (a, wortmannin, orange bars; b, LY294002, gold bars) was quantified as the average % pretreatment value over the entire duration of the pan-PI3K inhibitor treatment (30-95 min; the grey horizontal bar). Pooled responses are shown from experiments using individual cell preparations. Experiments with wortmannin (n = 15) and with LY294002 (n = 14) were performed with goldfish with either regressed gonads, gonads undergoing sexual recrudescence or matured (pre-spawning) gonads (wortmannin, September to April; LY294002, September to February and May to June). Within each trace, the grey horizontal line represents the 100% pre-treatment mark. Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

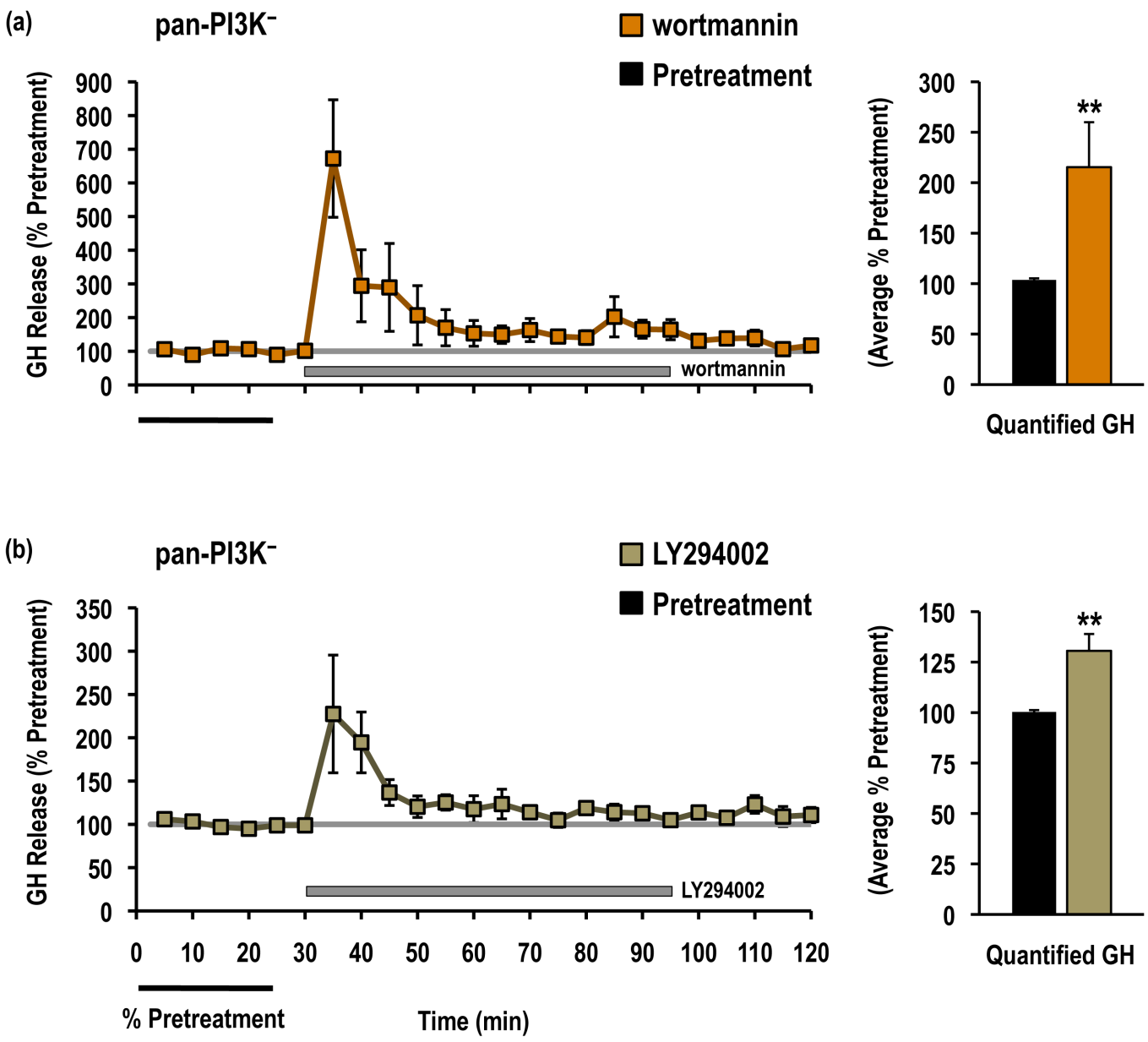

(a)
pan-PI3K⁻
wortmannin
Pretreatment
GH Release (% Pretreatment)
900
800
700
600
500
400
300
200
100
0
wortmannin
(Average % Pretreatment)
300
250
200
150
100
50
0
**
Quantified GH
(b)
pan-PI3K⁻
LY294002
Pretreatment
GH Release (% Pretreatment)
350
300
250
200
150
100
50
0
LY294002
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)
(Average % Pretreatment)
150
125
100
75
50
25
0
**
Quantified GH

Figure 3.9 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH2 (100 nM) induced $[Ca^{2+}]_i$ changes in morphologically identified gonadotropes. Ca^{2+} profiles are shown on the left panel and quantified net Ca^{2+} responses are shown on the right (mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Results were expressed as a percentage of pretreatment values (% pretreatment, average of the first five images collected; 114.33 ± 8.75 nM). Pooled responses are shown. The solid gray horizontal bar indicates duration of inhibitor treatment while the red horizontal bar indicates the 2-min GnRH2 exposure (gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor). Duration of net $[Ca^{2+}]_i$ response quantification is indicated by the vertical dotted lines. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). Gonadotropes were imaged from three to seven cell preparations and covered all stages of the seasonal reproductive year (n = 13, GnRH2 alone; 14, wortmannin alone; 21, GnRH2 + wortmannin; 10, LY294002 alone; 14, GnRH2 + LY294002). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

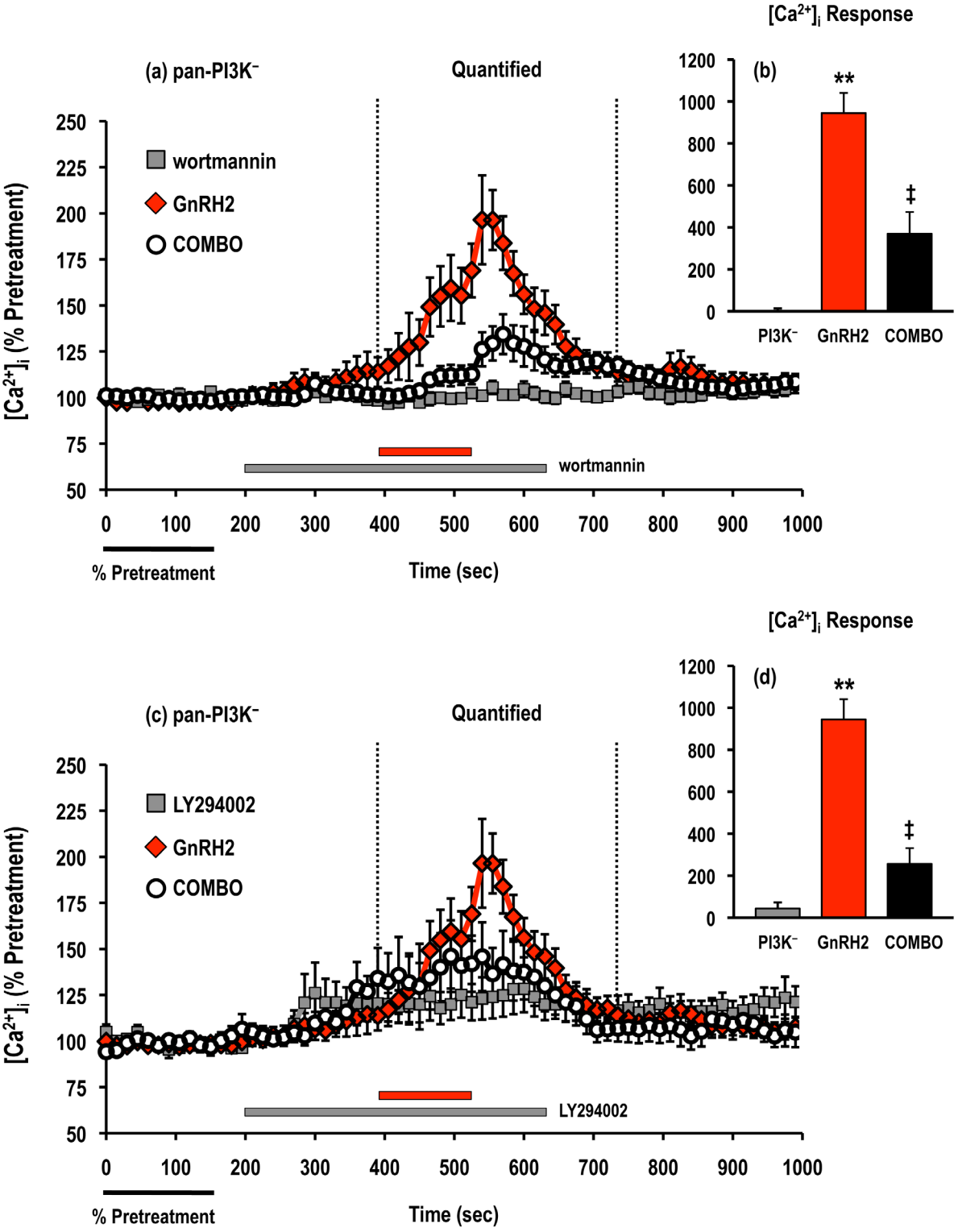

[Ca2+]i Response
(a) pan-PI3K-
Quantified
(b)
wortmannin
GnRH2
COMBO
[Ca2+]i (% Pretreatment)
Time (sec)
% Pretreatment
wortmannin
PI3K-
GnRH2
COMBO
**
‡
(c) pan-PI3K-
Quantified
(d)
LY294002
GnRH2
COMBO
LY294002

Figure 3.10 Representative pseudo-color images demonstrating the effects of the pan-PI3K inhibitor wortmannin (100 nM; b) on GnRH2 (100 nM; a) induced increases in $[Ca^{2+}]_i$ from a single morphologically identified gonadotrope. (a; top row) GnRH2-stimulated changes in the whole-cell Ca^{2+} signal and (b; bottom row) the effects of wortmannin pretreatment on GnRH2 induced increases in $[Ca^{2+}]_i$. Images (left to right) show the F340/380 ratio at basal (i; 0 min), the maximal amplitude of GnRH2 stimulation (ii; ~10 min), following the washout of treatments (iii; ~18 min), the maximal amplitude of a subsequent 30 mM KCl-induced depolarization (iv; ~24 min), and following a final washout (v; ~30 min). As quantified in Figure 3.9, similar results were observed using pretreatment with LY294002 (10 μM; not shown). Ratio values were collected every 15 sec and correspond to the $[Ca^{2+}]_i$ values provided in the color scheme on the far right (approximately 0-700 nM $[Ca^{2+}]_i$). A depolarizing dose of KCl (30 mM) at the end of each sequence ensures that cells remained healthy and responsive following experimental treatments (refer to Table 3.3 for the quantified KCl-induced $[Ca^{2+}]_i$ responses).

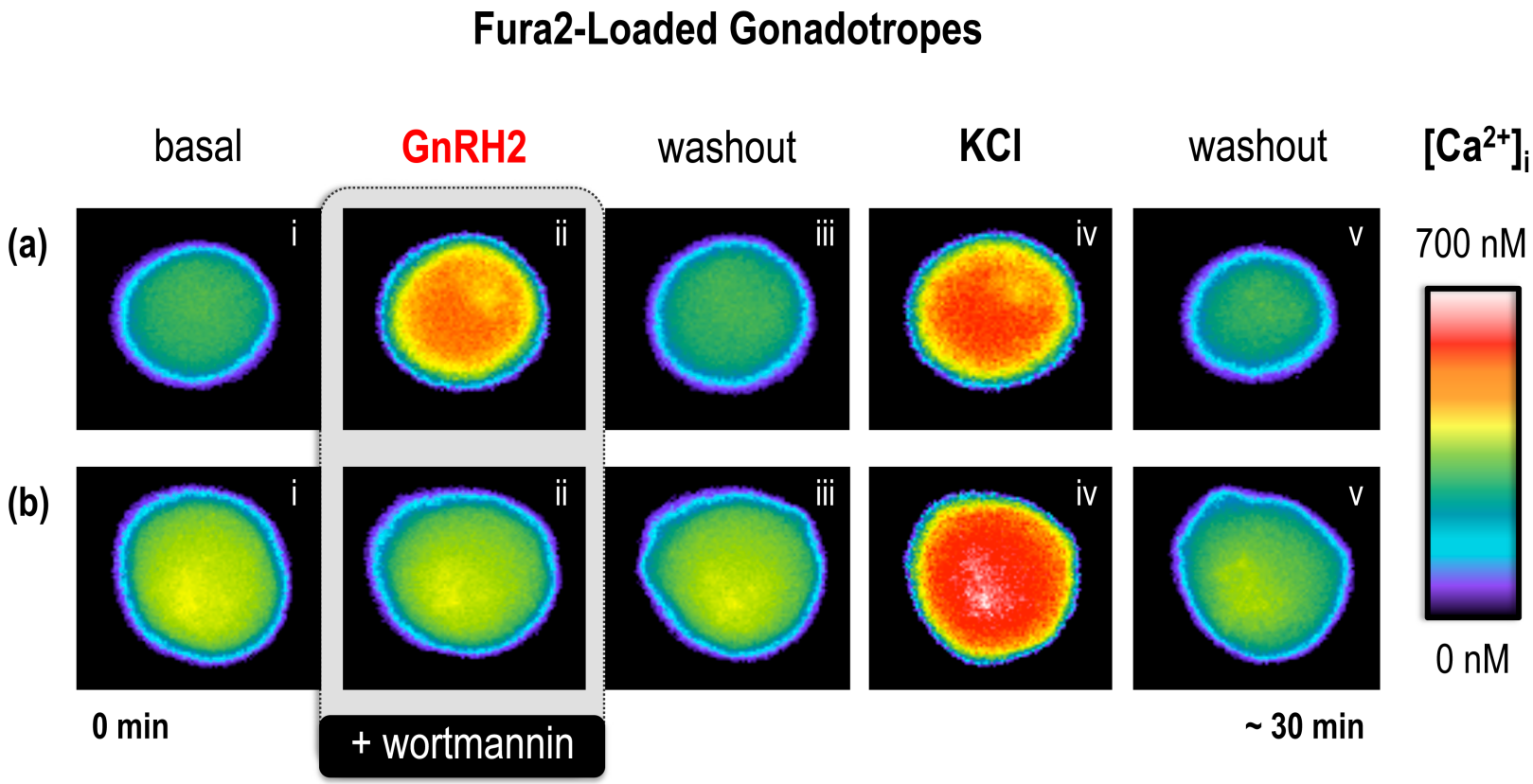

Fura2-Loaded Gonadotropes
basal
GnRH2
washout
KCl
washout
[Ca2+]i
(a)
(b)
i
ii
iii
iv
v
700 nM
0 nM
0 min
+ wortmannin
~ 30 min

Figure 3.11 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH3 (100 nM) induced $[Ca^{2+}]_i$ changes in morphologically identified gonadotropes. Ca^{2+} profiles are shown on the left panel and quantified net Ca^{2+} responses are shown on the right (mean ± SEM; $PI3K^-$ denotes inhibitor alone treatmen). Results were expressed as a percentage of pretreatment values (% pretreatment, average of the first five images collected; 113.92 ± 8.55 nM). Pooled responses are shown. The solid gray horizontal bar indicates duration of inhibitor treatment while the blue horizontal bar indicates the 2-min GnRH3 exposure (gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor). Duration of net $[Ca^{2+}]_i$ response quantification is indicated by the vertical dotted lines. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). Gonadotropes were imaged from three to seven cell preparations and covered all stages of the seasonal reproductive year (n = 13, GnRH3 alone; 14, wortmannin alone; 18, GnRH3 + wortmannin; 10, LY294002 alone; 13, GnRH3 + LY294002). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

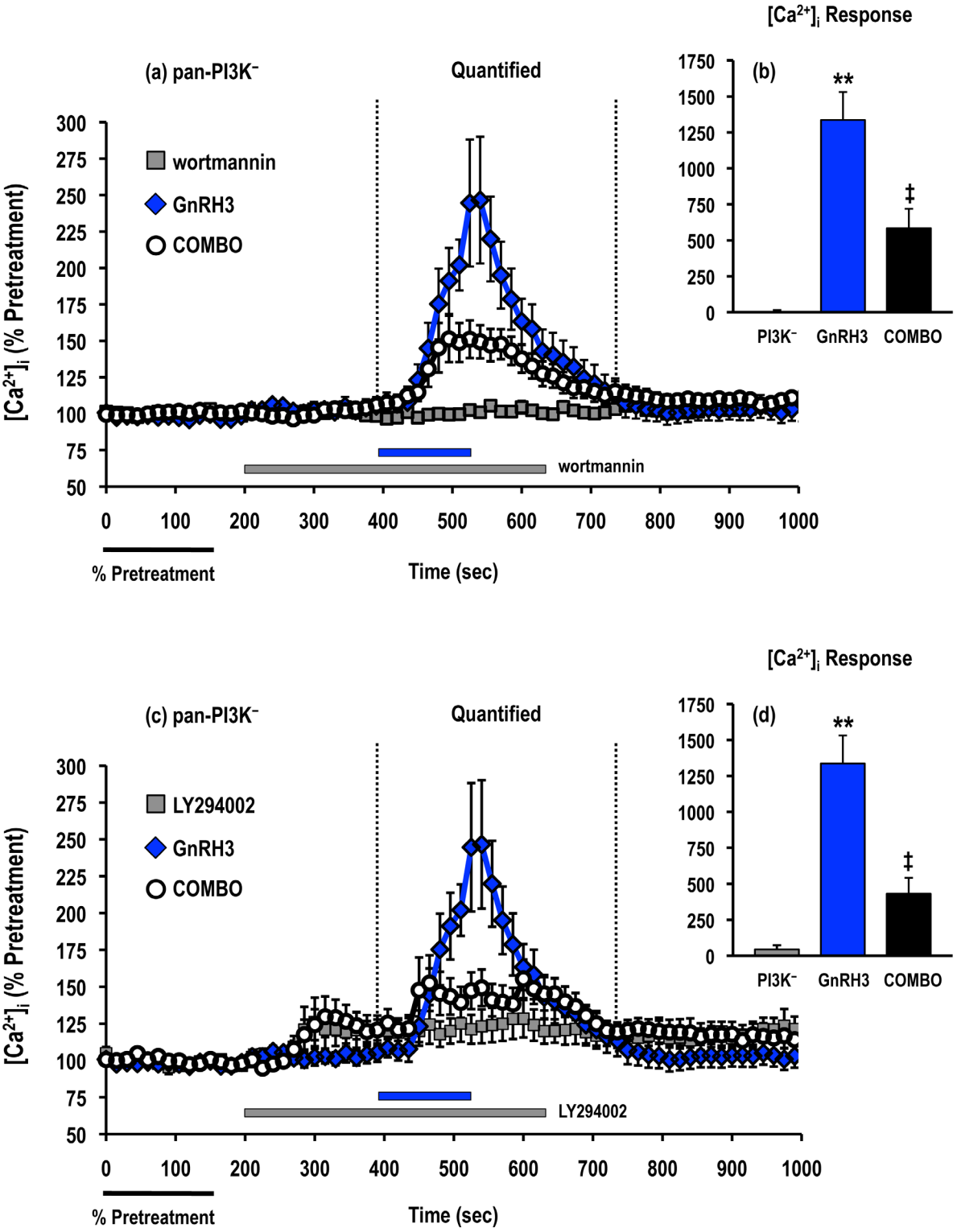

[Ca2+]i Response
(a) pan-PI3K-
Quantified
(b)
wortmannin
GnRH3
COMBO
wortmannin
[Ca2+]i (% Pretreatment)
% Pretreatment
Time (sec)
PI3K-
GnRH3
COMBO
**
‡
[Ca2+]i Response
(c) pan-PI3K-
Quantified
(d)
LY294002
GnRH3
COMBO
LY294002
[Ca2+]i (% Pretreatment)
% Pretreatment
Time (sec)
PI3K-
GnRH3
COMBO
**
‡

Figure 3.12 Representative pseudo-color images demonstrating the effects of the pan-PI3K inhibitor wortmannin (100 nM; b) on GnRH3 (100 nM; a) induced increases in $[Ca^{2+}]_i$ from a single morphologically identified gonadotrope. (a; top row) GnRH3-stimulated changes in the whole-cell Ca^{2+} signal and (b; bottom row) the effects of wortmannin pretreatment on GnRH2 induced increases in $[Ca^{2+}]_i$. Images (left to right) show the F340/380 ratio at basal (i; 0 min), the maximal amplitude of GnRH3 stimulation (ii; ~10 min), following the washout of treatments (iii; ~18 min), the maximal amplitude of a subsequent 30 mM KCl-induced depolarization (iv; ~24 min), and following a final washout (v; ~30 min). As quantified in Figure 3.11, similar results were observed using pretreatment with LY294002 (10 μM; not shown). Ratio values were collected every 15 sec and correspond to the $[Ca^{2+}]_i$ values provided in the color scheme on the far right (approximately 0-700 nM $[Ca^{2+}]_i$). A depolarizing dose of KCl (30 mM) at the end of each sequence ensures that cells remained healthy and responsive following experimental treatments (refer to Table 3.3 for the quantified KCl-induced $[Ca^{2+}]_i$ responses).

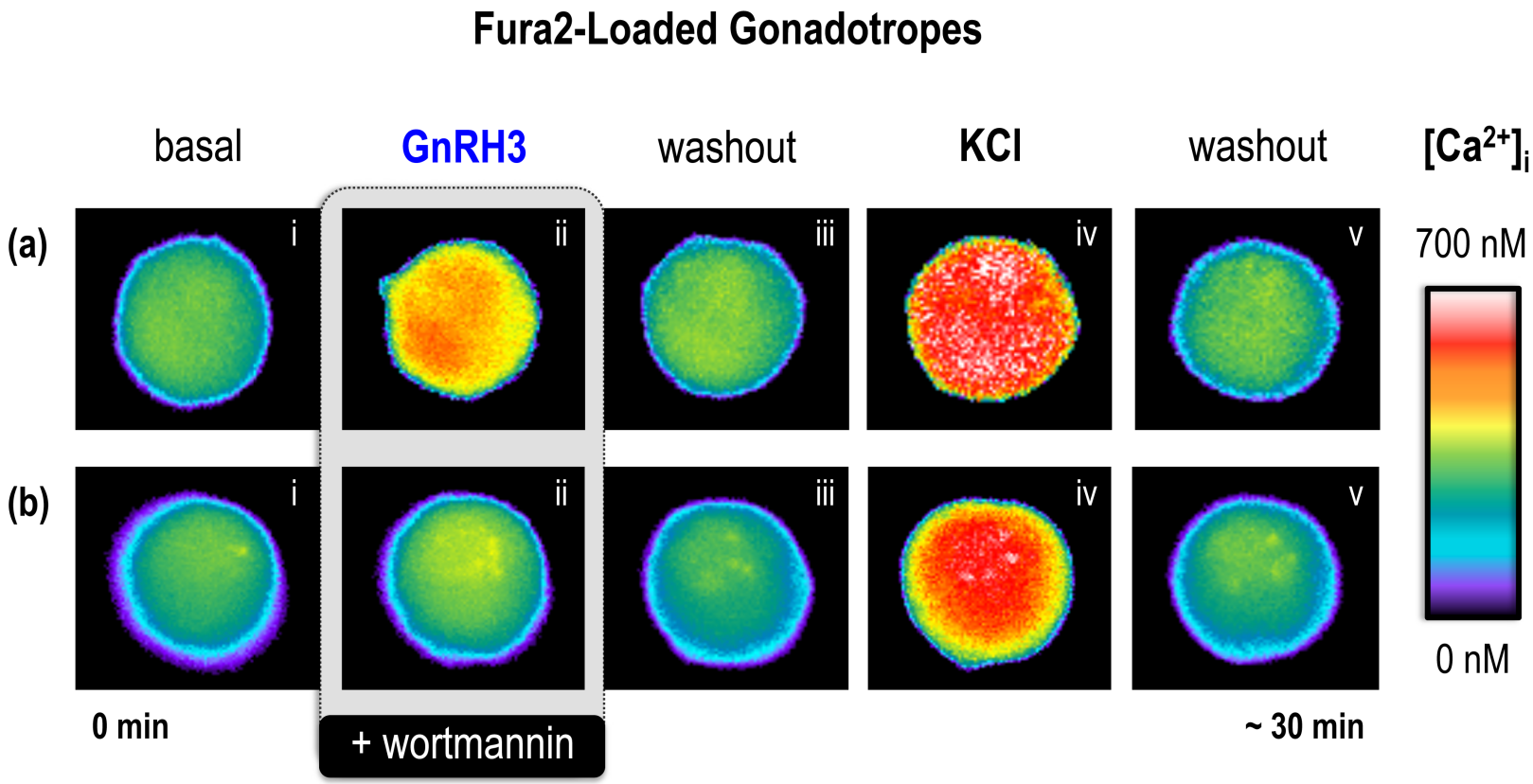

Fura2-Loaded Gonadotropes
basal
GnRH3
washout
KCl
washout
[Ca2+]i
(a)
(b)
i
ii
iii
iv
v
700 nM
0 nM
0 min
+ wortmannin
~ 30 min

Figure 3.13 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH2 (100 nM) induced $[Ca^{2+}]_i$ changes in morphologically identified somatotropes. Ca^{2+} profiles are shown on the left panel and quantified net Ca^{2+} responses are shown on the right (mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Results were expressed as a percentage of pretreatment values (% pretreatment, average of the first five images collected; 46.48 ± 4.67 nM). Pooled responses are shown. The solid gray horizontal bar indicates duration of inhibitor treatment while the red horizontal bar indicates the 2-min GnRH2 exposure (gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor). Duration of net $[Ca^{2+}]_i$ response quantification is indicated by the vertical dotted lines. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). Somatotropes were imaged from three to seven cell preparations and covered all stages of the seasonal reproductive year (n = 12, GnRH2 alone; 13, wortmannin alone; 9, GnRH2 + wortmannin; 8, LY294002 alone; 12, GnRH2 + LY294002). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

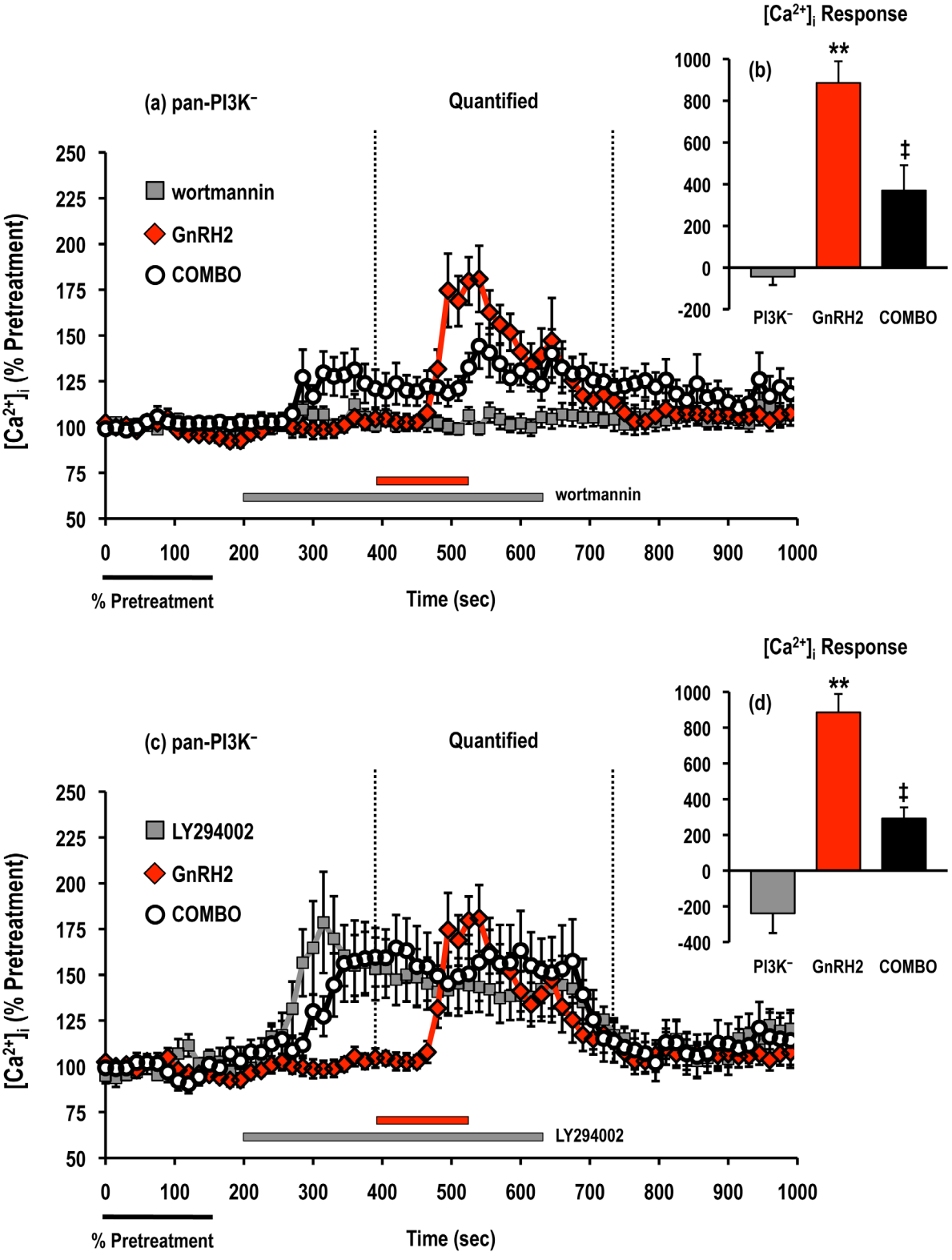

(a) pan-PI3K⁻
Quantified
wortmannin
GnRH2
COMBO
wortmannin
[Ca2+]i (% Pretreatment)
Time (sec)
% Pretreatment
[Ca2+]i Response
(b)
PI3K⁻
GnRH2
COMBO
(c) pan-PI3K⁻
Quantified
LY294002
GnRH2
COMBO
LY294002
(d)

Figure 3.14 Representative pseudo-color images demonstrating the effects of the pan-PI3K inhibitor wortmannin (100 nM; b) on GnRH2 (100 nM; a) induced increases in $[Ca^{2+}]_i$ from a single morphologically identified somatotrope. (a; top row) GnRH2-stimulated changes in the whole-cell Ca^{2+} signal and (b; bottom row) the effects of wortmannin pretreatment on GnRH2 induced increases in $[Ca^{2+}]_i$. Images (left to right) show the F340/380 ratio at basal (i; 0 min), the maximal amplitude of GnRH2 stimulation (ii; ~10 min), following the washout of treatments (iii; ~18 min), the maximal amplitude of a subsequent 30 mM KCl-induced depolarization (iv; ~24 min), and following a final washout (v; ~30 min). As quantified in Figure 3.13, similar results were observed using pretreatment with LY294002 (10 μM; not shown). Ratio values were collected every 15 sec and correspond to the $[Ca^{2+}]_i$ values provided in the color scheme on the far right (approximately 0-700 nM $[Ca^{2+}]_i$). A depolarizing dose of KCl (30 mM) at the end of each sequence ensures that cells remained healthy and responsive following experimental treatments (refer to Table 3.3 for the quantified KCl-induced $[Ca^{2+}]_i$ responses).

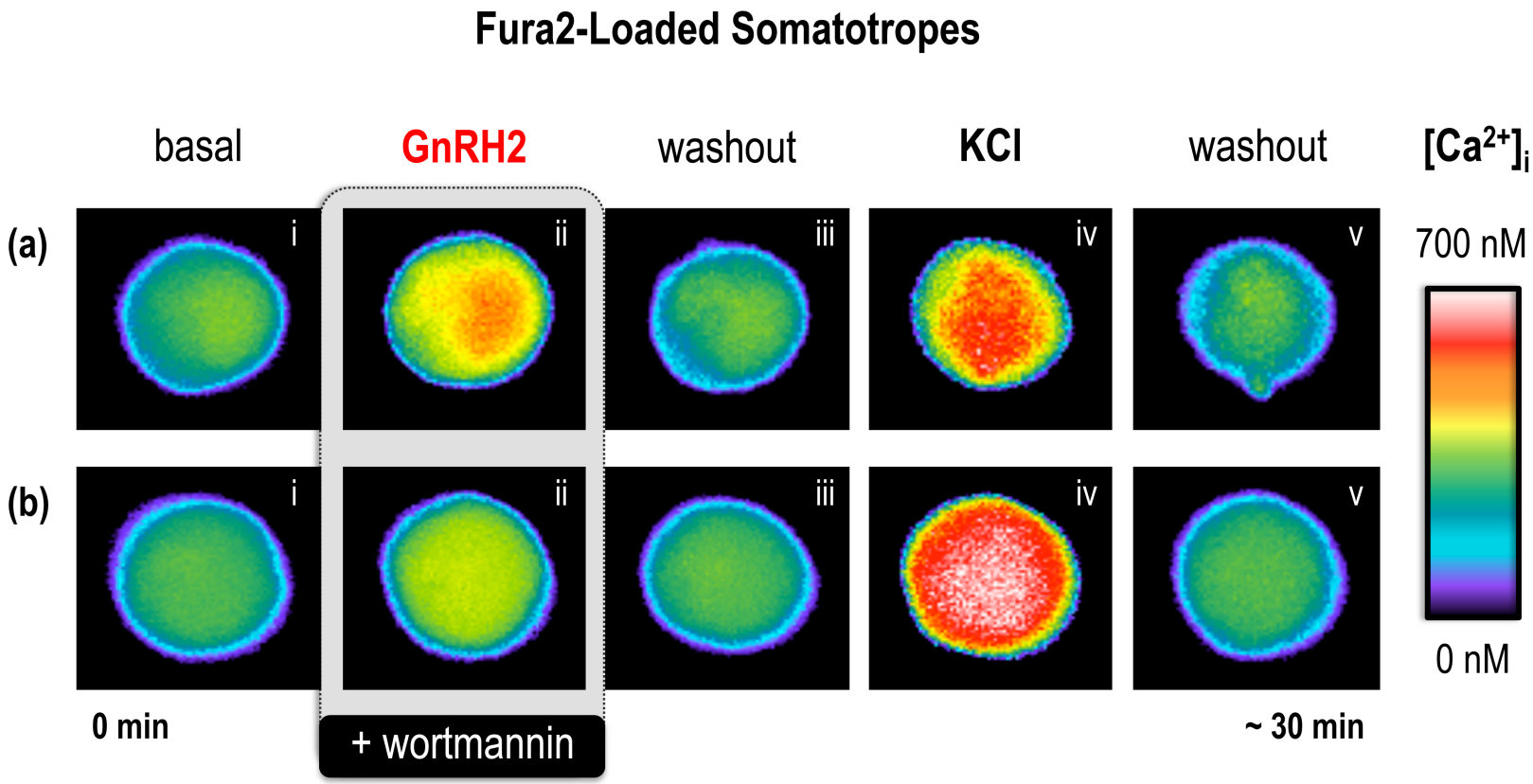

Fura2-Loaded Somatotropes
basal
GnRH2
washout
KCl
washout
[Ca2+]i
(a)
(b)
i
ii
iii
iv
v
700 nM
0 nM
0 min
+ wortmannin
~ 30 min

Figure 3.15 Effects of pan-PI3K inhibitors wortmannin (100 nM; a,b) and LY294002 (10 μM; c,d) on basal and GnRH3 (100 nM) induced $[Ca^{2+}]_i$ changes in morphologically identified somatotropes. Ca^{2+} profiles are shown on the left panel and quantified net Ca^{2+} responses are shown on the right (mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Results were expressed as a percentage of pretreatment values (% pretreatment, average of the first five images collected; 48.29 ± 4.18 nM). Pooled responses are shown. The solid gray horizontal bar indicates duration of inhibitor treatment while the blue horizontal bar indicates the 2-min GnRH3 exposure (gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor). Duration of net $[Ca^{2+}]_i$ response quantification is indicated by the vertical dotted lines. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). Somatotropes were imaged from three to seven cell preparations and covered all stages of the seasonal reproductive year (n = 8, GnRH3 alone; 13, wortmannin alone; 11, GnRH3 + wortmannin; 8, LY294002 alone; 14, GnRH3 + LY294002). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

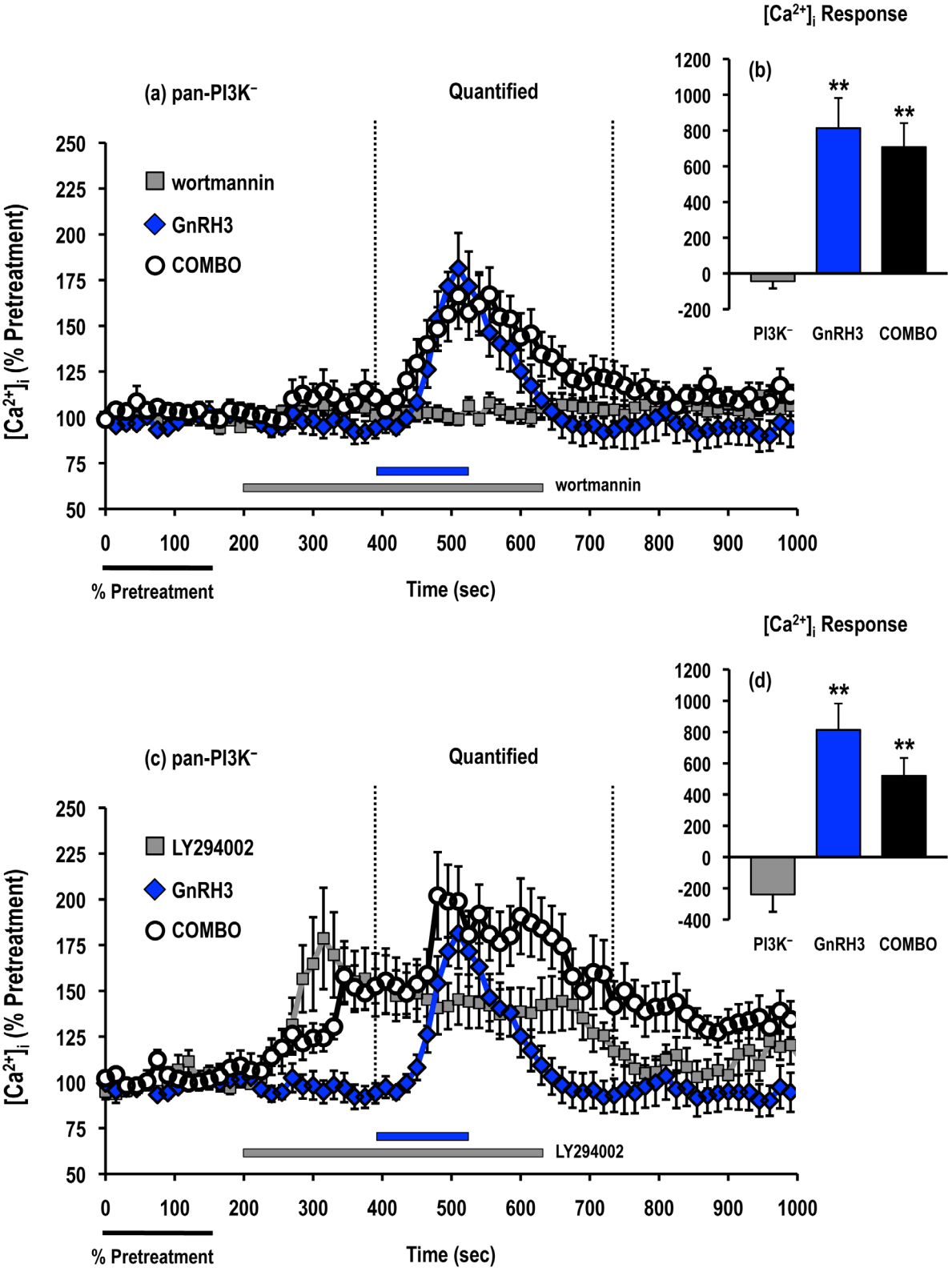

[Ca2+]i Response
(a) pan-PI3K⁻
Quantified
(b)
wortmannin
GnRH3
COMBO
[Ca2+]i (% Pretreatment)
wortmannin
% Pretreatment
Time (sec)
PI3K⁻
GnRH3
COMBO
**
**
(c) pan-PI3K⁻
Quantified
(d)
LY294002
GnRH3
COMBO
LY294002
% Pretreatment
Time (sec)

Figure 3.16 Representative pseudo-color images demonstrating the effects of the pan-PI3K inhibitor wortmannin (100 nM; b) on GnRH3 (100 nM; a) induced increases in $[Ca^{2+}]_i$ from a single morphologically identified somatotrope. (a; top row) GnRH3-stimulated changes in the whole-cell Ca^{2+} signal and (b; bottom row) the effects of wortmannin pretreatment on GnRH2 induced increases in $[Ca^{2+}]_i$. Images (left to right) show the F340/380 ratio at basal (i; 0 min), the maximal amplitude of GnRH3 stimulation (ii; ~10 min), following the washout of treatments (iii; ~18 min), the maximal amplitude of a subsequent 30 mM KCl-induced depolarization (iv; ~24 min), and following a final washout (v; ~30 min). As quantified in Figure 3.15, similar results were observed using pretreatment with LY294002 (10 μM; not shown). Ratio values were collected every 15 sec and correspond to the $[Ca^{2+}]_i$ values provided in the color scheme on the far right (approximately 0-700 nM $[Ca^{2+}]_i$). A depolarizing dose of KCl (30 mM) at the end of each sequence ensures that cells remained healthy and responsive following experimental treatments (refer to Table 3.3 for the quantified KCl-induced $[Ca^{2+}]_i$ responses).

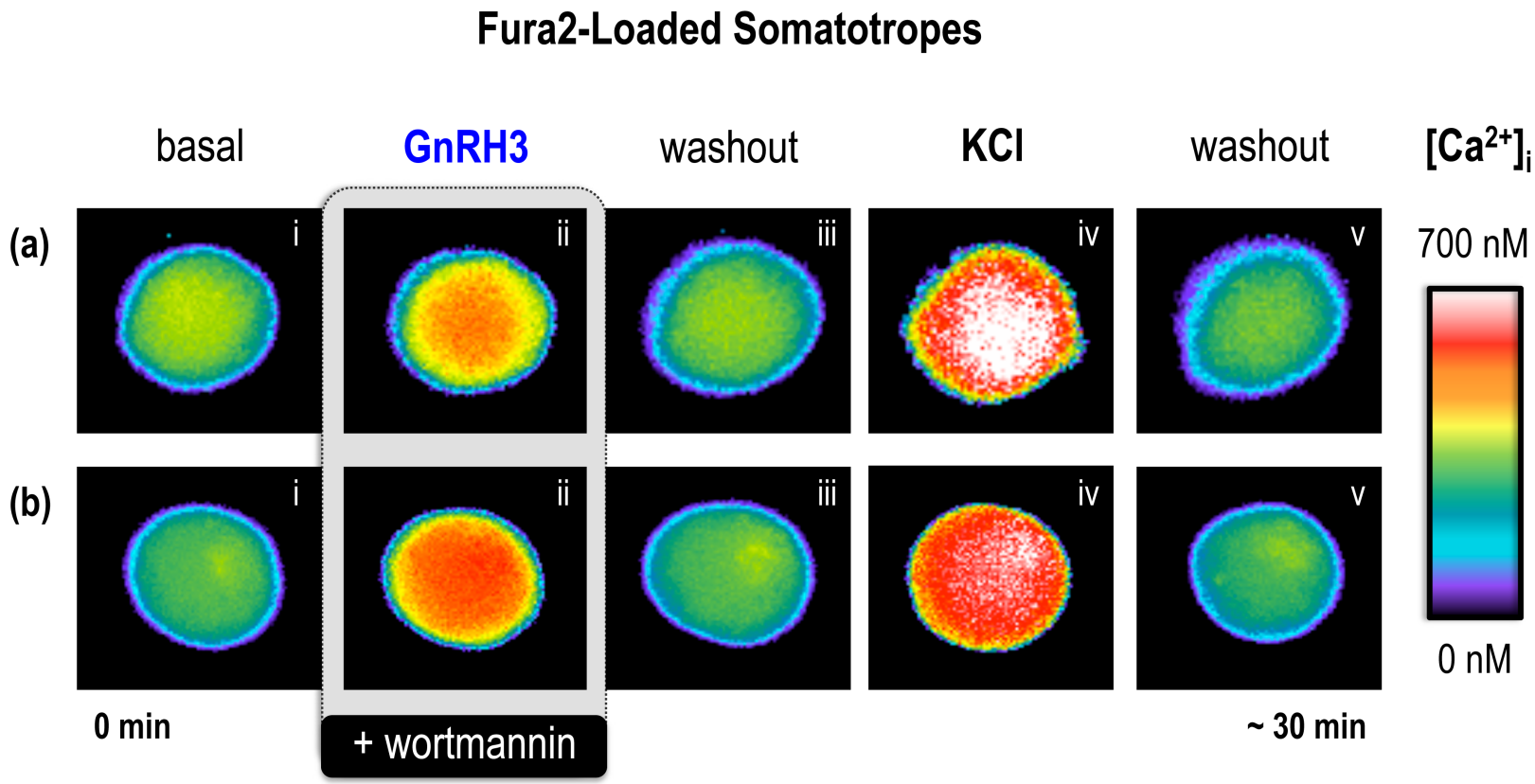

Fura2-Loaded Somatotropes
basal
GnRH3
washout
KCl
washout
[Ca2+]i
(a)
(b)
i
ii
iii
iv
v
700 nM
0 nM
0 min
+ wortmannin
~ 30 min

Figure 3.17 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on LH release responses stimulated by the novel and conventional PKC activators TPA (100 nM; a,b) and DiC8 (100 μM; c,d). LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; orange solid diamond, PKC activator alone; open circle, PKC activator + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 1.80 ± 0.10 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with either regressed gonads or gonads undergoing sexual recrudescence (June and August). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the orange horizontal bar represents the 5-min exposure to either PKC activator. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

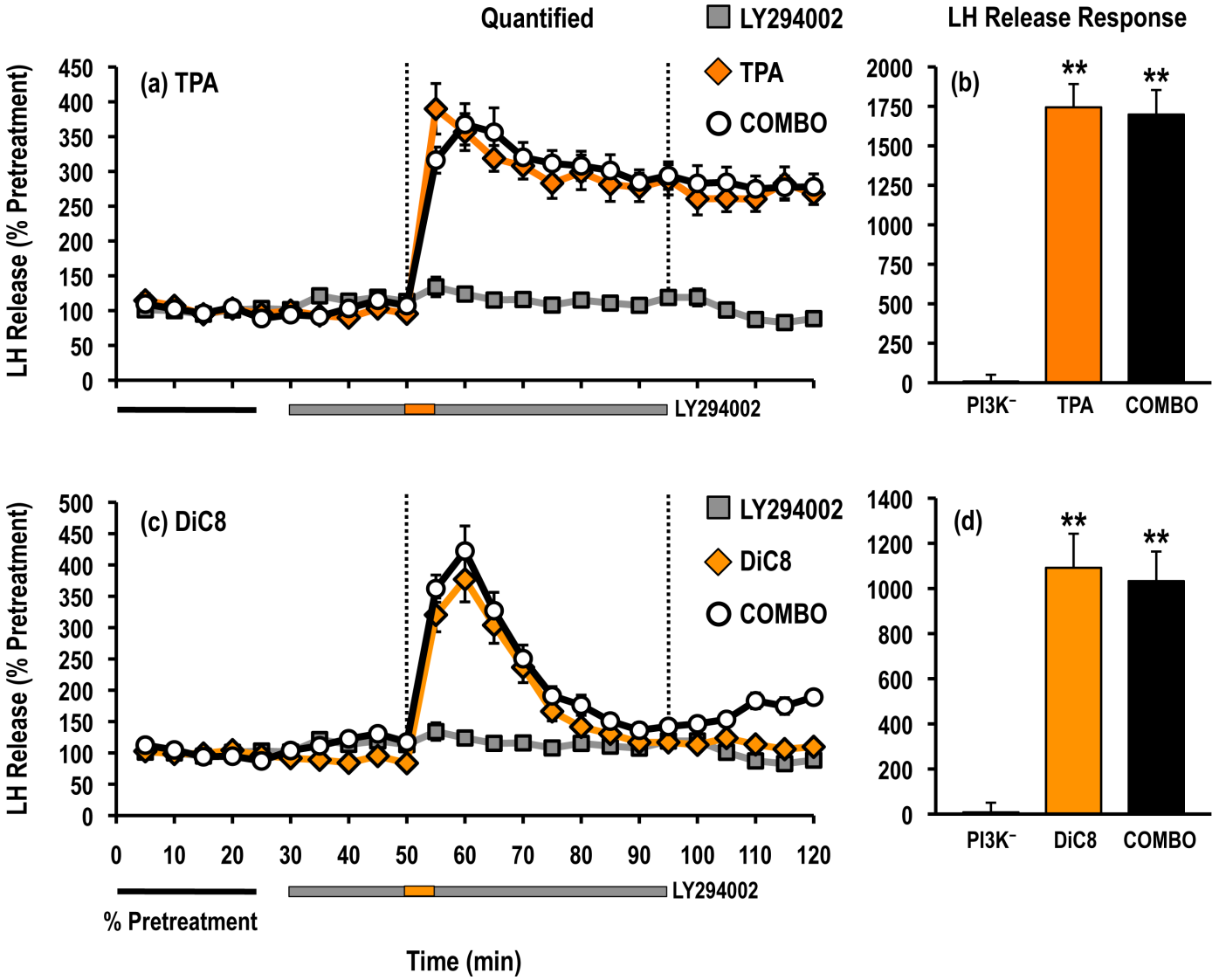

Quantified
LY294002
LH Release Response
(a) TPA
TPA
COMBO
LH Release (% Pretreatment)
LY294002
(b)
**
**
PI3K⁻
TPA
COMBO
(c) DiC8
LY294002
DiC8
COMBO
(d)
**
**
PI3K⁻
DiC8
COMBO
% Pretreatment
Time (min)

Figure 3.18 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on GH release responses stimulated by the novel and conventional PKC activators TPA (100 nM; a,b) and DiC8 (100 μM; c,d). GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; orange solid diamond, PKC activator alone; open circle, PKC activator + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; $PI3K^-$ denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 4.30 ± 0.30 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with either regressed gonads or gonads undergoing sexual recrudescence (June and August). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the orange horizontal bar represents the 5-min exposure to either PKC activator. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of the pan-PI3K inhibitors, refer to Table 3.1.

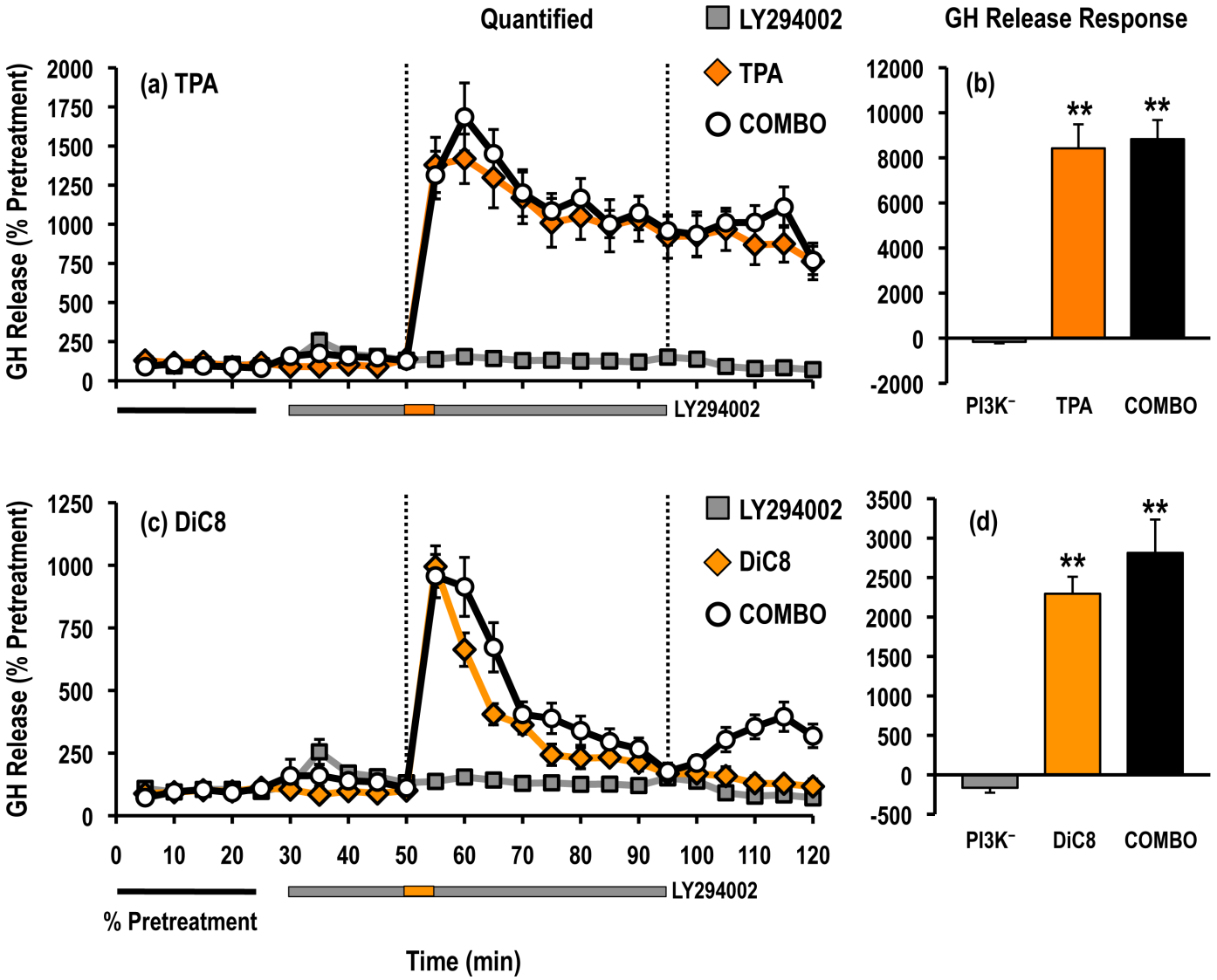

Quantified
LY294002
GH Release Response
(a) TPA
TPA
COMBO
GH Release (% Pretreatment)
2000
1750
1500
1250
1000
750
500
250
0
LY294002
(b)
12000
10000
8000
6000
4000
2000
0
-2000
**
**
PI3K⁻
TPA
COMBO
(c) DiC8
LY294002
DiC8
COMBO
1250
1000
750
500
250
0
0
10
20
30
40
50
60
70
80
90
100
110
120
LY294002
% Pretreatment
Time (min)
(d)
3500
3000
2500
2000
1500
1000
500
0
-500
**
**
PI3K⁻
DiC8
COMBO

Figure 3.19 Summary model depicting the integration of PI3Ks with PKC- and Ca^{2+}-dependent signalling during GnRH-stimulated LH release from goldfish gonadotropes. Results presented in this Chapter suggest that PI3Ks are involved in the transduction step(s) proximal to increases in $[Ca^{2+}]_i$ during GnRH2 and GnRH3 stimulation of LH release. Whether the PI3K-dependent step(s) affect extracellular Ca^{2+} entry and/or release of Ca^{2+} from intracellular stores in these cells is not known. However, the inability of pan-PI3K inhibitors to affect ionomycin-induced LH release suggests that PI3K involvement during basal secretion is not at steps downstream of $[Ca^{2+}]_i$ increases and, at this time, we cannot exclude the possibility that GnRHR-mediated activation of PI3Ks may also lead to intracellular events that are independent of Ca^{2+}-dependent signalling. Furthermore, activation of conventional or novel PKC isoforms is not upstream of PI3K-dependent signalling in goldfish gonadotropes during acute LH release responses; although, other relationships between PKC- and PI3K-dependent signalling have yet to be explored. Lastly, these data strongly implicate a role for the receptor-activated class I PI3Ks, although it is possible that the class II and/or class III PI3Ks may also contribute to the control of GnRH actions.

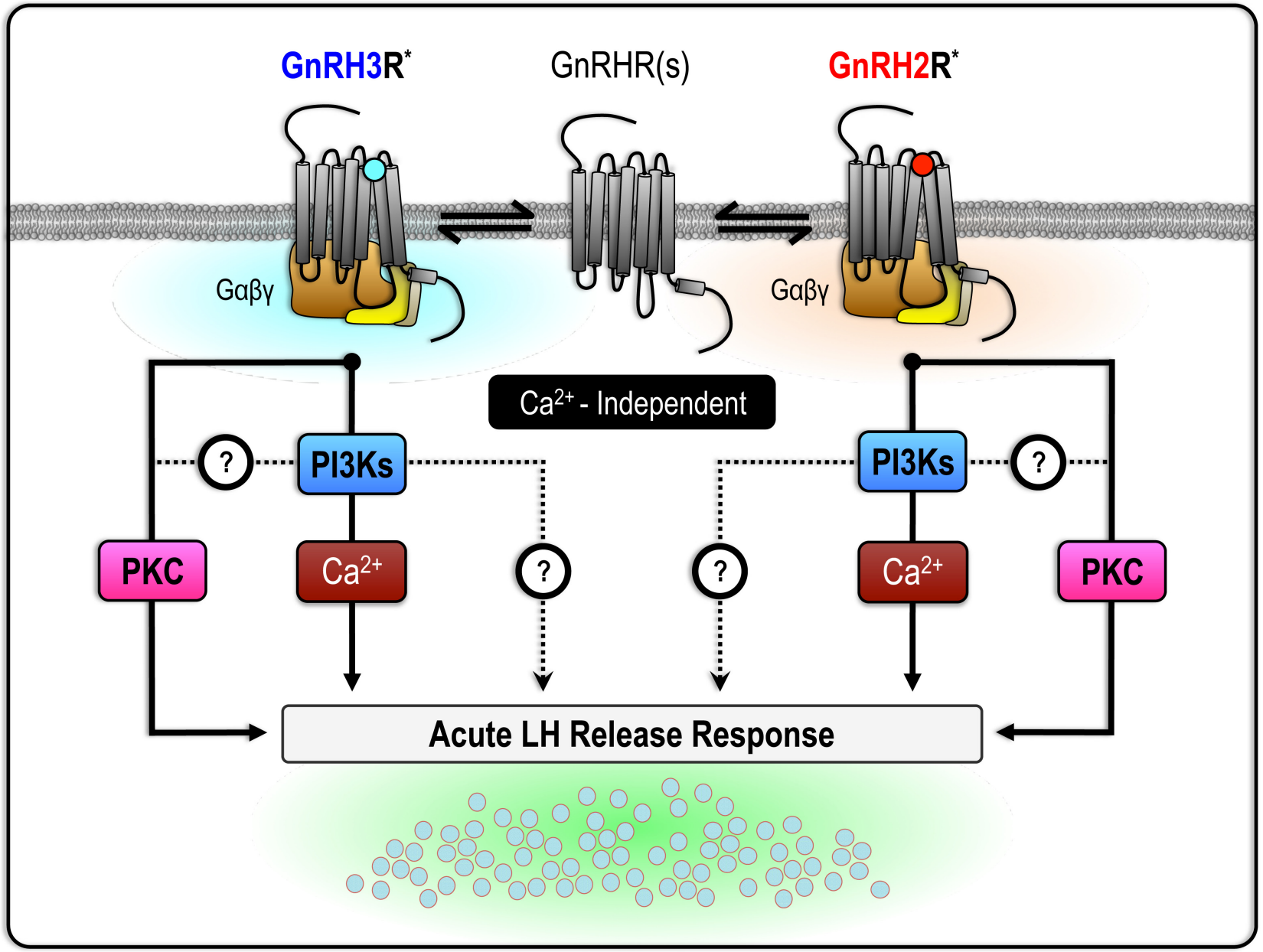
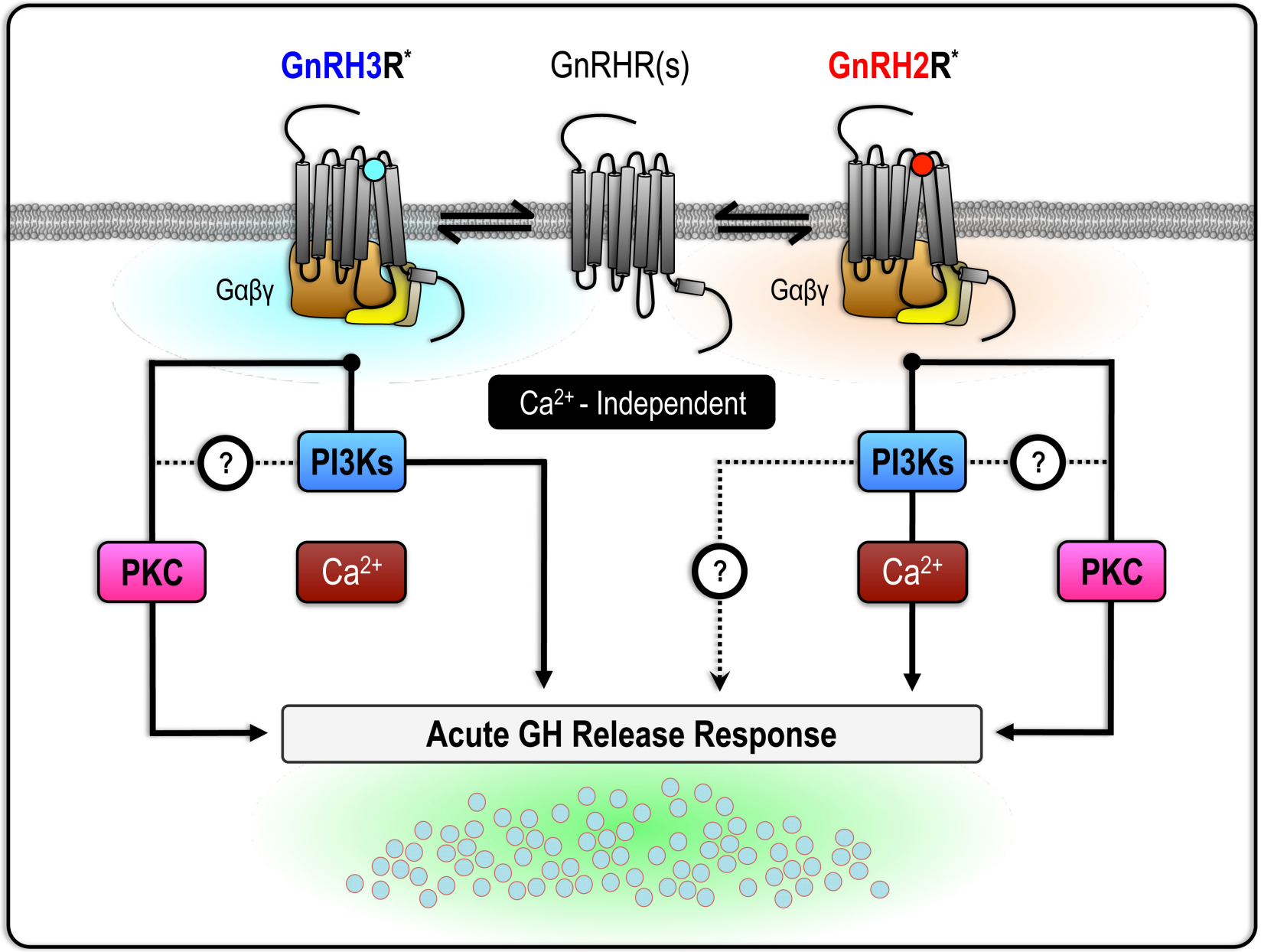
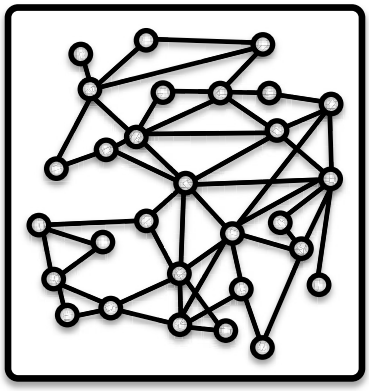

GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
Ca2+ - Independent
?
PI3Ks
PI3Ks
?
PKC
Ca2+
?
?
Ca2+
PKC
Acute LH Release Response

Figure 3.20 Summary model depicting the integration of PI3Ks with PKC- and Ca^{2+}-dependent signalling during GnRH-stimulated GH release from goldfish somatotropes. Results presented in this Chapter provide the first evidence for the differential activation of PI3K-dependent signalling in GnRH-induced hormone release responses. In particular, our findings suggest that PI3Ks are involved in the transduction step(s) proximal to increases in $[Ca^{2+}]_i$ during GnRH2, but not GnRH3, stimulation of GH release. Whether the PI3K-dependent step(s) affect extracellular Ca^{2+} entry and/or release of Ca^{2+} from intracellular stores in these cells is not known, . However, the inability of pan-PI3K inhibitors to affect ionomycin-induced GH release suggests that PI3K involvement during basal secretion is not at steps downstream of $[Ca^{2+}]_i$ increases and activation of PI3Ks may also lead to intracellular events that are independent of Ca^{2+}-dependent signalling. Furthermore, activation of conventional or novel PKC isoforms is not upstream of PI3K-dependent signalling in goldfish somatotropes during acute GH release responses; although, other relationships between PKC- and PI3K-dependent signalling have yet to be explored. Lastly, these data strongly implicate a role for the receptor-activated class I PI3Ks, although it is possible that the class II and/or class III PI3Ks may also contribute to the control of GnRH actions.


GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
Ca2+ - Independent
?
PI3Ks
PKC
Ca2+
?
PI3Ks
Ca2+
?
PKC
Acute GH Release Response

Chapter Four:

Distinct class I PI3K isoforms contribute to the differential control of basal and GnRH-stimulated hormone secretion



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

4.1 Introduction

Results from Chapter 3 provided the first evidence for the involvement of PI3Ks in the control of GnRH-stimulated LH and GH release. Although these observations strongly implicate the participation of classical receptor-mediated class I PI3K isoforms, they do not exclude the involvement of other intracellular targets of these pan-PI3K inhibitors in the control of GnRH actions, such as the lesser-studied class II and III PI3Ks or members of the PI3K-related kinase family including TOR. In this thesis Chapter, I investigated if GnRH-selective utilization of class I PI3K catalytic isoform(s), including those that are directly coupled to the activation of GPCRs and the dissociation of Gβγ heterodimers, is involved in the differential control of GnRH actions on hormone release. I first examined the expression of the regulatory and catalytic subunits of class I PI3Ks in primary cultures of goldfish pituitary cells using subunit-selective primary antibodies. Tyrosine phosphorylation of the p85-type regulatory subunit was also monitored as an index of the ability of GnRH to activate class IA PI3K signalling, and the effects of GnRH and PKC activators on p85 phosphorylation were compared. The effects of highly specific small molecule inhibitors of the class IA (p110α-selective; BYL719; Fritsch et al., 2014; p110β-selective; TGX221; Jackson et al., 2005; and p110δ-selective; IC87114; Sadhu et al., 2003) and class IB (p110γ-selective; CZC24832; Bergamini et al., 2012) catalytic subunits on basal, as well as GnRH2- and GnRH3-stimulated LH and GH release from primary cultures of goldfish pituitary cells were also evaluated in cell column perifusion hormone release experiments. Lastly, in order to determine if Gβγ heterodimers, the upstream regulators of p110β and p110γ (Dbouk et al., 2012; Vadas et al., 2013), contributed to GnRH-dependent signal transduction in goldfish, GnRH-

stimulated hormone release was monitored in the presence of a selective inhibitor of Gβγ-effector interactions (NSC8668; Lehmann et al., 2008; Bonacci et al., 2006).

4.2 Results

4.2.1 Immunoreactive regulatory and catalytic subunits of both the class IA and IB PI3Ks are expressed in goldfish pituitary cell cultures

To our knowledge, expression of immunoreactive class I PI3K regulatory or catalytic subunits has never been examined in goldfish tissues; although orthologs of p85, p55, p101, p110α, p110β, p110γ, and p110δ subunits are present in all vertebrate models examined, including the closely related cyprinid zebrafish (Engelman et al., 2006; Brown and Auger, 2011). As an initial indication of the class IA and IB isoforms that might be involved in goldfish pituitary cell signalling, the presence of the p85, p55, and p101 regulatory subunits, as well as the p110α, p110β, p110γ, and p110δ catalytic subunits was examined by Western blot using subunit-selective primary antibodies. As expected, protein extracts from a representative mammalian cell line, RBL-2H3 mast cells, known to express all of the p110 catalytic subunits as well as p85-, p55-, and p101-type regulatory subunits (Andrade et al., 2004; Ali et al., 2004), reacted positively to all seven primary antibodies (Figure 4.1 and 4.2). Immunoreactive bands for p110β, p110γ, and p110δ, but not p110α, were observed in goldfish pituitary cell extracts and corresponded with the bands observed in the RBL-2H3 cells at approximately 110 kDa (Figure 4.1b and Figure 4.2b). Goldfish pituitary cell extracts also reacted positively to the antibody against the p85-type regulatory subunits that couple to the class IA PI3K catalytic subunits p110α, p110β, and p110δ (Figure 4.1a). Due to sequence similarity,

immunoreactivity detected using the pan-p85 antibody would represent either p85α (PIK3R1) or p85β (PIK3R2) regulatory subunits. Immunoreactivity to p55-type (PIK3R3) class IA regulatory subunits was not detected in goldfish pituitary extracts (Figure 4.1a). Immunoreactive p85 was of an appropriate molecular mass (estimated at approximately 85 kDa) and corresponded to the bands observed for the RBL-2H3 cells and the recombinant full-length p85α peptide (N-terminal His tag; expected 86 kDa; Figure 4.3a). Pre-incubation of the primary antibody with a competing antigenic p85α peptide confirmed the specificity of p85α-like immunoreactivity in the goldfish pituitary (Figure 4.3b). Goldfish pituitary extracts also reacted positively to the antibody against the p110γ-specific class IB regulatory subunit p101 (PIK3R5; Figure 4.2a).

4.2.2 Endogenous GnRHs, but not synthetic PKC activators, increase phosphorylation of p85-type regulatory subunits in excised goldfish pituitary fragments

Phosphorylation of tyrosine residues within the p85 regulatory subunit is thought to contribute to the activation and localization of class IA PI3K signalling (Cuevas et al., 2001; Mellor et al., 2012). The capacity of endogenous GnRHs to induce tyrosine phosphorylation of immunoreactive goldfish p85 was investigated. Phosphorylated-p85 levels were determined using a primary antibody that recognizes human p85 phosphorylated at residue Tyr458 (Rush et al., 2005). Interestingly, phosphorylated p85 was present in unstimulated goldfish pituitary fragments (Figure 4.4a and 4.5a). In preliminary experiments, both endogenous GnRHs were consistently effective at increasing phosphorylation of p85 (Tyr458) at 30 min; while at 1 and 2 hr treatments,

GnRH2 was generally less effective than GnRH3 (data not shown). Thus, for quantification of GnRH actions, we chose a 30 min static treatment protocol. Treatment of excised pituitary fragments with either GnRH3 (100 nM) or GnRH2 (100 nM) for 30 min significantly increased phosphorylation of p85 (Figure 4.4). To evaluate the possibility of receptor-independent regulation of class IA PI3K signalling, we also investigated whether synthetic activators of PKC regulated phosphorylation of p85 in goldfish pituitary fragments. Unlike the endogenous GnRHs, treatment with maximal stimulatory doses of either TPA (100 nM; Chang et al., 1991) or DiC8 (100 μM; Chang et al., 1991) did not significantly change the levels of phosphorylation of p85 (Tyr458) in 30 min static incubations (Figure 4.5). This is in agreement with the hormone release data collected in Chapter 3; confirming that that activation of class IA PI3Ks is unlikely to occur downstream of conventional or novel PKCs during acute ligand-stimulated LH and GH release responses.

4.2.3 GnRH2- and GnRH3-stimulated LH and GH release involves the ligand-biased regulation of p110β, p110δ, and p110γ class I PI3K catalytic isoforms

Few studies have examined the involvement of PI3K-dependent signalling in GnRH actions on hormone release, and whether differential activation of unique PI3K isoforms mediates GnRH signal transduction is unknown. To assess the potential for differential involvement of class I PI3K signalling in GnRH isoform-selective actions, I examined the effects of subunit-specific small molecule inhibitors of the class I PI3K catalytic subunits p110α, p110β, p110δ, and p110γ on GnRH2- and GnRH3-stimulated LH and GH release responses. The p110 subunit specificity and experimental

concentrations used for each of the selective p110 isoform inhibitors are provided in Table 4.1. In general, the inhibitor concentrations used for perifusion studies were chosen based on previous studies of the efficacy and p110 subunit-selectivity in cell-based assays. Since Akt is a common downstream target of PI3K-dependent signalling and Akt activation is at least in part regulated by phosphorylation at Ser473 (reviewed in Chapter 1), the effectiveness of the selected inhibitors in goldfish pituitary cells were examined using phosphorylated Akt (Ser473) as an index of class I PI3K activation (Figure 4.6). Results show that isoform-selective class I PI3K inhibitors selectively alter basal phosphorylation of Akt (Ser473) in 2 hr static incubations. Briefly, selective inhibitors of p110β (TGX221; 0.05 μM; Figure 4.6b) and p110δ (IC87114, 5 μM; Figure 4.6b) both significantly reduced the basal levels of phosphorylated Akt (Ser473). Treatment with with the p110α-specific inhibitor (BYL719; 0.05 μM) tended to increase the expression of immunoreactive phosphorylated Akt(Ser473) but the responses were variable; no significant effects were observed with either BYL719 or the p110γ-selective inhibitor CZC24832 (0.5 μM) relative to untreated cells (Figure 4.6b).

As expected, maximally effective concentrations of GnRH2 (100 nM; Chang et al., 1990) and GnRH3 (100 nM; Chang et al., 1990) significantly stimulated acute LH (Figures 4.7 and 4.8) and GH release (Figures 4.9 and 4.10). The p110α-specific inhibitor (BYL719; 0.05 μM) did not alter GnRH2- or GnRH3-induced LH release responses (Figures 4.7 and 4.8). GnRH2-induced LH release was significantly reduced by selective inhibitors of p110β (TGX221; 0.05 μM; Figure 4.7c,d) and p110δ (IC87114; 5 μM; Figure 4.7e,f), but was not affected by the p110γ inhibitor (CZC24832; 0.5 μM; Figure 4.7g,h). In contrast, GnRH3-stimulated LH release was significantly reduced by selective

inhibitors of p110γ and p110δ (Figure 4.8e-h), but not by the p110β inhibitor (Figure 4.8c,d). GnRH3 and GnRH2 stimulation of GH release were unaffected by the p110α inhibitor (Figure 4.9a,b and 4.10a,b) while the GnRH2-stimulated GH release was reduced by the selective p110β (Figure 4.9c,d) and p110γ (Figure 4.9g,h) inhibitors, but not by the p110δ inhibitor (Figure 4.9e,f). On the other hand, the selective inhibitors of p110β (Figure 4.10c,d), p110δ (Figure 4.10e,f), and p110γ (Figure 4.11g,h) all significantly reduced GnRH3-stimulated GH release responses.

4.2.4 Involvement of PI3K p110 catalytic activity in the control of basal LH release and GH release

In goldfish pituitary cells, basal secretion and agonist-stimulated LH and GH release are regulated differently (Johnson et al., 2002; Johnson et al., 2003; Pemberton et al., 2013, 2014). In addition to being involved in GnRH-stimulated hormone release responses, basal LH and GH release was altered in a p110 subunit-selective manner. Inhibition of p110α, p110β, p110γ, and p110δ all significantly reduced basal LH release by between 15-20% (Figure 4.11). However, basal GH release was selectively reduced by inhibition of p110γ and p110δ, and by 20% and 13%, respectively (Figure 4.12).

4.2.5 Basal and GnRH-stimulated hormone release involves Gβγ-dependent signal transduction

GnRH actions have been speculated to involve Gβγ heterodimers (Naor and Huhtaniemi, 2013) but few studies have directly examined the involvement of Gβγ-dependent signal transduction downstream of GnRHR activation. To characterize the role

played by Gβγ heterodimers in the control of basal and GnRH-stimulated hormone release, we utilized the allosteric Gβγ inhibitor NSC8668 (Gallein; Lehmann et al., 2008). NSC8668 is a small molecule that can bind to Gβγ with sub-micromolar affinity to selectively modulate Gβγ-protein interactions (Lehmann et al., 2008; Bonacci et al., 2006). Importantly, NSC8668 can inhibit the interactions between Gβγ and holomeric p110γ, resulting in a loss of p110γ catalytic activity both *in vitro* and *in vivo* (Lehmann et al., 2008). Treatment with NSC8668 (10 μM) significantly reduced acute GnRH2- and GnRH3-stimulated LH and GH release responses measured in cell column perifusion (Figure 4.13 and 4.14). In addition, inhibition of Gβγ-effector interactions significantly decreased the average basal LH and GH release from goldfish pituitary cells by approximately 17% and 15%, respectively (Figure 4.15).

4.3 Discussion

Results presented in this study not only confirm previous findings that PI3Ks play a role in GnRH-induced LH and GH release (Chapter 3), but also demonstrates ligand-selective involvement of class I PI3K isoforms downstream of GnRHR activation for the first time, in any cell type. These novel findings suggest that GnRH2 and GnRH3 binding to GnRHRs can bias the activation of class I PI3K-dependent signalling to mediate hormone release responses. The involvement of both class IA and IB PI3Ks implicates Gβγ subunits, as well as other known regulators of class I PI3Ks, as important components of GnRHR-mediated responses that could also influence GnRH-selective signalling in other cell types. Lastly, hormone release data using NSC8668 provides the first direct physiological evidence for Gβγ-dependent signalling downstream of GnRHR

activation in any pituitary cell system, including mammals. This finding, coupled to the GnRH-selective reliance on p110β and p110γ observed during the control of hormone release responses, leads to the novel hypothesis that Gβγ-directed signalling bias might represent an unique mechanism for controlling ligand-selective signalling downstream of shared GPCRs. Just as importantly, results in this Chapter represent the first time that the presence and/or activity of holomeric class IA and IB PI3K isoforms has been demonstrated in any teleost endocrine tissues. Insights into the relationship between class I PI3Ks and Akt activation in intracellular signalling within goldfish pituitary cells are also gained.

4.3.1 Presence of holomeric class IA and IB PI3Ks in goldfish pituitary tissues and their differential activation by GnRH but not PKC

The regulatory subunits of class I PI3Ks are responsible for stabilizing the basal p110 catalytic activity and contribute to the subcellular localization as well as activation of PI3K-dependent signalling (Hawkins et al., 2006; Burke and Williams, 2015). The presence of p85-type and p101-type immunoreactivity, when taken together with results using catalytic subunit-selective antibodies and inhibitors, suggest that holomeric class IA and IB PI3Ks are present and are catalytically active under basal conditions in the goldfish pituitary. Additionally, both GnRH2 and GnRH3 increased phosphorylation of tyrosine residues within the p85 regulatory subunits expressed in goldfish pituitary fragments, suggesting elevated class IA p110 catalytic activity following GnRHR activation. These results are in agreement with studies using ATP-dependent p110 subunit inhibitors showing that GnRH3 and GnRH2 both utilized one or both of the class

IA p110β or p110δ catalytic subunits to stimulated LH and GH release responses (see Discussion sections below). Interestingly, although GnRH actions on hormone release from goldfish gonadotropes and somatotropes are dependent on PKC (reviewed in Chapter 1; Chang et al., 2009, 2012), activators of novel and conventional PKCs failed to alter tyrosine phosphorylation of p85-type regulatory subunits in goldfish pituitary tissues. These findings using TPA and DiC8 not only indicate that activation of class IA PI3K is stimuli-specific, but also support the conclusion put forth in Chapter 3 that PI3K-dependent signalling is upstream or independent of PKC activation in goldfish pituitary gonadotropes and somatotropes.

4.3.2 Involvement of p110β and p110γ in GnRH actions

Despite significant progress in characterizing the importance of isoform-selective PI3K signalling in regulating cellular functions, information regarding the involvement of PI3Ks in the control of endocrine secretion is sparse and is derived primarily from studies of insulin release using pancreatic β-cells (Taniguchi et al., 2006; Hirsch et al., 2007). However, the involvement of specific class I PI3K isoforms in the acute actions of GnRH on pituitary hormone release, and in the neuroendocrine control of pituitary hormone secretion, in general, has not been investigated. Results from the present Chapter indicate that GnRH-selective activation of LH and GH release responses involved the biased activation of class I PI3K isoforms. More specifically, GnRH2 stimulation of LH release involves p110β and p110δ catalytic activity but utilizes p110β and p110γ in GH release. In contrast, GnRH3 stimulation of LH release involves p110γ and p110δ, while it requires the kinase-dependent actions of p110β, p110γ, and p110δ in inducing GH

secretion. Differential activation of p110-dependent signalling is similar to studies of pancreatic β-cells that have outlined a prominent role for class I PI3K isoforms, including p110β and p110γ, in the control of insulin secretion. In particular, p110γ is an important determinant of insulin granule trafficking and exocytosis, in part by negatively regulating cortical F-actin density to promote secretory granule recruitment to the plasma membrane (Pigeau et al., 2009). In contrast, unlike p110γ, p110β facilitates glucose-stimulated insulin exocytosis independently of its catalytic activity, suggesting unique a kinase-independent role for p110β (Kolic et al., 2013). Furthermore, recent studies have demonstrated that insulin secretion induced by a GPCR agonist is p110γ-dependent (Kolic et al., 2014); however, the direct connection between GPCR activation and p110β-mediated signalling in insulin-secreting β-cells is still unknown.

How GnRHR activation leads to differential recruitment of class I PI3K isoforms has not been directly examined but heterotrimeric Gαβγ complexes are likely involved. GnRHRs interact predominantly with $G\alpha_{q/11}$ subunits in the majority of pituitary and extra-pituitary sites examined; however, there is also evidence for the use of $G\alpha_s$ and $G\alpha_i$ subunits in some models (Naor, 2009). In contrast to the influence of Gα subunits, the importance of Gβγ heterodimers in GnRH signalling has received little attention until now (Naor and Huhtaniemi, 2013; also see Discussion section 4.3.6 below). Work in goldfish pituitary cells has shown that GnRH actions on LH and GH release are dependent on PKC and intracellular Ca^{2+} signalling, supporting GnRHR coupling to $G\alpha_{q/11}$ and classical activation of PLCβ (reviewed in Chapter 1). Regardless of the Gα subunit family involved, Gβγ heterodimers will dissociate from the Gαβγ complex following GPCR activation and two of the established, downstream signalling targets of

Gβγ are the class I PI3K isoforms p110β and p110γ. Consistent with this hypothesis, GnRH2 and GnRH3 actions on hormone secretion are attenuated by inhibition of Gβγ-effector interactions using NSC8668. In addition, the catalytic activity of Gβγ-dependent class I PI3K isoforms, p110β and p110γ, is involved in GnRH2- and GnRH3-stimulated LH and GH release responses in a cell type-, and ligand-specific manner. These findings are also in agreement with previous work demonstrating that p110β and p110γ can couple to the same GPCR agonist (Guillermet-Guibert et al., 2008).

The molecular mechanisms controlling the agonist-selective activation of p110β and p110γ signalling downstream of GPCRs are unknown, but the subunit composition of the Gβγ heterodimer is thought to affect the coupling of Gβγ subunits to downstream signalling in other GPCR models (Albert and Robillard, 2002). This has been demonstrated for p110γ/p101 complexes, which display differential sensitivity to combinations of Gβ and Gγ subunits with the signalling specificity driven primarily by the identity of the Gγ subunit within the heterodimer (Kerchner et al., 2004). Similar data regarding the Gγ subunit selectivity of p110β/p85 complexes is not available, although the majority of cellular studies of p110β activation have used recombinant $\beta_1\gamma_2$ heterodimers. That said, in a more physiological setting, p110γ/p101 complexes show enhanced sensitivity for purified mixtures of bovine brain Gβγ when compared to p110β/p85; suggesting that p110γ/p101 also possesses higher affinity for endogenous Gβγ subunits (Maier et al., 2000). Recent reports examining Gβγ interaction surfaces of holomeric class IA p110β/p85 and class IB p110γ/p101 complexes provide important insights into the potential basis for selective activation of these PI3K isoforms by distinct Gβγ subunits. Direct contact of Gβγ with two lysines (K532 and K533) in the C2

domain-helical domain linker region of the p110β catalytic subunit is required for activation of p110β signalling (Dbouk et al., 2012). On the other hand, the homologous Gβγ interaction surface in the C2-helical loop of the p110γ catalytic subunit consists of a slightly different pair of basic residues (R552 and K553) and, unlike p110β/p85 complexes, the p101 regulatory subunit also interacts with Gβγ to reinforce the membrane-affinity of p110γ (Vadas et al., 2013). The presence of the ^{532}KK motif in p110β and the ^{552}RK motif in p110γ is highly conserved among different vertebrate species, including zebrafish (Dbouk et al., 2012; Vadas et al., 2013; Bradford et al., 2011; Figure 4.16). A recent sequence analysis of Gβ and Gγ subunits from teleost fish and mammalian species showed that when compared with plant and invertebrate models, vertebrate lineages share a larger and more diverse set of Gβ and Gγ subunits and, with minor exceptions, the Gβ and Gγ subunits from both fish and mammalian species appear to have evolved from the same common ancestor (Khan et al., 2013). When taken together, these findings support the idea that p110 isoform-specific coupling resulting from differential sensitivities to dissimilar Gβγ heterodimers could potentially lead to GnRH-selective activation of p110β and p110γ in goldfish.

Numerous reports have suggested that the closely related class IB regulatory subunits p101 (PIK3R5) and p84 (PIK3R6) can selectively control p110γ catalytic activity (Hawkins et al., 2006; Burke and Williams, 2015). Whether the differential activities of holomeric p110γ complexes are due to selective interactions with Gβγ heterodimers is controversial. The sensitivity of p110γ/p84 complexes to activation by Gβγ is much lower than that observed for p110γ/p101 (Suire et al., 2005), and some reports even suggest that the p110γ/p84 complex is actually insensitive to Gβγ (Kurig et

al., 2009). In the goldfish pituitary, we detected immunoreactive p101 and p110γ, but did not examine the expression of p84. However, based on the available data, p101 appears to be essential for Gβγ-dependent regulation of p110γ catalytic activity downstream of GPCR activation. Future studies examining the expression and cellular localization of these unique regulatory isoforms in gonadotropes and somatotropes will be important for understanding biased signalling by p110γ. That said, the unique regulation of p110γ-dependent signalling by p101 and p84 adds another layer of signal resolution that might contribute to discrete function-specific cellular responses.

Isoform-selective class I PI3K signalling has also been shown to involve distinct families of small GTPases that bind to the amino-terminal Ras-binding domain (RBD) present in all p110 catalytic subunits (Burke and Williams, 2015). Direct interactions between membrane-localized Ras and p110γ activates the lipid kinase activity of p110γ cooperatively with input from Gβγ heterodimers (Pacold et al., 2000; Suire et al., 2002). However, p110β is selectively activated by the Rho family small GTPases, Rac1 and Cdc42, but not Ras (Fritsch et al., 2013). Whether GnRH2 and GnRH3 selectively activate Ras or Rho family members is not known, although the involvement of Ras and Cdc42/Rac1 have been implicated in the control of GnRH actions in multiple cell lines (Naor, 2009). The idea of coordinate stimulation of both the p110β and p110γ isoforms by Gβγ heterodimers and small GTPases by GnRH is also consistent with the role of PI3Ks as important integrators within signal transduction networks (Pacold et al., 2000). Additionally, when co-expressed, p110β and p110γ have been shown to act in concert to provide full PI3K signalling activity downstream of a single GPCR agonist (Guillermet-Guibert et al., 2008). Overall, the present set of experiments constitute one of only a few

studies to suggest the involvement of Gβγ-dependent signalling, as well as the only study to implicate the participation of p110β and p110γ, in GnRH actions.

4.3.3 Participation of p110δ in GnRH actions

GnRH2 and GnRH3 actions on LH release, as well as GnRH3 stimulation of GH release, also require the class IA p110δ isoform that does not directly interact with Gβγ heterodimers. However, p110δ is activated downstream of chemokine receptors to control GPCR-dependent chemotaxis in natural killer cells and B cells, demonstrating that p110δ-dependent signalling can be regulated by agonist-stimulated class A GPCRs (Reif et al., 2004; Saudemont et al., 2009). In general, p110δ functions have been widely studied within the mammalian immune system since expression of p110δ is enriched in leukocytes and was once thought to be restricted to the hematopoietic system (Vanhaesebroeck et al., 1997). However, more recent work has demonstrated that the tissue expression pattern of p110δ is broader, including high levels of expression within the central nervous system (Eickholt et al., 2007; Gross and Bassell, 2014). Irrespective of its tissue expression pattern, few studies have examined the functions of p110δ signalling outside of well-characterized roles in the regulation of innate and adaptive immune responses (Okkenhaug, 2013), including, important effects on exocytic events during degranulation and cytokine release (Vanhaesebroeck et al., 2005; Ali et al., 2004; Soond et al., 2010). In contrast to mammalian systems, the physiological role(s) of p110δ in lower vertebrates is largely uncharacterized. Results in this Chapter demonstrate, for the first time, the presence and involvement of p110δ in the control of GnRH-stimulated signal transduction responses, in general, as well as in the regulation of pituitary hormone

release in any vertebrate model. Understanding how GnRHRs activate p110δ signalling is complicated by the fact that p110δ catalytic activity is regulated primarily by tyrosine-phosphorylated peptides and the Ras family small GTPases R-Ras and R-Ras2 (Rodriguez-Viciana et al., 2004; Burke et al., 2011). In mammalian models, GnRHR-mediated activation of tyrosine kinase-dependent signalling pathways and/or Ras family GTPases, can occur through transactivation of the epidermal growth factor (EGF) receptor (Grosse et al., 2000; Shah et al., 2006). As a result, regulation of p110δ signalling by GnRHR-mediated transactivation of receptor tyrosine kinases, activation of intracellular non-receptor tyrosine kinases, or by members of the Ras superfamily in goldfish gonadotropes and somatotropes are important avenues for future investigation. Additionally, it is important to realize that these mechanisms are not mutually exclusive and Gβγ-independent signalling could also contribute to activation of the class IA p110β/p85 or p110δ/p85 complexes to mediate GnRH-selective actions. On the other hand, there is mounting evidence suggesting that p110β is not a major effector downstream of tyrosine kinase signalling, but couples ubiquitously to GPCRs through interactions with Gβγ (Guillermet-Guibert et al., 2008).

4.3.4 Class I PI3Ks and basal secretion

Results from Chapter 3 have shown that basal PI3K activity exist in goldfish pituitary cells. Observations in the present Chapter indicate that the p110β, p110γ, and p110δ subunits selectively regulate basal LH and GH secretion. Although p110γ and p110δ are involved in the control of basal LH and GH release, p110β only participates in regulating basal LH secretion. The differential involvement of p110β, p110δ, and p110γ

signalling in mediating basal and GnRH-induced hormone release in both gonadotropes and somatotropes further indicates that the effects of isoform-selective class I PI3K inhibitors on pituitary hormone release responses are not due solely to functional redundancy in p110-dependent downstream signalling. Interestingly, despite the failure to detect immunoreactive p110α in primary cultures of goldfish pituitary cells p110α inhibition by BYL719 reduces basal LH release suggesting that p110α-like protein could be present in goldfish gonadotropes. Considering that BYL719 is unable to alter GnRH-stimulated LH or GH release, it is unlikely that the reduction of basal LH release observed is due to it targeting other p110 catalytic subunits. Given that both the p110α and p110β class IA PI3K isoforms are ubiquitously expressed in mammals (Engelman et al., 2006; Vanhaesebroeck et al., 2005), this discrepancy is more likely due to species-specific limitations with regards to the cross-reactivity of the p110α subunit-selective antibody. The involvement of p110α in the control of basal LH release is consistent with the known ability of p110α catalytic activity to alter agonist-independent insulin and neurotensin secretion (Kolic et al., 2013; Li et al., 2012). In pancreatic β-cells, p110α acts in part by affecting Ca^{2+}-dependent exocytosis to alter insulin release (Kolic et al., 2013). Whether the catalytic activity of p110α, or any other p110 isoform, exerts a similar effect on basal Ca^{2+} signalling in pituitary gonadotropes will be an important area of future investigation.

4.3.5 Class I PI3Ks and other known signalling components in biased GnRH action

The results presented in this study lend further support to the hypothesis that the intracellular mechanisms contributing to GnRH2 and GnRH3 actions on hormone release

in goldfish gonadotropes and somatotropes are not identical; differing in terms of their use of Ca^{2+} stores, as well as the relationships between PI3K-dependent signalling and changes in $[Ca^{2+}]_i$. General inhibition of PI3Ks prevented GnRH2- and GnRH3-elicited increases in $[Ca^{2+}]_i$ in gonadotropes, but only reduced the $[Ca^{2+}]_i$ responses to GnRH2, and not GnRH3, in somatotropes (Chapter 3). It is plausible that the GnRH-selective utilization of class I PI3K isoforms identified in this study contributes to these differences in how PI3Ks integrate with downstream Ca^{2+}-dependent signalling. In goldfish gonadotropes and somatotropes, GnRH actions also involve PKCs and activation of conventional, as well as novel, PKC isozymes increases $[Ca^{2+}]_i$ (Chang et al., 2009, 2012). However, the LH and GH release responses induced by synthetic PKC activators are unaffected by pre-treatment with LY294002 (Chapter 1), suggesting that PI3K-dependent intracellular events, including actions on $[Ca^{2+}]_i$, are upstream or independent of PKC-dependent signalling. Additionally, although PtdIns(3,4,5)P_3 production resulting from class I PI3K catalytic activity can contribute to PKC maturation through the membrane-recruitment of PDK1, PDK1-mediated phosphorylation of PKC is not directly correlated with acute agonist-induced changes in PKC catalytic activity (Newton et al., 2010; Antal et al., 2014). Thus, it is unlikely that PI3K-dependent signalling regulates PKC catalytic activity to modulate $[Ca^{2+}]_i$ changes involved in acute GnRH-stimulated LH and GH release in goldfish. Nonetheless, GnRH-induced dissociation of Gα subunits and Gβγ heterodimers, downstream of GnRHR activation, likely plays a central role in the differential signalling utilized by GnRH2 and GnRH3 to regulate LH and GH secretion. It is reasonable to hypothesize that $G\alpha_{q/11}$-mediated activation of PLCβ leads to PKC- and Ins(1,4,5)P_3-dependent events, while dissociated Gβγ heterodimers are part of

the mechanism(s) leading to selective PI3K-dependent signal transduction by differential actions on p110β and p110γ. While the relationships between specific class I PI3K isoforms and $[Ca^{2+}]_i$ remain to be investigated, differences in the usage of p110β, p110γ, and p110δ catalytic activities have been identified as part of the integrated control of GnRH-selective signal transduction in goldfish gonadotropes and somatotropes leading to hormone release (Figure 4.16).

4.3.6 Gβγ-dependent signal transduction regulates basal and GnRH-stimulated hormone release

As discussed earlier, results using a small molecule inhibitor (NSC8668) of the shared Gβγ interaction surface provides the first evidence for the involvement of Gβγ-dependent signalling downstream of GnRHR activation; as well as provides a molecular basis for the selective activation of class I PI3K isoforms by GnRH2 and GnRH3 in goldfish pituitary gonadotropes and somatotropes. Outside of activating p110β and p110γ, Gβγ subunits can also modulate the signalling activity of numerous cellular effectors via direct interactions. Other canonical Gβγ-dependent signalling targets include isoforms of PLCs, GRKs, VSCCs, inwardly-rectifying K^+ channels, ACs, and MAPKs (Smrcka et al., 2008; Khan et al., 2013). Many of these effectors use the same highly conserved binding surface to interact with Gβγ (Scott et al., 2001; Bonacci et al., 2006). Interestingly, GnRH stimulation of LH secretion, as well as LH subunit and GH mRNA expression, are known to also involve the MEK1/2 family of MAPKKs (Klausen et al., 2008; Chang et al., 2009). Whether and how Gβγ participates in mediating MEK1/2-

dependent signalling following GnRHR activation in goldfish pituitary cells requires further investigation.

In addition to its effects on agonist-induced LH and GH secretion, inhibition of Gβγ-effector interactions significantly reduced both basal LH and GH release. The magnitude of NSC8668 effects on basal LH release mirrors those observed using selective inhibitors of p110β and p110γ catalytic activity, and that of p110γ inhibition on unstimulated GH secretion. However, basal GH release is not sensitive to inhibition of p110β. Taken together, these results suggest that p110β and p110γ may be redundant Gβγ-sensitive signals during the constitutive control of basal LH release. Conversely, Gβγ heterodimers selectively target p110γ to control basal GH release. Interestingly, the pattern of involvement observed for Gβγ-dependent effectors in basal hormone release differs from those for GnRH-stimulated LH and GH responses. Thus, the hypothesis that the repertoire of heterodimeric Gβγ subunits involved in the control of basal and GnRH-stimulated actions are different will be an important topic for future studies.

4.3.7 Relationship between class I PI3K activation and Akt in goldfish pituitary cell

Many of the downstream actions of class I PI3Ks are linked to the activation of Akt, and phosphorylation of Akt at Ser473 is often used an index for class I PI3K activity (reviewed in Chapter 1; Manning and Cantley, 2007). Interestingly, TGX221 (p110β-selective) and IC87114 (p110δ-selective), but not BYL719 (p110α-selective) and CZC24832 (p110γ-selective), attenuated basal Akt phosphorylation (Ser473); although all four inhibitors are effective in altering GnRH-stimulated and/or basal hormone release. In many instances, Akt phosphorylation still occurs in the presence of significant

reductions in the availability of PtdIns(3,4,5)P_3 induced by isoform-selective class I PI3K inhibitors (Vahaesebroeck et al., 2010; Fruman and Rommel, 2014). Furthermore, in class I PI3K-knockout mice models, there are also no strict correlations between the activation of individual isoforms of class I PI3Ks and the activation of Akt (Vanhaesebroeck et al., 2005, 2010). Taken together, these results suggest that Akt may not be an obligatory element of PI3K-dependent signalling in goldfish pituitary cells, but implicate p110β and p110δ as upstream regulators of Akt-dependent signalling under basal conditions.

4.3.8 Summary

Using dynamic measures of acute hormone release responses, we have demonstrated GnRH-selective utilization of class I PI3K isoforms for the first time in any cell type. Our findings also represent the first example of ligand-bias in GPCR activation of p110α, p110β, p110γ, and p110δ catalytic activity. GnRH utilization of p110β and p110γ signalling, as well as the ability of NSC8668 to block GnRH actions on hormone release, also provides physiological evidence for the functional involvement of Gβγ-dependent transduction downstream of GnRHR activation. This is extremely novel considering that the relative importance of signalling initiated by Gβγ heterodimers in the control of GnRH actions, as well as the identity of Gβγ-dependent cellular effectors regulated by GnRHR activation, is a long-standing question in GnRH biology. Furthermore, the possibility of Gβγ-mediated signalling bias is largely uncharacterized (Lin and Smrcka, 2011; Smrcka, 2013), and this study represents the first demonstration of biased signalling by Gβγ-sensitive class I PI3Ks in any model.

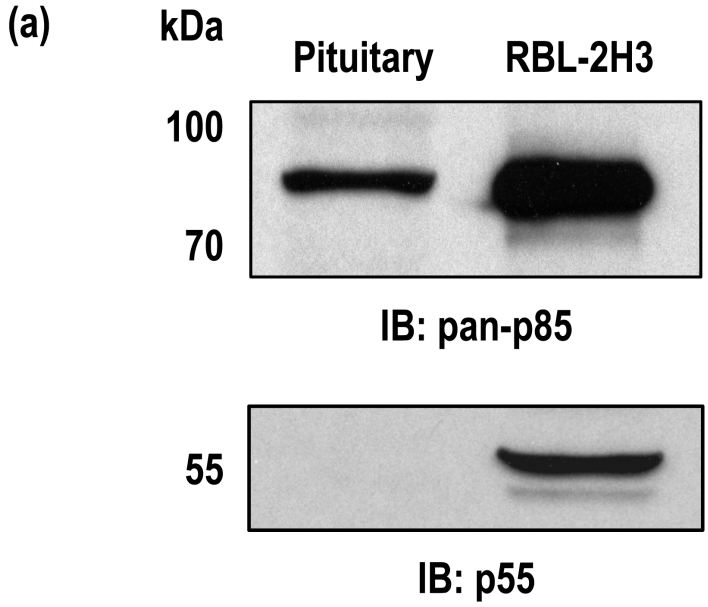
Additionally, in general, by studying naturally biased GnRH isoforms in a lower vertebrate model, our results provide important evolutionary insights into the molecular mechanisms that couple biased GnRHR activation to signal transduction responses, while also highlighting how intracellular signalling dynamics within the neuroendocrine system ultimately contribute to the regulation of whole-organism physiology. Just as importantly, the involvement of p110α in the control of basal, but not GnRH-stimulated, LH release is not only consistent with the previously demonstrated phenomenon of dissociated regulation of basal and agonist-stimulated hormone release in goldfish gonadotropes, but it also identifies an intracellular mechanism by which this can be achieved. Taken together, these findings reveal the complexity of class I PI3K-dependent signalling in GnRH-stimulated and basal hormone secretion, as well as add to our understanding of how biased activation of intracellular signalling networks contribute to GnRH-selective hormone release responses.

Table 4.1 Isoform specificity of the class I PI3K inhibitors used in this study.

Inhibitor	IC_{50} Values (μM) for p110-Selective Class I PI3K Inhibitors					[Used]	Specificity
	Class IA PI3K			Class IB PI3K	PI4Ks		
	p110α	p110β	p110δ	p110γ			
BYL719[a]	**0.0046**	1.156	0.290	0.250	0.581	**0.05 μM**	**p110α**
TGX221[b]	5	**0.005**	> 10	0.1	> 10	**0.05 μM**	**p110β**
IC87114[c,d]	> 100	75	**0.5**	29	> 100	**5 μM**	**p110δ**
CZC24832[e]	> 10	1.1	8194	**0.027**	> 10	**0.5 μM**	**p110γ**

Values are drawn from published reports ([a]Fritsch et al., 2014; [b]Jackson et al., 2005; [c]Sadhu et al., 2003; [d]Knight et al., 2006; [e]Bergamini et al., 2012).

Figure 4.1 Expression of immunoreactive class IA PI3K regulatory (p85 and p55) and catalytic (p110α, p110β, and p110δ) subunits in primary goldfish pituitary cell cultures. Representative immunoblots (from one of three independent experiments) of whole cell lysates isolated from mixed goldfish pituitary cell cultures using subunit-selective primary antibodies raised against the (a) class IA regulatory subunits p85 (PIK3R1 and PIK3R2; expected molecular mass of 85 kDa) and p55 (PIK3R3; expected molecular mass of 55 kDa), as well as the (b) class IA catalytic subunits p110α (PIK3CA), p110β (PIK3CB), and p110δ (PIK3CD). For each of the class I PI3K catalytic subunits, the expected molecular mass is approximately 110 kDa. Positive controls for immunoblotting were performed using whole cell lysates from the RBL-2H3 rat basophilic leukaemia mast cell line. Importantly, mammalian mast cells are known to express all the class IA PI3K subunits. Goldfish pituitary cell lysates also failed to cross-react with an additional p110α primary antibody (sc-7174; results not shown).


(a)
kDa
Pituitary
RBL-2H3
100
70
IB: pan-p85
55
IB: p55

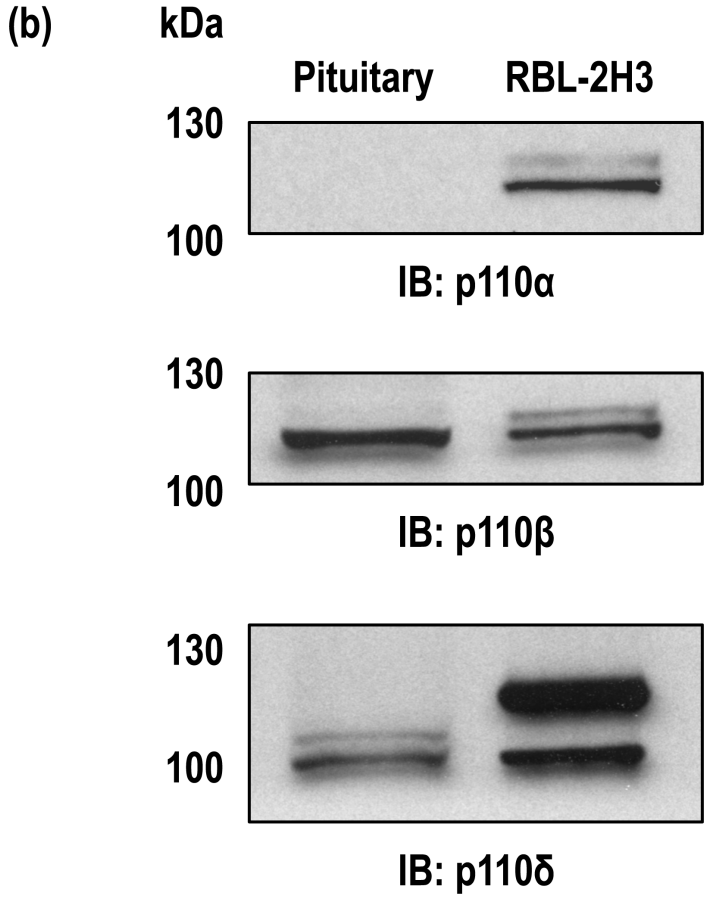

(b)
kDa
Pituitary
RBL-2H3
130
100
IB: p110α
130
100
IB: p110β
130
100
IB: p110δ

Figure 4.2 Expression of immunoreactive class IB PI3K regulatory (p101) and catalytic (p110γ) subunits in primary goldfish pituitary cell cultures. Representative immunoblots (from one of three independent experiments) of whole cell lysates isolated from mixed goldfish pituitary cell cultures using subunit-selective primary antibodies raised against the (a) class IB regulatory subunit p101 (PIK3R5; expected molecular mass of ~100 kDa) and the (b) class IB catalytic subunit p110γ (PIK3CG; expected molecular mass of 110 kDa). Positive controls for immunoblotting were performed using whole cell lysates from the RBL-2H3 rat basophilic leukaemia mast cell line. Importantly, mammalian mast cells are known to express both the p101, although at low levels, and p110γ subunits.

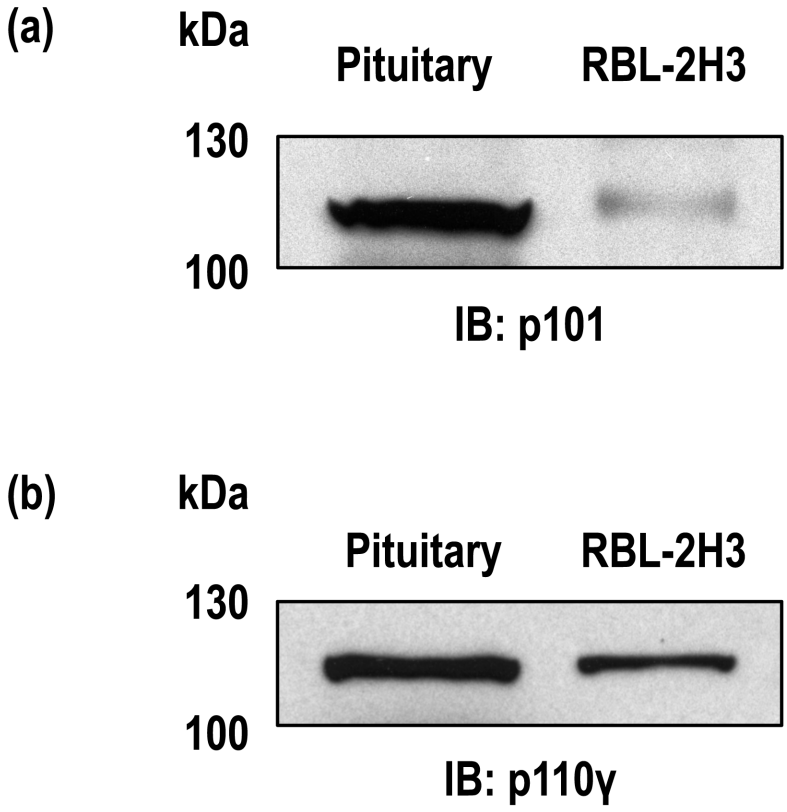

(a)
kDa
Pituitary
RBL-2H3
130
100
IB: p101
(b)
kDa
Pituitary
RBL-2H3
130
100
IB: p110γ

Figure 4.3 Preabsorption of immunoreactive bands corresponding to the class IA PI3K p85-type regulatory subunits in goldfish pituitary tissue extracts. (a) Immunoblots of RBL-2H3 (RBL) cell lysate and goldfish pituitary tissue lysate probed with a pan-p85 primary antibody (expected molecular mass of 85 kDa). (b) Pre-absorption of primary anti-human pan-p85 monoclonal antibody with full-length recombinant p85α peptide abolishes or reduces immunostaining of full-length recombinant human PI3K p85α (N terminal His tag), RBL cell lysate, and goldfish pituitary tissue lysate.

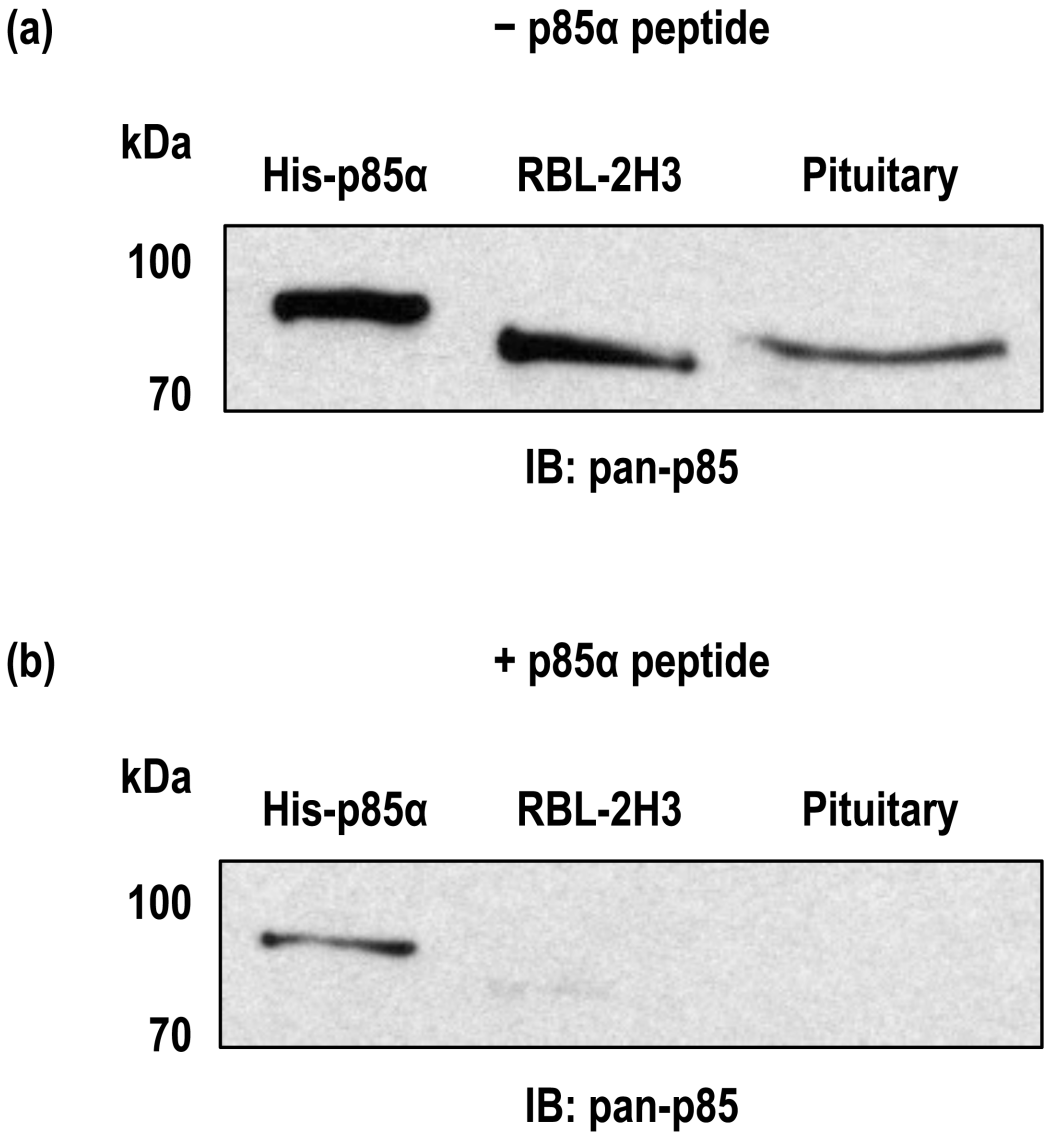
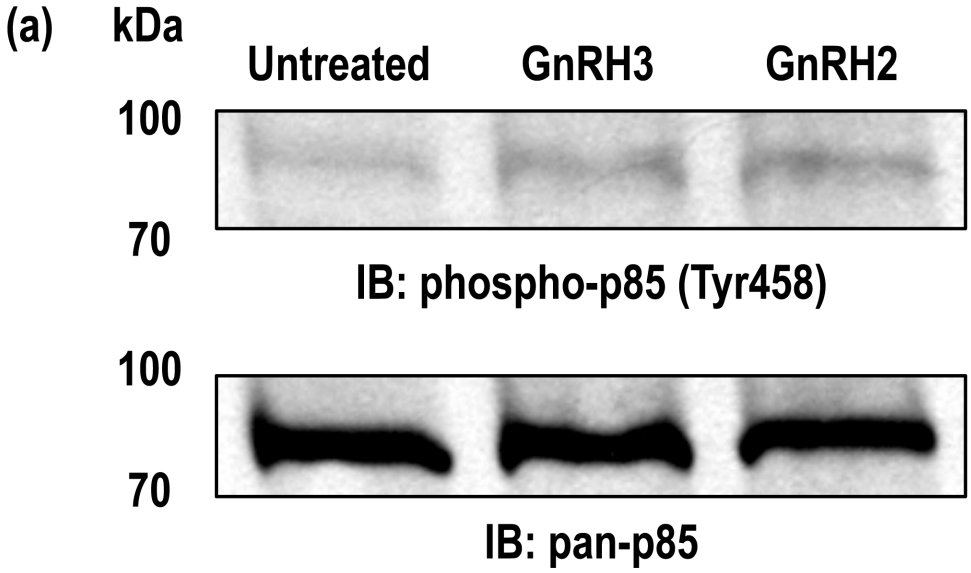

(a)
− p85α peptide
kDa
His-p85α
RBL-2H3
Pituitary
100
70
IB: pan-p85
(b)
+ p85α peptide
kDa
His-p85α
RBL-2H3
Pituitary
100
70
IB: pan-p85

Figure 4.4 Effects of GnRH2 (100 nM) or GnRH3 (100 nM) on the expression of phosphorylated p85-type regulatory subunits in goldfish pituitary fragments following 30 min in static incubation. (a) Representative immunoblots of goldfish pituitary tissue lysate probed with a primary antibody that recognizes human p85 phosphorylated at residue Tyr458 and a blot of the same membrane probed with a pan-p85 antibody are shown (expected molecular mass of 85 kDa). (b) Quantified ratios of phosphorylated to total immunoreactive p85 in GnRH-treated groups, expressed as a percentage of unstimulated controls are presented (mean ± SEM, $n = 3$; with goldfish undergoing sexual recrudescence in February and March). Treatments that are significantly different from one another are identified by different symbols (**; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).


(a)
kDa
Untreated
GnRH3
GnRH2
100
70
IB: phospho-p85 (Tyr458)
100
70
IB: pan-p85

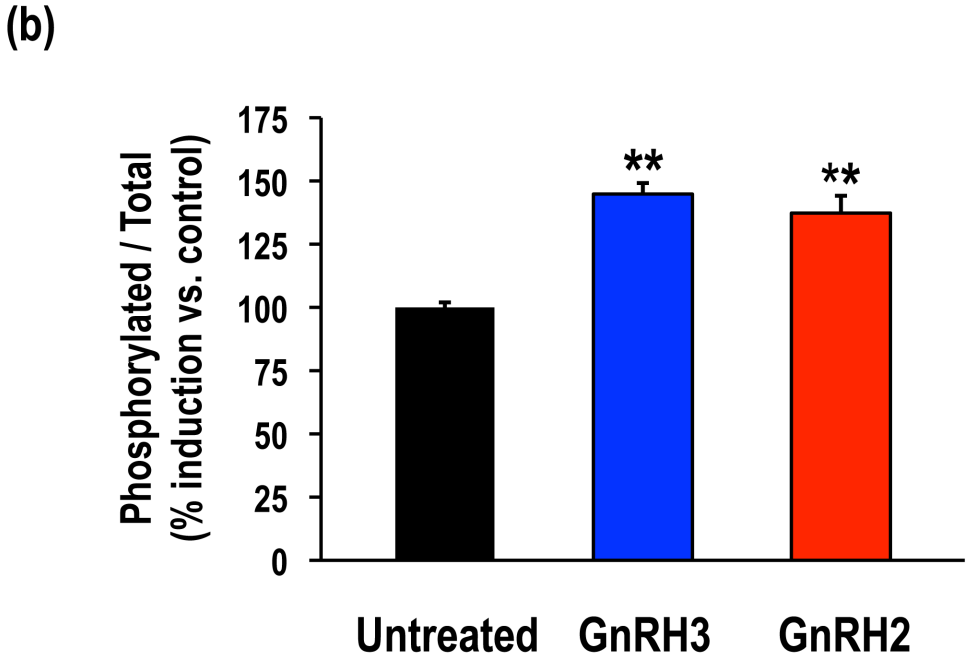
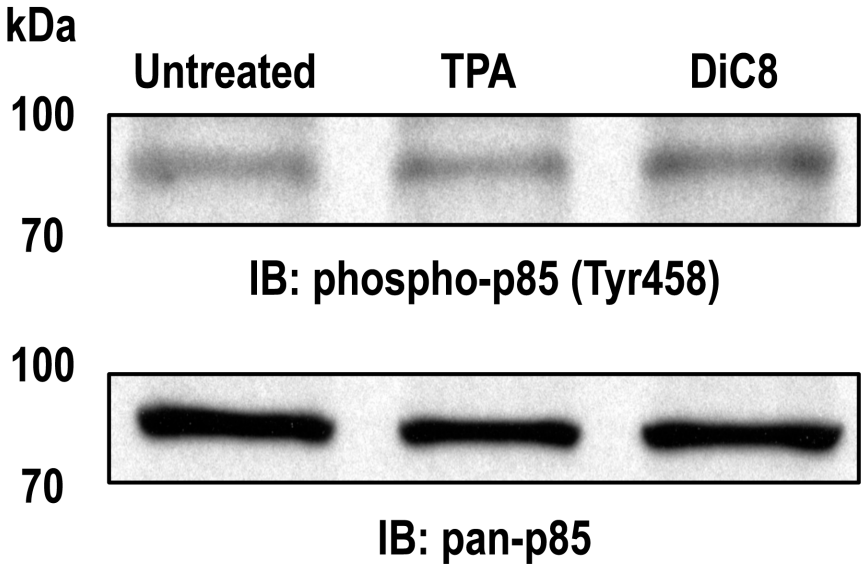

(b)
Phosphorylated / Total
(% induction vs. control)
175
150
125
100
75
50
25
0
**
**
Untreated
GnRH3
GnRH2

Figure 4.5 Effects of TPA (100 nM) or DiC8 (100 μM) on the expression of phosphorylated p85-type regulatory subunits in goldfish pituitary fragments following 30 min in static incubation. (a) Representative immunoblots of goldfish pituitary tissue lysate probed with a primary antibody that recognizes human p85 phosphorylated at residue Tyr458 and a blot of the same membrane probed with a pan-p85 antibody are shown. (b) Quantified ratios of phosphorylated to total immunoreactive p85 in GnRH-treated groups, expressed as a percentage of unstimulated controls are presented (mean ± SEM, n = 3; with goldfish in April, a time of year when the gonads are at a sexually matured/pre-spawning stage). Treatments that are significantly different from one another are identified by different symbols (ANOVA followed by Fisher's LSD multiple comparisons where applicable; $p < 0.05$).

(a)


kDa
Untreated
TPA
DiC8
100
70
IB: phospho-p85 (Tyr458)
100
70
IB: pan-p85

(b)

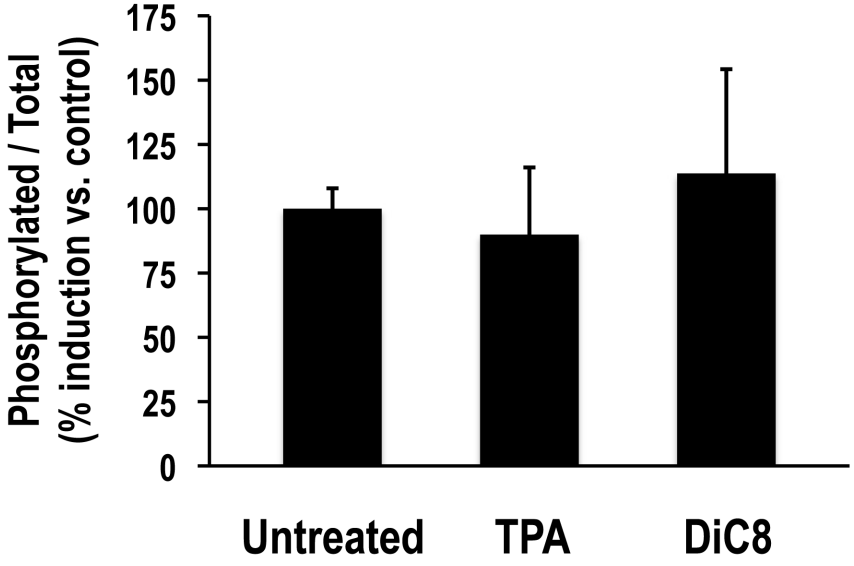
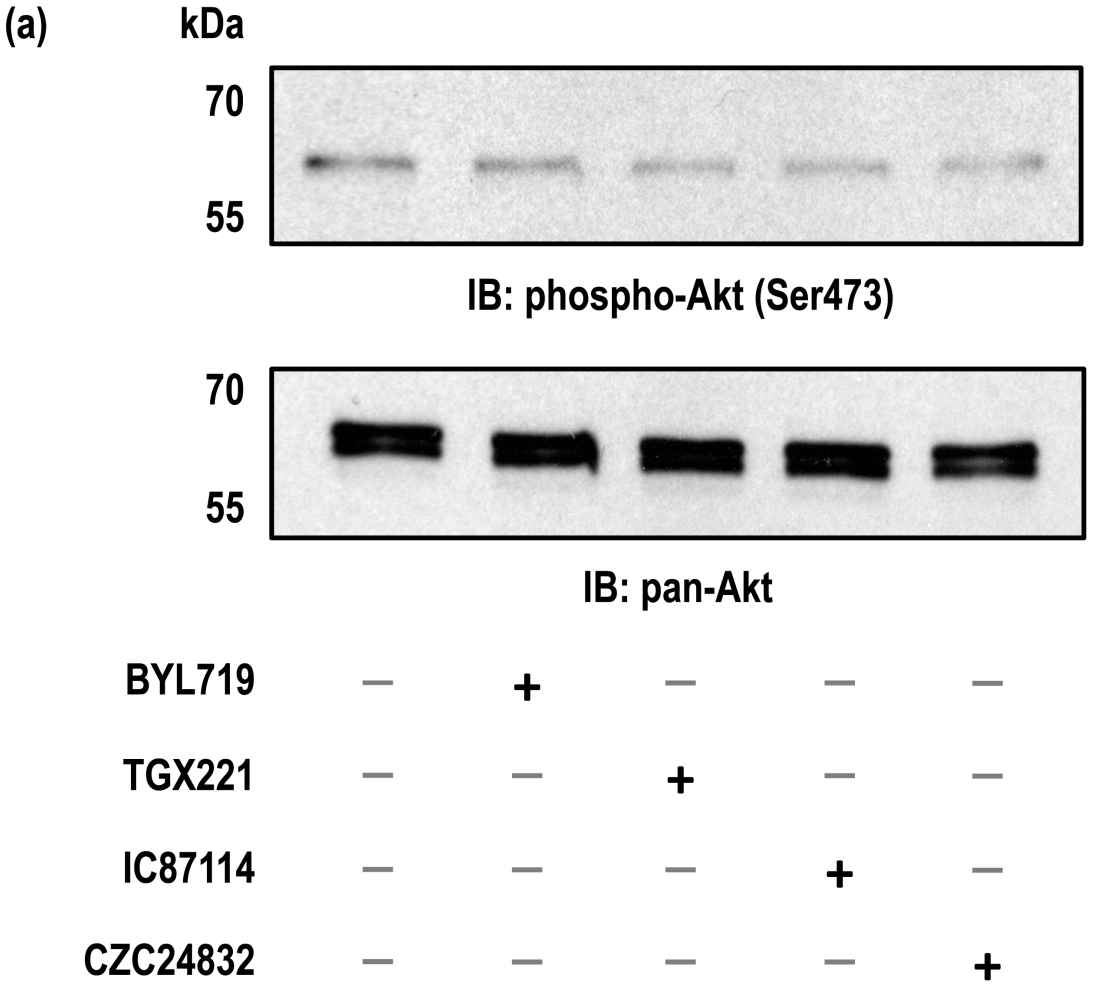

Phosphorylated / Total
(% induction vs. control)
175
150
125
100
75
50
25
0
Untreated
TPA
DiC8

Figure 4.6 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM), p110β (TGX221; p110β^{-}; 0.05 μM), p110δ (IC87114; p110δ^{-}; 5 μM), and p110γ (CZC24832; p110γ^{-}; 0.5 μM) on the expression of phosphorylated Akt (Ser473) in dispersed goldfish pituitary cells following 2 hrs in static incubation. (a) Representative immunoblots of a goldfish pituitary lysate probed with a primary antibody that recognizes Akt phosphorylated at residue Ser473, and immunoblotting of the same membrane with a pan-Akt antibody. (b) Quantified ratios of phosphorylated Akt (Ser473) to total immunoreactive pan-Akt in inhibitor-treated groups, expressed as a percentage of unstimulated controls are presented (mean ± SEM, n = 3; with goldfish in April and May, a time of year when the gonads are at a sexually matured/pre-spawning stage). Positive controls for Akt immunoblotting were performed using whole cell lystates isolated from a rat basophilic leukaemia cell line (RBL-2H3; data not shown). For both the phospho-Akt (Ser473) and pan-Akt immunoblotting experiments, the antibodies used cross-react with conserved sequences within Akt1, Akt2, and Akt3 (molecular mass is expected to be ~ 60 kDa). Treatments that are significantly different from one another are identified by different symbols (**; Kruskal-Wallis ANOVA followed by pairwise comparison using the Mann-Whitney U test; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.


(a)
kDa
70
55
IB: phospho-Akt (Ser473)
70
55
IB: pan-Akt
BYL719 – + – – –
TGX221 – – + – –
IC87114 – – – + –
CZC24832 – – – – +

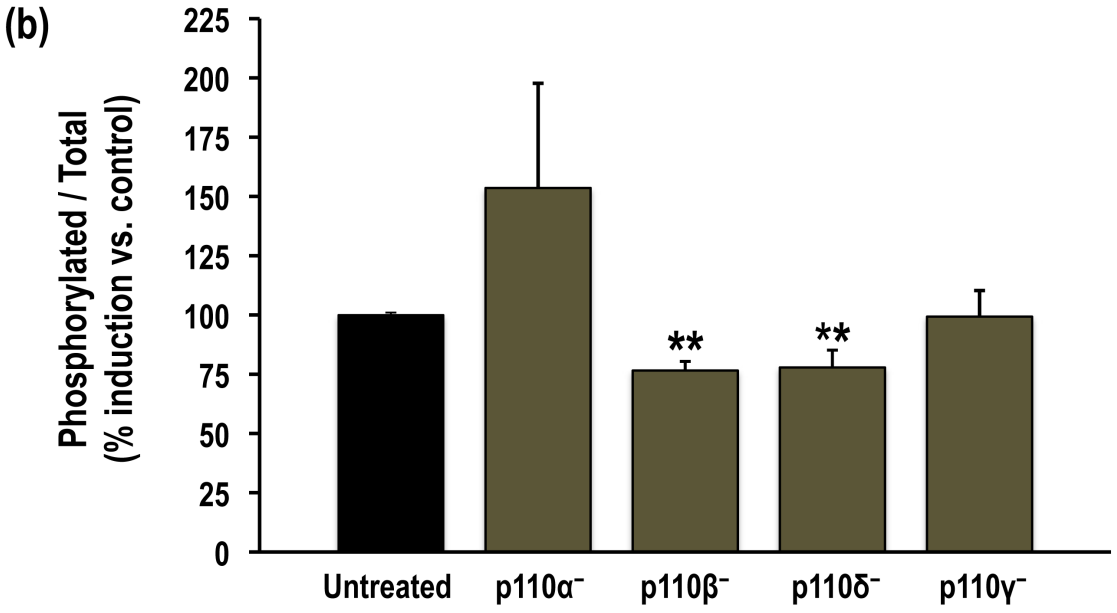

(b)
Phosphorylated / Total
(% induction vs. control)
225
200
175
150
125
100
75
50
25
0
**
**
Untreated
p110α⁻
p110β⁻
p110δ⁻
p110γ⁻

Figure 4.7 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM; a,b), p110β (TGX221; p110β^{-}; 0.05 μM; c,d), p110δ (IC87114; p110δ^{-}; 5 μM; e,f), and p110γ (CZC24832; p110γ^{-}; 0.5 μM; g,h) on basal and GnRH2-stimulated LH release responses. For each panel, LH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified LH release responses are shown on the right (b,d,f,h). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 3.22 ± 0.13 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in April to June (BYL719, completed in May-June; TGX221, completed in April; IC87114, completed in May; CZC24832, completed in April-May) are shown. The gray horizontal bar indicates the duration of the p110 inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

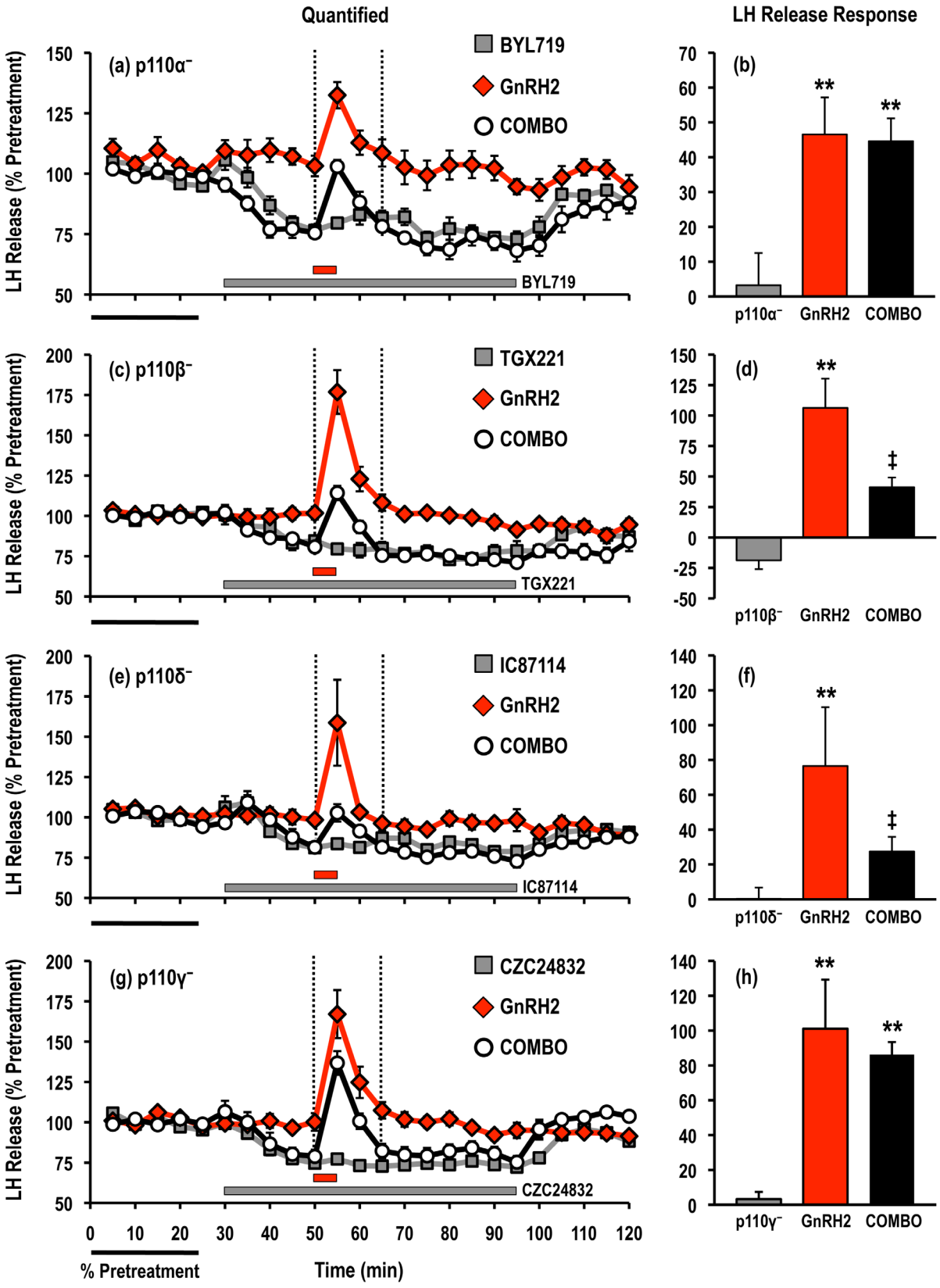

Quantified
LH Release Response
(a) p110α⁻
BYL719
GnRH2
COMBO
LH Release (% Pretreatment)
(b)
p110α⁻ GnRH2 COMBO
(c) p110β⁻
TGX221
(d)
p110β⁻ GnRH2 COMBO
(e) p110δ⁻
IC87114
(f)
p110δ⁻ GnRH2 COMBO
(g) p110γ⁻
CZC24832
(h)
p110γ⁻ GnRH2 COMBO
% Pretreatment
Time (min)

Figure 4.8 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM; a,b), p110β (TGX221; p110β^{-}; 0.05 μM; c,d), p110δ (IC87114; p110δ^{-}; 5 μM; e,f), and p110γ (CZC24832; p110γ^{-}; 0.5 μM; g,h) on basal and GnRH3-stimulated LH release responses. For each panel, LH release profiles are shown on the left (a,c,e,g; grey solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified LH release responses are shown on the right (b,d,f,h). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 3.07 ± 0.13 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in April to June (BYL719, completed in May-June; TGX221, completed in April; IC87114, completed in May; CZC24832, completed in April-May) are shown. The grey horizontal bar indicates the duration of the p110 inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

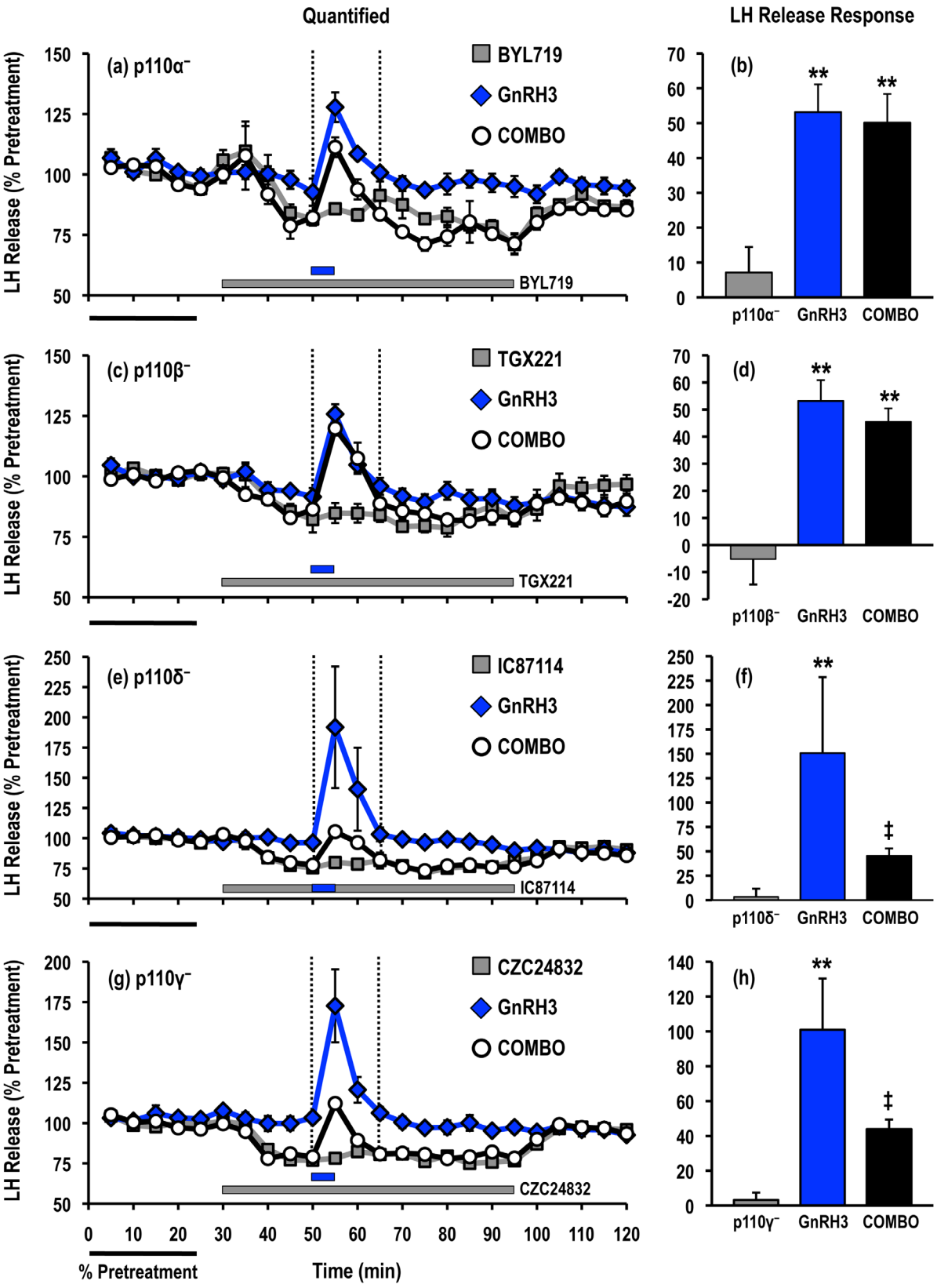

Quantified
LH Release Response
(a) p110α⁻
BYL719
GnRH3
COMBO
BYL719
LH Release (% Pretreatment)
(b)
p110α⁻
GnRH3
COMBO
(c) p110β⁻
TGX221
GnRH3
COMBO
TGX221
(d)
p110β⁻
GnRH3
COMBO
(e) p110δ⁻
IC87114
GnRH3
COMBO
IC87114
(f)
p110δ⁻
GnRH3
COMBO
(g) p110γ⁻
CZC24832
GnRH3
COMBO
CZC24832
(h)
p110γ⁻
GnRH3
COMBO
% Pretreatment
Time (min)

Figure 4.9 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM; a,b), p110β (TGX221; p110β^{-}; 0.05 μM; c,d), p110δ (IC87114; p110δ^{-}; 5 μM; e,f), and p110γ (CZC24832; p110γ^{-}; 0.5 μM; g,h) on basal and GnRH2-stimulated GH release responses. For each panel, GH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified GH release responses are shown on the right (b,d,f,h). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 8.96 ± 1.00 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in April to June (BYL719, completed in May-June; TGX221, completed in April; IC87114, completed in May; CZC24832, completed in April-May) are shown. The grey horizontal bar indicates the duration of the p110 inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

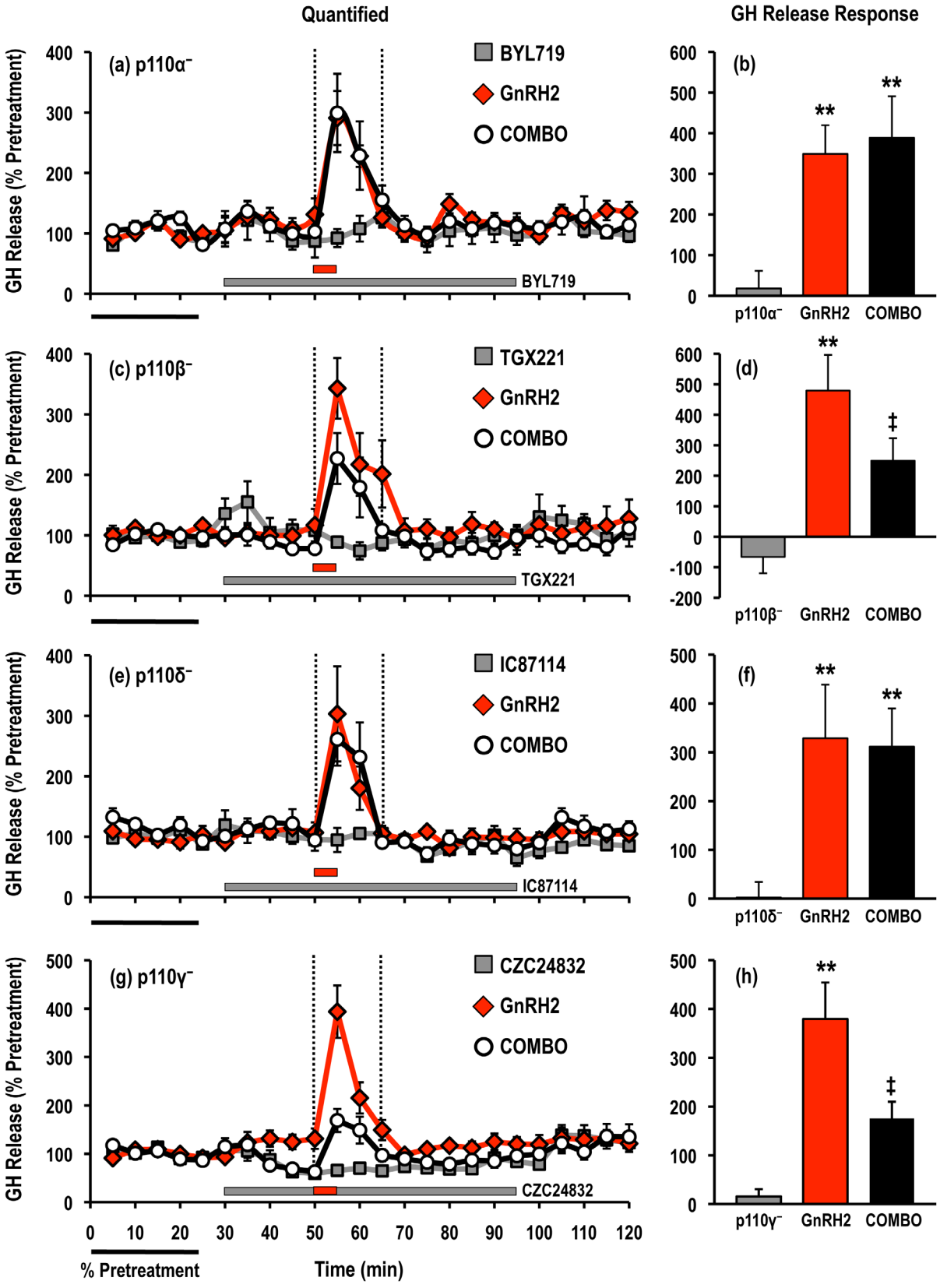

Quantified
GH Release Response
(a) p110α⁻
BYL719
GnRH2
COMBO
BYL719
(b)
p110α⁻ GnRH2 COMBO
(c) p110β⁻
TGX221
GnRH2
COMBO
TGX221
(d)
p110β⁻ GnRH2 COMBO
(e) p110δ⁻
IC87114
GnRH2
COMBO
IC87114
(f)
p110δ⁻ GnRH2 COMBO
(g) p110γ⁻
CZC24832
GnRH2
COMBO
CZC24832
(h)
p110γ⁻ GnRH2 COMBO
GH Release (% Pretreatment)
% Pretreatment
Time (min)

Figure 4.10 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM; a,b), p110β (TGX221; p110β^{-}; 0.05 μM; c,d), p110δ (IC87114; p110δ^{-}; 5 μM; e,f), and p110γ (CZC24832; p110γ^{-}; 0.5 μM; g,h) on basal and GnRH3-stimulated GH release responses. For each panel, GH release profiles are shown on the left (a,c,e,g; grey solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified GH release responses are shown on the right (b,d,f,h). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 11.08 ± 1.80 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in April to June (BYL719, completed in May-June; TGX221, completed in April; IC87114, completed in May; CZC24832, completed in April-May) are shown. The grey horizontal bar indicates the duration of the p110 inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

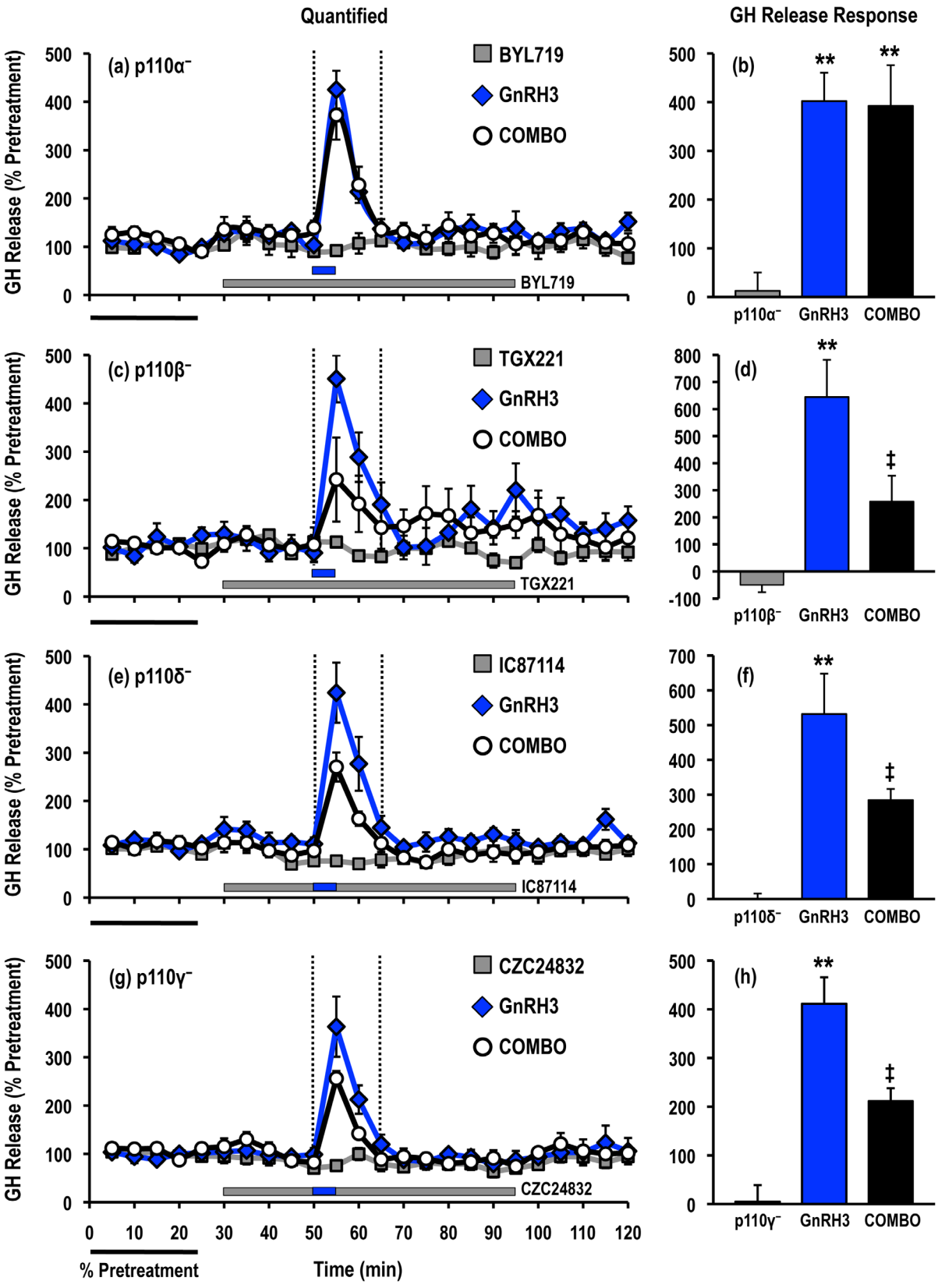

Quantified
GH Release Response
(a) p110α⁻
BYL719
GnRH3
COMBO
BYL719
GH Release (% Pretreatment)
(b)
p110α⁻
GnRH3
COMBO
(c) p110β⁻
TGX221
(d)
p110β⁻
(e) p110δ⁻
IC87114
(f)
p110δ⁻
(g) p110γ⁻
CZC24832
(h)
p110γ⁻
% Pretreatment
Time (min)

Figure 4.11 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM), p110β (TGX221; p110β^{-}; 0.05 μM), p110δ (IC87114; p110δ^{-}; 5 μM), and p110γ (CZC24832; p110γ^{-}; 0.5 μM) on basal LH release. LH release profiles are shown in the top panel (a; orange solid square, BYL719 alone; red solid diamond, TGX221 alone; green solid circle, IC87114 alone; blue solid diamond, CZC24832 alone) and quantified responses are shown on the bottom (b; mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (coloured bars; orange, BYL719 alone; red, TGX221 alone; green, IC87114 alone; blue, CZC24832 alone) was quantified as the average % pretreatment value over the entire duration of the p110-selective inhibitor treatment (30-95 min; the grey horizontal bar; n = 16, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

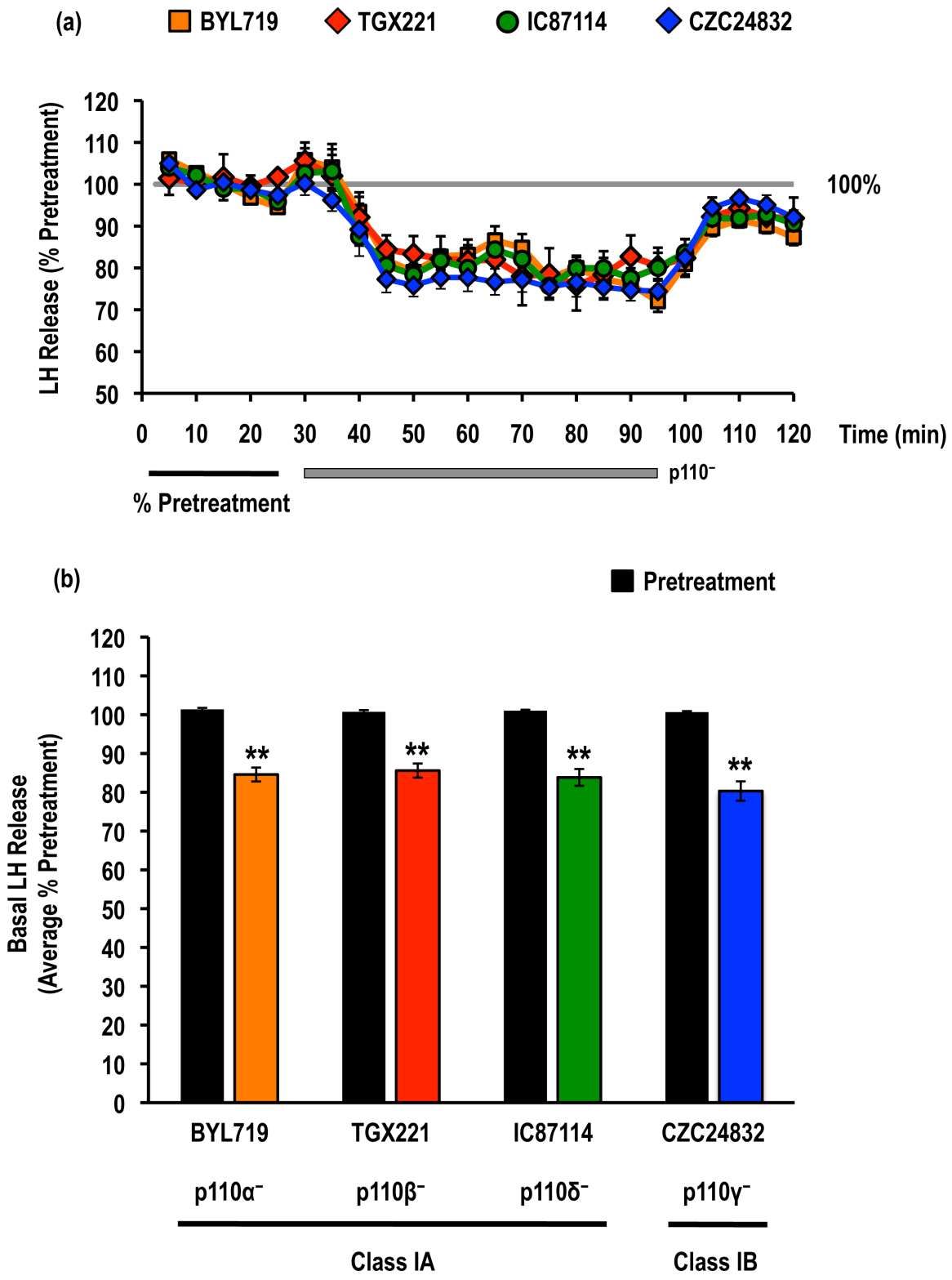

(a)
BYL719
TGX221
IC87114
CZC24832
LH Release (% Pretreatment)
120
110
100
90
80
70
60
50
100%
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
p110⁻
% Pretreatment
(b)
Pretreatment
Basal LH Release
(Average % Pretreatment)
120
110
100
90
80
70
60
50
40
30
20
10
0
**
**
**
**
BYL719
TGX221
IC87114
CZC24832
p110α⁻
p110β⁻
p110δ⁻
p110γ⁻
Class IA
Class IB

Figure 4.12 Effects of selective small molecule inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM), p110β (TGX221; p110β^{-}; 0.05 μM), p110δ (IC87114; p110δ^{-}; 5 μM), and p110γ (CZC24832; p110γ^{-}; 0.5 μM) on basal GH release. GH release profiles are shown in the top panel (a; orange solid square, BYL719 alone; red solid diamond, TGX221 alone; green solid circle, IC87114 alone; blue solid diamond, CZC24832 alone) and quantified responses are shown on the bottom (b; mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (coloured bars; orange, BYL719 alone; red, TGX221 alone; green, IC87114 alone; blue, CZC24832 alone) was quantified as the average % pretreatment value over the entire duration of the p110-selective inhibitor treatment (30-95 min; the grey horizontal bar; n = 16, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$). For information about the specificity of the class I PI3K isoform inhibitors, refer to Table 4.1.

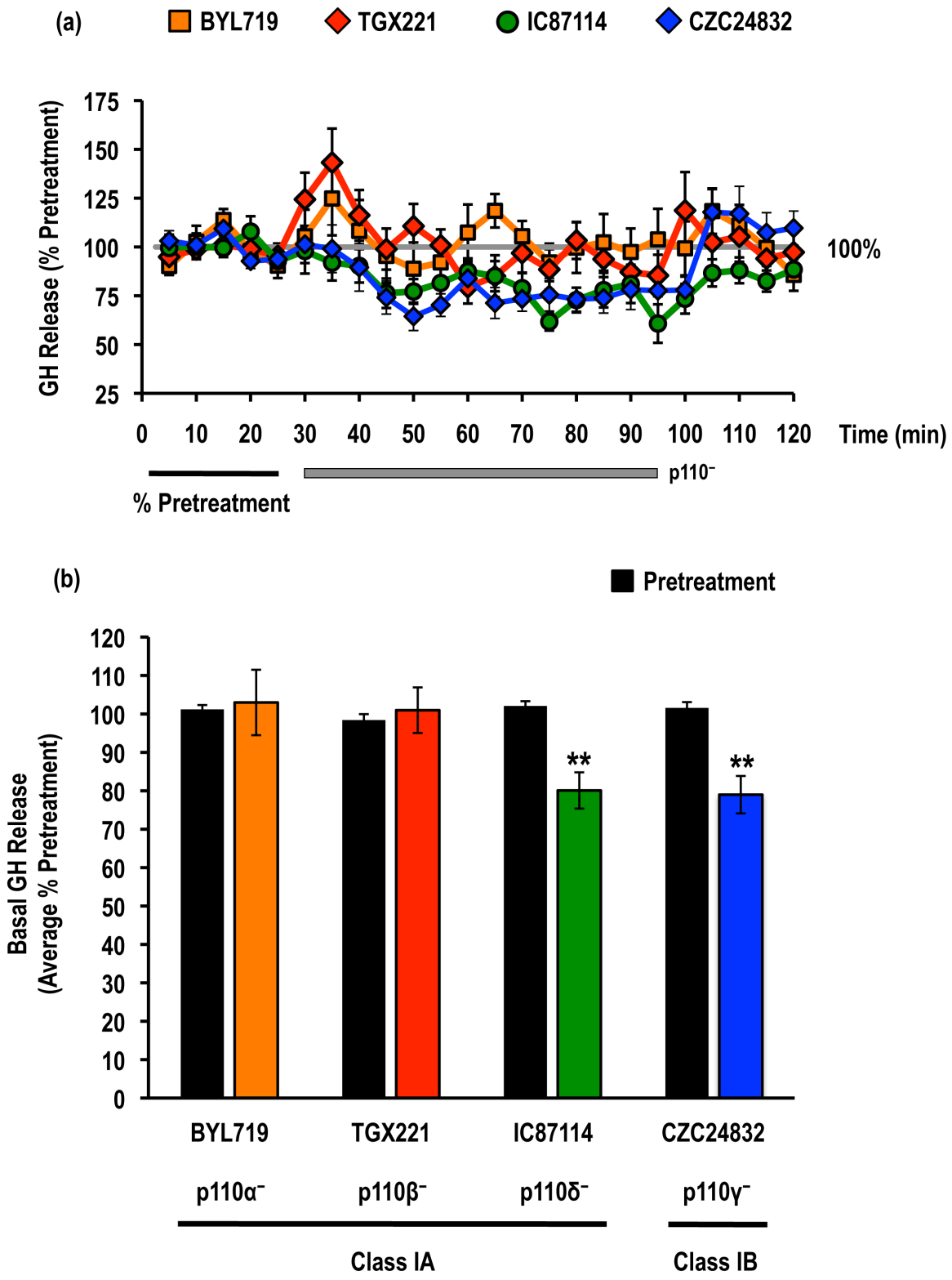

(a)
BYL719
TGX221
IC87114
CZC24832
GH Release (% Pretreatment)
175
150
125
100
75
50
25
100%
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
p110⁻
% Pretreatment
(b)
Pretreatment
Basal GH Release
(Average % Pretreatment)
120
110
100
90
80
70
60
50
40
30
20
10
0
**
**
BYL719
TGX221
IC87114
CZC24832
p110α⁻
p110β⁻
p110δ⁻
p110γ⁻
Class IA
Class IB

Figure 4.13 Effects of the allosteric Gβγ inhibitor NSC8668 (Gβγ^{-}; 10 μM) on LH release responses stimulated by GnRH2 (100 nM; a,b) or GnRH3 (100 nM; c,d). LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; blue solid diamond, GnRH3 alone; open circle, GnRH + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 4.08 ± 0.14 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with gonads that are at a sexually matured/pre-spawning stage (April). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the red (GnRH2) or blue (GnRH3) horizontal bar represents the 5-min exposure to either GnRH. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

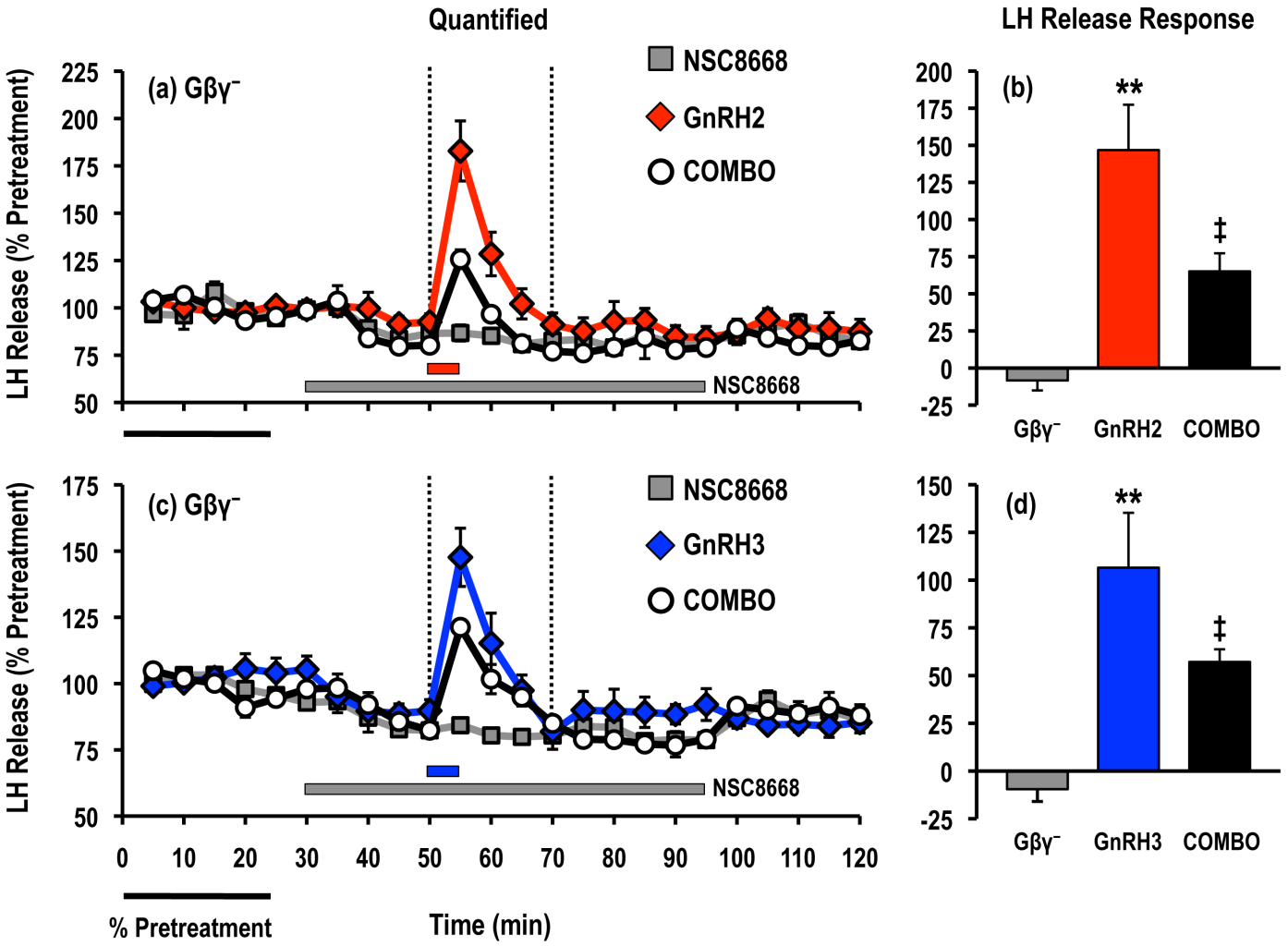

Quantified
LH Release Response
(a) Gβγ⁻
NSC8668
GnRH2
COMBO
NSC8668
LH Release (% Pretreatment)
(b)
**
‡
Gβγ⁻
GnRH2
COMBO
(c) Gβγ⁻
NSC8668
GnRH3
COMBO
NSC8668
(d)
**
‡
Gβγ⁻
GnRH3
COMBO
% Pretreatment
Time (min)

Figure 4.14 Effects of the allosteric Gβγ inhibitor NSC8668 (Gβγ^{-}; 10 μM) on GH release responses stimulated by GnRH2 (100 nM; a,b) or GnRH3 (100 nM; c,d). GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; blue solid diamond, GnRH3 alone; open circle, GnRH + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 9.18 ± 0.43 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with gonads that are at a sexually matured/pre-spawning stage (April). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the red (GnRH2) or blue (GnRH3) horizontal bar represents the 5-min exposure to either GnRH. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

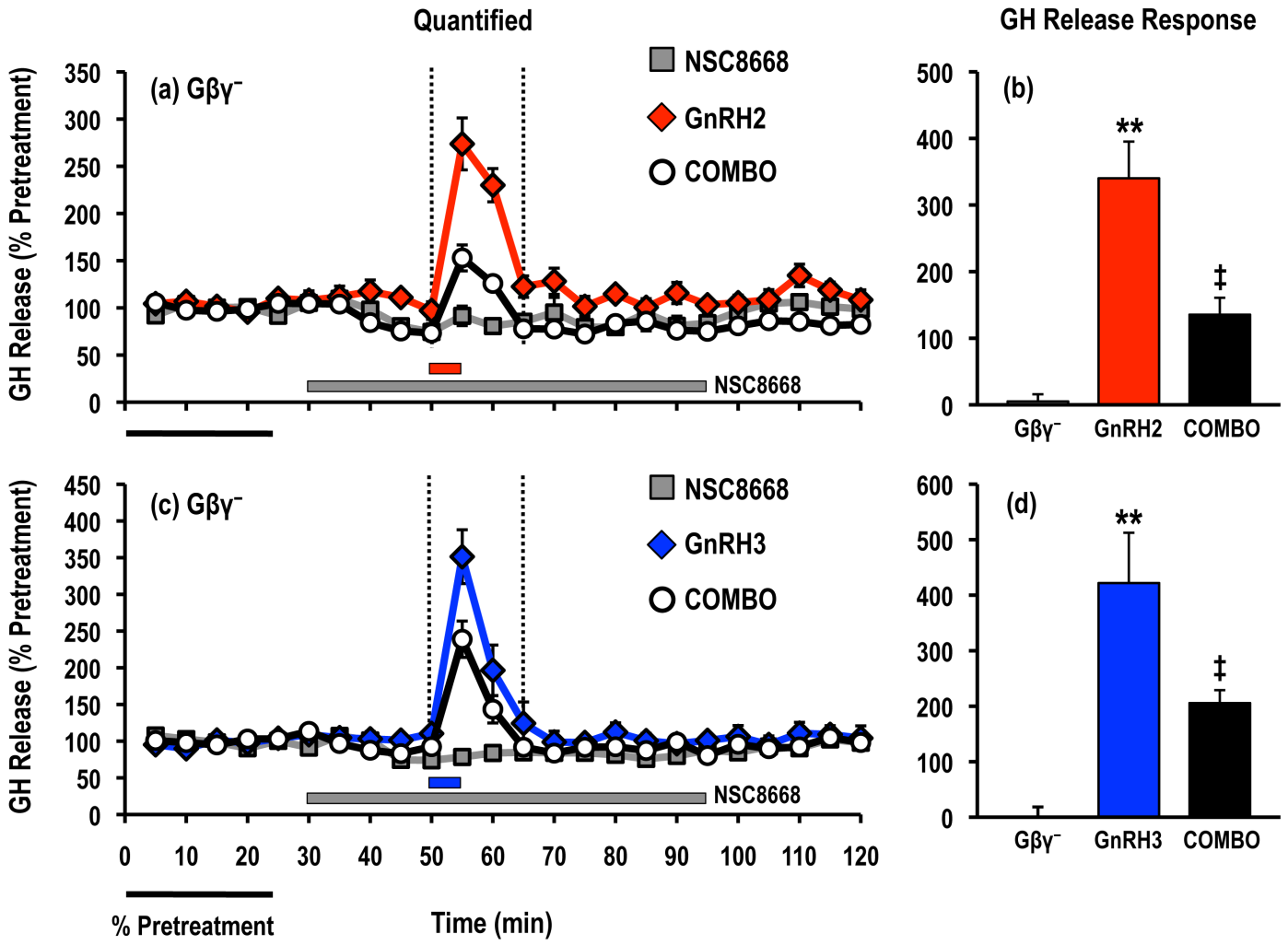

Quantified
GH Release Response
(a) Gβγ⁻
NSC8668
GnRH2
COMBO
GH Release (% Pretreatment)
NSC8668
(b)
**
‡
Gβγ⁻
GnRH2
COMBO
(c) Gβγ⁻
NSC8668
GnRH3
COMBO
NSC8668
(d)
**
‡
Gβγ⁻
GnRH3
COMBO
0 10 20 30 40 50 60 70 80 90 100 110 120
% Pretreatment
Time (min)

Figure 4.15 Effects of the allosteric Gβγ inhibitor NSC8668 (Gβγ^{-}; 10 μM) on basal (a) LH and (b) GH release responses. Hormone release profiles are shown on the left panel and quantified responses are shown on the right (mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (gold bars) was quantified as the average % pretreatment value over the entire duration of NSC8668 treatment (30-95 min; the grey horizontal bar; n = 16, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$).

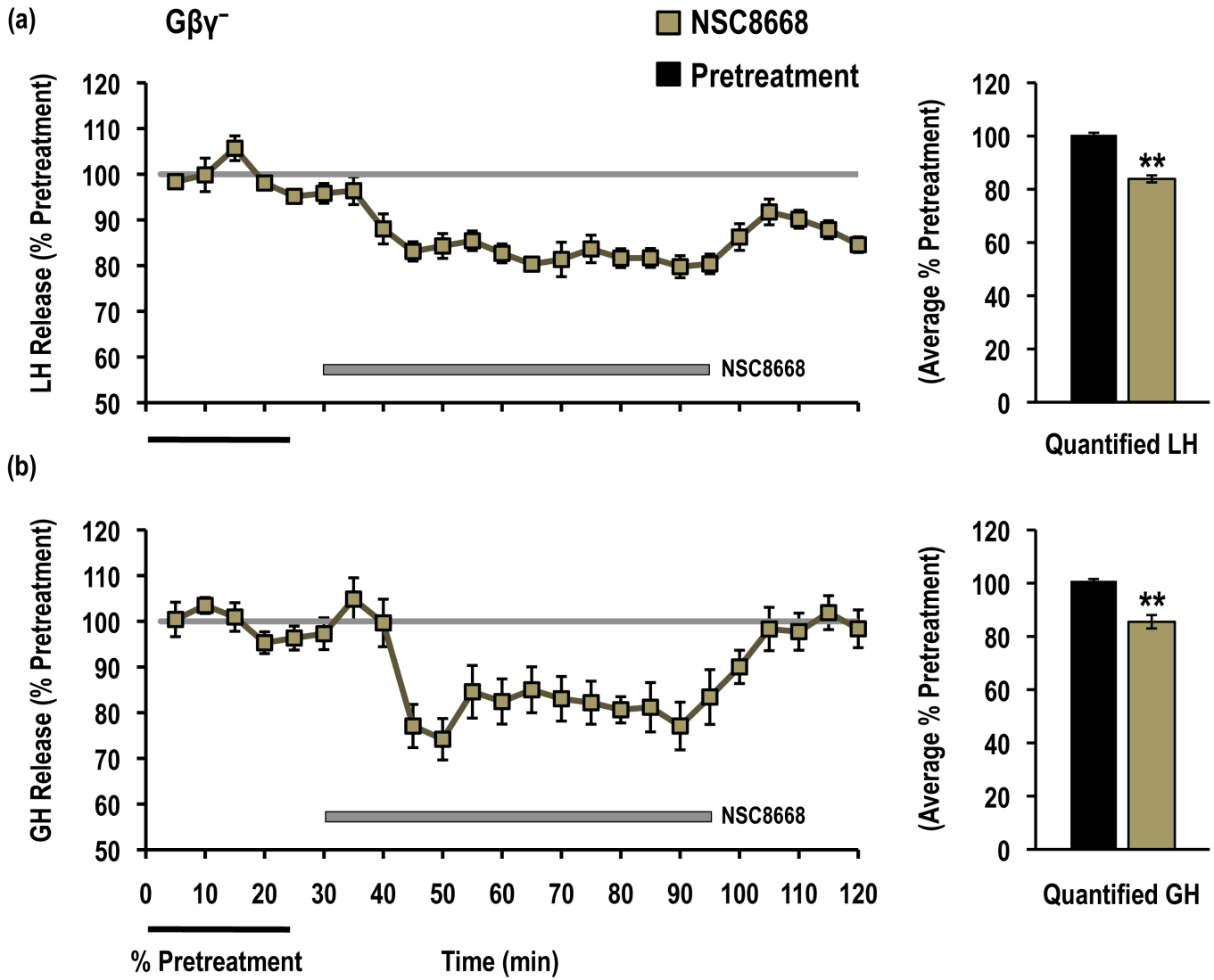

(a)
Gβγ⁻
NSC8668
Pretreatment
LH Release (% Pretreatment)
120
110
100
90
80
70
60
50
NSC8668
(Average % Pretreatment)
120
100
80
60
40
20
0
**
Quantified LH
(b)
GH Release (% Pretreatment)
120
110
100
90
80
70
60
50
NSC8668
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)
(Average % Pretreatment)
120
100
80
60
40
20
0
**
Quantified GH

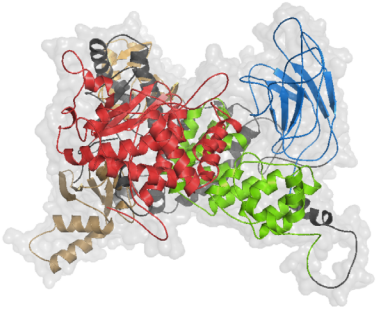
Figure 4.16 Mapping of the Gβγ-binding surface within the C2 domain-helical domain linker region of zebrafish class I PI3K catalytic subunits. Class I phosphoinositide 3-kinase (PI3K) catalytic subunits (p110α, p110β, p110δ, and p110γ) have a core structure consisting of an adaptor-binding domain (ABD), a Ras-binding domain (RBD), a C2 phospholipid-binding domain, a helical domain, and a catalytic domain. Phylogenetic analysis of the class I PI3K catalytic subunits has identified two distinct subclusters of duplicated vertebrate sequences; including, one set of paralogs consisting of PIK3CA (p110α; a) and PIK3CG (p110γ; a) as well as another paralog set comprised of of PIK3CD (p110δ; b) and PIK3CB (p110β; b). Sequence alignments of the loop region between the C2 domain and the helical domain of p110α, p110β, p110δ, and p110γ are shown on the left; whereas, homology models of the structural features of the zebrafish p110α, p110β, p110δ, and p110γ orthologs are provided on the right (prepared using PyMol). Within each paralog subcluster, the Gβγ interaction surface within the C2 domain–helical domain linker region of p110γ (^{552}RK motif; dark blue lettering; a) and p110β (^{532}KK motif; dark red lettering; b) is highly conserved among vertebrate species. In addition, the homologous dibasic motifs (yellow arrowhead) are surface accessible in the zebrafish orthologs of p110γ (residues highlighted as yellow spheres; a) and p110β (residues highlighted as yellow spheres; b), but are not present in either p110α (a; grey lettering) or p110δ (b; grey lettering). The following symbols denoting the degree of conservation observed in each sequence are provided below each alignment: an * (asterisk) indicates positions which have a single, fully conserved residue, the **:** (colon) indicates conservation between groups of strongly similar properties, while a **.** (period) indicates conservation between groups of weakly similar properties. Within each homology model, domains of p110 catalytic subunits are outlined and colored according to the legend provided at the top right (ABD, orange; RBD, brown; C2 domain, blue; helical domain, green; catalytic domain, red). Adapted from Brown and Auger, *BMC Evol. Biol.*, 2011, Dbouk et al., *Sci. Signaling*, 2012, and Vadas et al., *Proc. Natl. Acad. Sci. USA*, 2013.

(a) PIK3CA/PIK3CG Paralog Subcluster

p110α

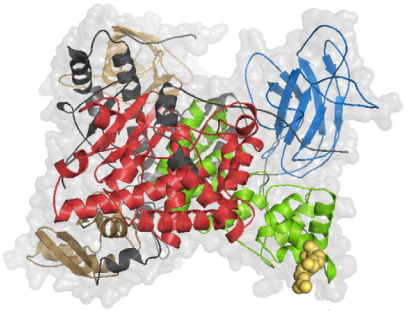
p110γ-like dibasic motif

```
Human     521 NELRENDKEQLKAIS 535
Chicken       NELRESDKEQLRAIC
Xenopus       NEVRESDKEQLKAIS
Zebrafish     HAMTEADIEQLRQLG
                :  *  *  * * *:  :
```



p110γ

```
Human     545 AEMPNQLRKQLEAII 559
Chicken       AEMPNQLRKQLEEII
Xenopus       TEMPNQLRKQLEEII
Zebrafish     REMPNHLRKQFEQII
                * * * * : * * * * : *  **
```



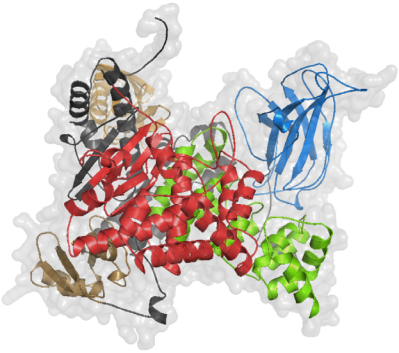
^{552}RK

(b) PIK3CD/PIK3CB Paralog Subcluster

p110δ

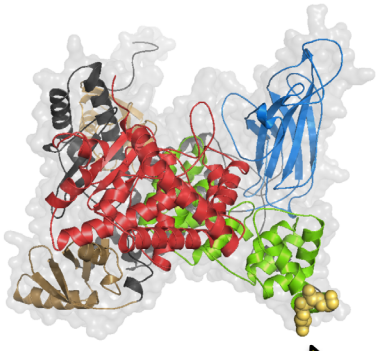
p110β-like dibasic motif

```
Human     501 VHVTEEEQLQLREIL 515
Chicken       APEDPEEKLQLKEIL
Xenopus       -----------------------
Zebrafish     VIATKEEHQKLKEIV
                      *.   : * : **:
```



p110β

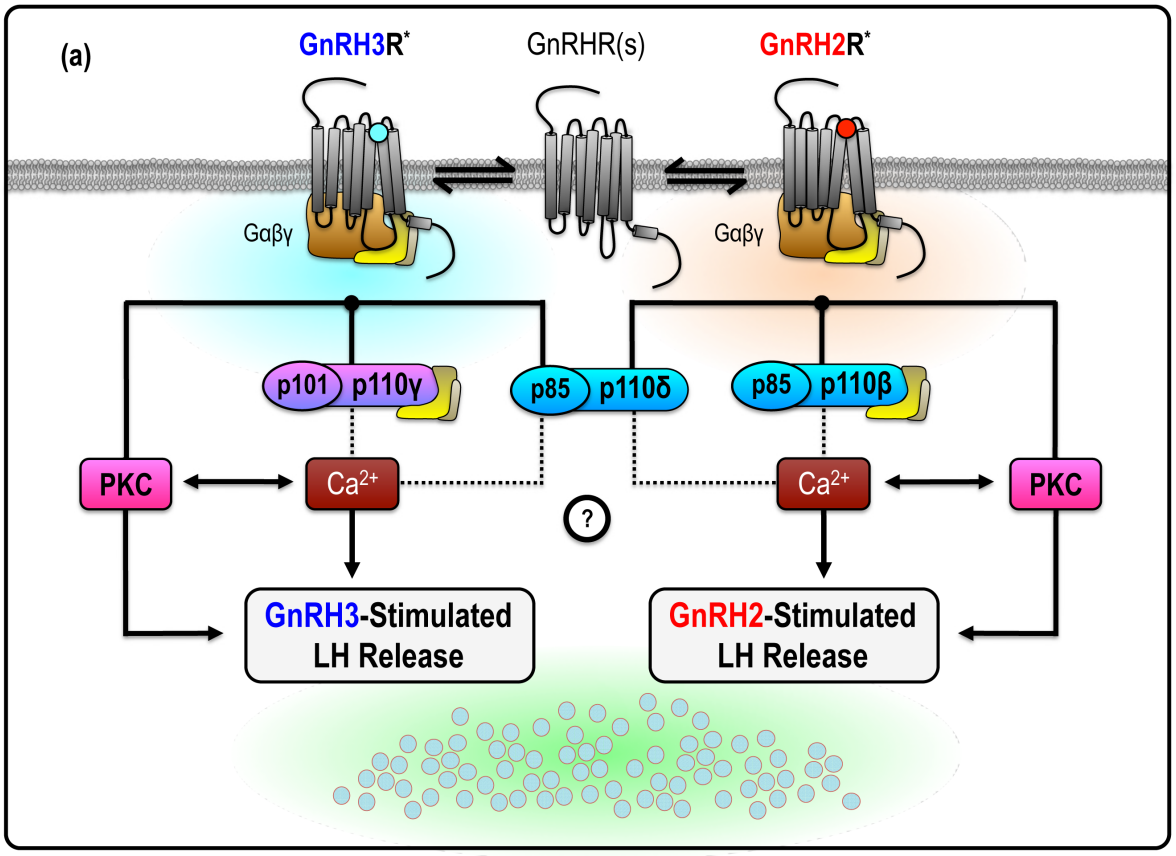
```
Human     528 SRGGKKFLPVLKEIL 542
Chicken       GRGGKKFYIVLKEIM
Xenopus       GRGGKKLNIELKEIM
Zebrafish     GRGGKKFYIELKEIM
                : * * * * * :     * * * * :
```

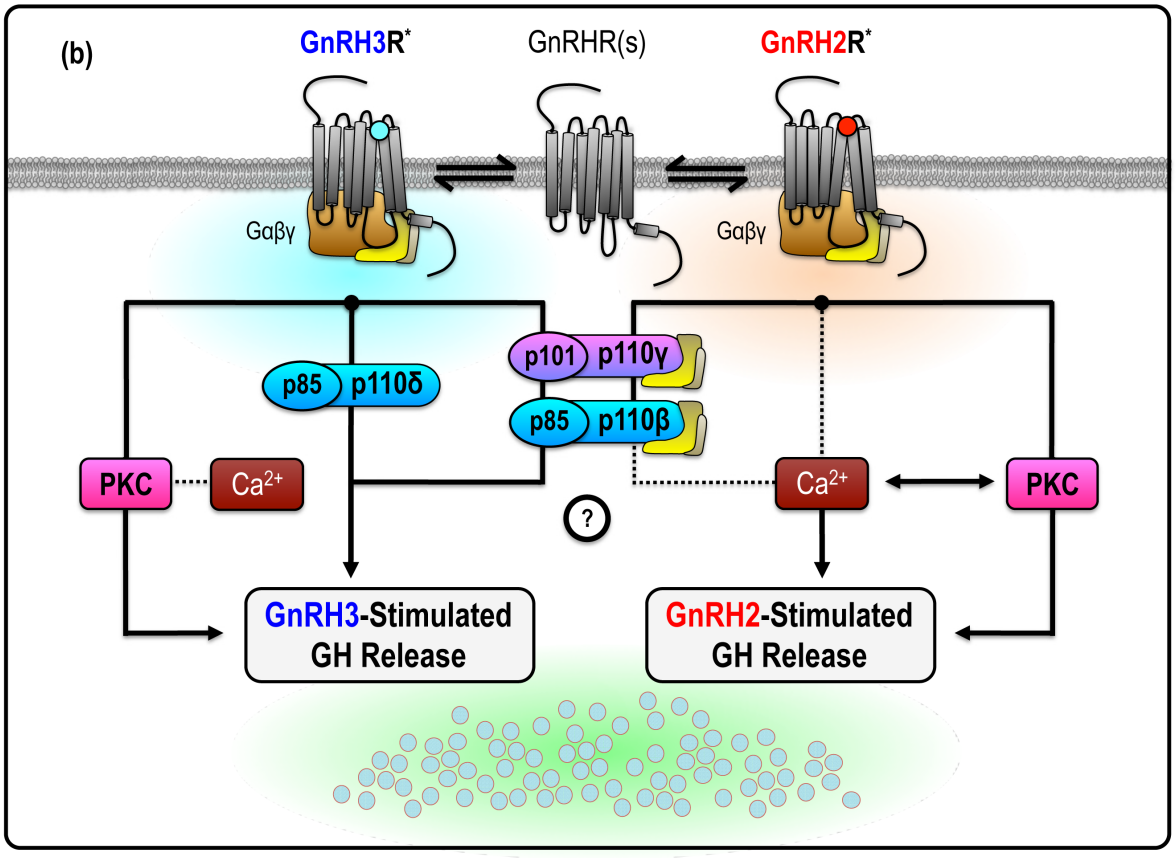


^{532}KK

C2-Helical Linker

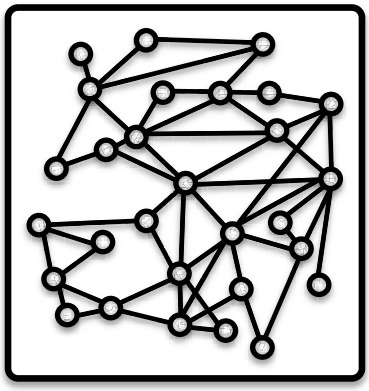
Figure 4.17 Summary model depicting the integration of class I PI3K-dependent signal transduction in GnRH2- and GnRH3-selective stimulation of acute hormone release from goldfish gonadotropes and somatotropes. In general, GnRH actions on LH and GH release did not require activation of the class IA p110α catalytic subunit. (a) GnRH2 and GnRH3 stimulation of LH release depend on the catalytic activity of p110δ. However, these endogenous GnRH isoforms also utilize unique Gβγ-dependent class I PI3K isoforms, with GnRH2 requiring p110β and GnRH3 utilizing p110γ catalytic activity. In pituitary gonadotropes, GnRH-stimulated increases in the $[Ca^{2+}]_i$ were in-part dependent upon PI3K catalytic acitivity and PI3K-dependent signalling was shown to be upstream or independent of PKC activation. (b) GnRH2- and GnRH3-stimulated acute GH release required p110β and p110γ catalytic activity; while GnRH3, but not GnRH2, actions on GH release also required the class IA isoform p110δ. As in pituitary gonadotropes, previous studies have shown that PI3K catalytic activity is either upstream or independent of PKC-dependent signalling in the control of GH release. However, only GnRH2-stimulated increases in the $[Ca^{2+}]_i$ are dependent upon PI3K catalytic activity.


(a)
GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
p101
p110γ
p85
p110δ
p85
p110β
PKC
Ca2+
Ca2+
PKC
?
GnRH3-Stimulated
LH Release
GnRH2-Stimulated
LH Release


(b)
GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
p85
p110δ
p101
p110γ
p85
p110β
PKC
Ca2+
Ca2+
PKC
?
GnRH3-Stimulated
GH Release
GnRH2-Stimulated
GH Release

Chapter Five:

PtdIns(3,4,5)P_3-dependent signalling is selectively involved in the integrated control of basal and GnRH-stimulated hormone secretion



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

5.1 Introduction

Results presented in this thesis show that the PI3K superfamily of lipid kinases in general (Chapter 3), and class I PI3K isoforms in particular (Chapter 4), are differentially involved in the control of basal, as well as GnRH2- and GnRH3-stimulated, LH and GH secretion. As reviewed in Chapter 1, all four isoforms of class I PI3K produce the lipid second-messenger PtdIns(3,4,5)P_3 despite serving distinct intracellular signalling roles (Vanhaesebroeck et al., 2010). Through interactions with PH domains, PtdIns(3,4,5)P_3 production regulates the activity of many downstream signalling proteins, including members of the AGC family protein kinases, such as PDK1 and Akt, as well as the Tec family of non-receptor tyrosine kinases such as BTK (Hawkins et al., 2006). The typical affinities measured for PtdIns(3,4,5)P_3-PH domain interactions are in the nanomolar range, and PtdIns(3,4,5)P_3-binding is a universal step required for the initiation of class I PI3K-dependent signal transduction (McNamara and Degterev, 2011). Thus, it is reasonable to hypothesize that ligand-selective activation of PtdIns(3,4,5)P_3-dependent effectors contributes to the agonist-specific control of hormone release responses by GnRH2 and GnRH3. However, whether and how PtdIns(3,4,5)P_3-sensitive downstream signalling components are required for GnRH actions on hormone release pituitary cells in goldfish, as well as in other animal study models, is not known. For reference, detailed descriptions of the canonical signalling systems activated downstream of PtdIns(3,4,5)P_3 production by class I PI3Ks are provided in Chapter 1 (also see Figures 1.10 and 1.11) and a schematic of the experimental design used in this Chapter is provided in Figure 5.1.

In this chapter, whether PtdIns(3,4,5)P_3-sensitive signalling effectors participate in the control of GnRH2- and GnRH3-stimulated LH and GH release from goldfish

pituitary cells was investigated. To do this, I first evaluated the presence of PDK1 and Akt immunoreactivity in goldfish pituitary cell protein extracts, and then performed pharmacological investigations of the possible roles PtdIns(3,4,5)P_3-dependent signal transduction effectors may play in the control of basal and GnRH-induced LH and GH secretion in cell column perifusion experiments. Specifically, perturbation of signal transduction downstream of PtdIns(3,4,5)P_3 production was achieved using a small molecule antagonist of PtdIns(3,4,5)P_3-PH domain interactions in general (PITenin (PIT)-1), as well as through the use of allosteric or ATP-competitive inhibitors of the classical PtdIns(3,4,5)P_3-dependent signalling effectors PDK1, Akt, and BTK (GSK2334470, Akt_i VIII, and CGI1746, respectively). Unlike direct inhibition of class I PI3K catalytic activity, targeting PtdIns(3,4,5)P_3-PH domain interactions represents a unique inhibition strategy that allows for the investigation of the non-catalytic roles played by PtdIns(3,4,5)P_3-dependent signalling effectors (Meuillet, 2011; Miao and Degterev, 2011). Consequently, in addition to insights regarding the regulation of PDK1-, Akt-, and BTK-dependent signalling, results from this Chapter have the potential to implicate novel PtdIns(3,4,5)P_3-sensitive effectors that control hormone release responses independently of the canonical class I PI3K transduction targets.

5.2 Results

5.2.1 Immunoreactive PDK1 and Akt are present in goldfish pituitary cell extracts and are phosphorylated under basal conditions

Immunoblotting with a rabbit anti-human PDK1 antibody revealed multiple immunoreactive PDK1 bands between 55 and 70 kDa in goldfish pituitary lysates that

were of a similar molecular weight as those observed in mammalian RBL-2H3 cell lysates (known to be between 58 and 68 kDa in size; Alessi et al., 1997; Figure 5.2b). Likewise, protein extracts from unstimulated goldfish pituitary cells also reacted positively with a primary rabbit anti-human antibody that recognizes PDK1 phosphorylated at Ser241 (Figure 5.2a). As mentioned in Chapter 1, PDK1 is thought to be constitutively active in eukaryotic cells following autophosphorylation of Ser241 within the T-loop domain (Alessi et al., 1997; Casamayor et al., 1999).

Immunoblotting for pan-Akt in goldfish pituitary cell extracts revealed two distinct bands of which the central band was both of the appropriate size (60 kDa; Frank et al., 1995) and corresponded with the immunoreactive bands observed in the mammalian RBL-2H3 cell line (positive control; Figure 5.3b). Pre-absorption of the primary antibody with the pan-Akt blocking peptide abolished the ability of the antibody to produce an immunoreactive signal (data not shown). Isoform-selective antibodies raised against human Akt1, Akt2, and Akt3 produced immunoreactive bands of the appropriate size for each of the Akt isoforms in goldfish pituitary cells (Figure 5.4). Importantly, RBL-2H3 cells have been previously shown not to express Akt3 (Cortes et al., 2014), and immunoreactivity for this Akt isoform in RBL-2H3 cells lysates was not detected (Figure 5.4). Protein extracts from unstimulated goldfish pituitary cells reacted positively to antibodies raised against Akt phosphorylated at either Ser473 or Thr308 (Figure 5.3a). Pre-absorption with an antigenic peptide abolished the ability of the phosphorylated Akt (Ser473) antibody to label morphologically identified pituitary gonadotropes (Figure 5.5) and somatotropes (Figure 5.6) within mixed pituitary cell populations. Identified pituitary gonadotropes and somatotropes could also be

immunostained using pan-Akt primary antibodies (Figures 5.6c and 5.7c). Furthermore, co-staining of phosphorylated Akt (Ser473) within LH-expressing gonadotopes (Figure 5.7) and GH-expressing somatotropes (Figure 5.8) was visualized using an imaging flow cytometry technique under unstimulated conditions. Importantly, mixed pituitary cell populations analyzed by imaging flow cytometry were not stained when incubated with the secondary antibodies alone (Figures 5.8 and 5.9).

Since PDK1 activity is dependent upon its phosphorylation at Ser241, whereas that of Akt is dependent on the coordinate regulation of phosphorylation of Ser473 and/or Thr308, these results when taken together are indicative of the presence of PDK1 and Akt activation in goldfish pituitary cells under unstimulated conditions.

5.2.2 Pharmacological mapping of PtdIns(3,4,5)P_3-dependent signalling

In general, the drug concentrations selected for use in these column perifusion studies are based on published studies of the efficacy and selectivity of these reagents in mammalian cell-based assays. However, to facilitate the understanding of the pharmacological manipulations used, I will briefly outline the specificity and mode of action of each compound before describing the experimental results.

PIT-1 is a nonphosphoinositide antagonist of PtdIns(3,4,5)P_3-PH domain interactions that was identified as part of a screen of ~50,000 small molecules using a PtdIns(3,4,5)P_3-Akt PH domain binding assay (Miao et al., 2010). PIT-1 was subsequently shown to selectively disrupt binding of PtdIns(3,4,5)P_3 to the PH domains of Akt, PDK1, general receptor for phosphoinositides 1 (GRP1), and ADP-ribosylation factor (Arf) nucleotide-binding-site opener (ARNO; Miao et al., 2010). Importantly, PITs

failed to affect membrane translocation of PtdIns(4,5)P_2- or PtdIns(3,4)P_2-specific PH domain-containing effectors and treatment with PIT-1 did not alter cellular levels of PtdIns(3,4,5)P_3 (Miao et al., 2010). Taken together, these studies identify PIT-1 as a unique and powerful tool for examining the cellular actions of PtdIns(3,4,5)P_3-selective PH domain-containing proteins. Furthermore, due to the high degree of conservation present in functionally-important protein superfolds like PH domains (Lemmon et al., 2008), selective targeting protein-lipid interaction domains by PIT-1 is an especially useful tool for studies with non-classical model organisms. On the other hand, PIT-1 does not inhibit binding of PtdIns(3,4,5)P_3 to the PtdIns(3,4,5)P_3-specific PH domain of BTK, suggesting that PIT-1 exhibits selectivity towards a subset of PtdIns(3,4,5)P_3-selective PH domains (Miao et al., 2010).

The highly selective ATP-competitive PDK1 inhibitor, GSK2334470 was used to examine the role of PDK1-mediated signalling. This compound has been shown to have little or no effect on the activity of 95 protein kinases and 15 lipid kinases assayed in cell-free systems at concentrations 100-fold greater than the reported IC_{50} (Najafov et al., 2011). However, due to the unique Akt activation mechanism employed by PDK1 that facilitates efficient activation of Akt even in the presence of a PDK1 inhibitor (Najafov et al., 2012; reviewed in Chapter 1), Akt activation is often much less sensitive to inhibition using this PDK1 inhibitor when compared with the activation of other PDK1-dependent effectors such as S6K, SGK, or RSK (Najafov et al., 2011; Knight, 2011).

To characterize the involvement of Akt, the allosteric Akt inhibitor Akt_i VIII was used (Lindsley et al., 2005). Since Akt_i VIII acts by binding to an unique PH domain-kinase domain interface, it is extremely specific for Akt isozymes over other AGC

kinases and has been reported to show minimal inhibitory effects on the catalytic activity of a representative panel of 70 protein kinases (Logie et al., 2007; Calleja et al., 2009).

Involvement of BTK catalytic activity in the control of GnRH actions on hormone release was examined using the novel ATP-dependent inhibitor of BTK called CGI1746 (Di Paolo et al., 2011). This compound has been reported to be extremely selective for BTK (IC_{50} = 1.9 nM), with over 1000-fold selectivity over similar Tec and Src family tyrosine kinases, 205 other protein kinases, as well as negligible off-target activity against 82 non-kinase targets at 1 μM (Di Paolo et al., 2011).

5.2.2.1 PtdIns(3,4,5)P_3-sensitive PH domain-containing proteins in general, but not PDK1, Akt and BTK, are important regulators of GnRH-stimulated LH release

Treatment with PIT-1 (100 μM) significantly reduced acute GnRH2- and GnRH3-stimulated hormone release responses measured in cell column perifusion (Figures 5.9 and 5.10). In contrast, inhibition of PDK1 (GSK2334470; 3 μM), Akt (Akt_i VIII; 10 μM), or BTK (CGI1746; 1 μM) did not alter the LH release responses stimulated by GnRH2 or GnRH3 (Figures 5.9 and 5.10).

5.2.2.2 PtdIns(3,4,5)P_3-sensitive PH domain-containing proteins are important regulators of GnRH-stimulated GH release

Pretreatment with PIT-1 abolished GH release responses induced by both GnRH2 and GnRH3 (Figures 5.11 and 5.12), while application of the BTK-selective inhibitor CGI1746 also reduced GnRH2-elicited GH secretion (Figure 5.11). However, the GnRH2-stimulated GH release was unaffected by both GSK2334470 and Akt_i VIII

(Figure 5.11). GnRH3-induced GH secretion was not altered by GSK2334470 or CGI1746, but was enhanced in the presence of Akt_i VIII (Figure 5.12).

5.2.3 Differential involvement of PtdIns(3,4,5)P_3-dependent signal transduction effectors in the control of basal LH and GH release

Data from perifusion studies using PIT-1, GSK2334470, Akt_i VIII, and CGI1746 treatments alone were pooled and the average hormone release observed during the inhibitor exposure were compared to those prior to inhibitor application. Inhibition of Akt catalytic activity or antagonism of PtdIns(3,4,5)P_3-PH domain interactions significantly increased basal LH by approximately 87% and 39%, respectively (Figure 5.13). Similar effects were observed for pituitary somatotropes, whereby Akt_i VIII (223% pretreatment) and PIT-1 (175% pretreatment) significantly increased basal GH release (Figure 5.14). Treatment with the selective PDK1 inhibitor GSK2334470 selectively increased basal GH release (Figure 5.14). Inhibition of BTK using CGI 1746 significantly reduced basal LH and GH release by approximately 20% (Figures 5.13 and 5.14). These observations suggest that basal LH and GH release were selectively altered by distinct PtdIns(3,4,5)P_3-sensitive effectors.

5.3 Discussion

This is the first study to examine the role of PtdIns(3,4,5)P_3-sensitive PH domain-containing intracellular signalling proteins in the regulation of pituitary hormone secretion in any vertebrate system. In addition, this is the first demonstration that PDK1 and Akt are present in goldfish pituitary cells and are catalytically active under

unstimulated conditions. Consistent with the known involvement of class I PI3Ks in GnRH action outlined in Chapter 4, kinetic analysis of GnRH-stimulated hormone release responses indicates that recruitment of PtdIns(3,4,5)P_3-sensitive PH domain-containing proteins, in general, is important for mediating GnRH effects on goldfish gonadotropes and somatotropes. Although the candidate PtdIns(3,4,5)P_3-sensitive signalling protein(s) that participates in GnRH stimulation of LH release has not been identified, Akt and BTK differentially modulates acute GH release responses in a GnRH-selective manner. Lastly, independent of agonist-stimulated actions, unique pituitary cell type-specific differences in the involvement of PtdIns(3,4,5)P_3-dependent signalling in the control of basal hormone release responses are also revealed.

5.3.1 PtdIns(3,4,5)P_3-sensitive PH domain-containing proteins are important regulators of GnRH-stimulated hormone release

PH domains are found in many proteins, but only a small number of them bind phosphoinositides such as PtdIns(3,4.5)P_3 with high affinity (Lemmon et al., 2008; reviewed in Chapter 1). Although the amino acid sequence of the PH domains within PtdIns(3,4.5)P_3-sensitive intracellular signalling proteins in goldfish are not known, sequence analyses and protein homology modelling indicate a high degree of conservation between the PtdIns(3,4,5)P_3-coordinating residues identified in mammals and those found within the teleost homologs of GRP1, ARNO, PDK1, Akt, and BTK (Figure 5.15; NCBI Conserved Domain Database; Marchler-Bauer et al., 2015). Additional support for the conservation of PtdIns(3,4,5)P_3 selectivity across vertebrates comes from a genome-wide examination of zebrafish PH domains using a recursive-

learning algorithm designed for predicting PtdIns(3,4,5)P_3-specific binding sequences (Park et al., 2008). This approach clearly identified roughly 40 highly-conserved PtdIns(3,4,5)P_3-specific PH domains in zebrafish, including those from GRP1, ARNO, PDK1, Akt, and BTK (Park et al., 2008). Taken together, these *in silico* analyses strongly suggest that the selectivity and efficacy of PIT-1 described in mammalian cells is maintained across vertebrate PH domains. Furthermore, in mammals, PIT-1 antagonizes PtdIns(3,4,5)P_3-PH domain interactions but does not affect cellular levels of PtdIns(3,4,5)P_3, or the translocation of PtdIns(4,5)P_2-sensitive PH domain containing effectors such as PLCs (Miao et al., 2010). Thus, PIT-1 is an especially powerful tool for examining the role of PtdIns(3,4.5)P_3-dependent signalling downstream of $G\alpha_{q/11}$-coupled GPCRs. The present findings with PIT-1 strongly suggest that signalling effectors containing PtdIns(3,4,5)P_3-sensitive PH domains contribute to signal transduction responses stimulated by agonist-stabilized GnRHRs. Furthermore, these results are consistent with earlier findings showing significant reductions in GnRH-stimulated hormone release using broad-spectrum (Chapter 3) or isoform-selective (Chapter 4) catalytic inhibitors of class I PI3Ks. Taken together, these results support the hypothesis that class I PI3K activation and downstream PtdIns(3,4,5)P_3-PH domain interactions mediate GnRH stimulation of acute LH and GH release in goldfish.

The identification of signalling roles for Akt and BTK during GnRH-stimulated GH release, as well as the lack of PDK1 involvement during GnRH actions on acute hormone release, are all dependent on the exquisite specificity of the inhibitors used. GSK2334470 (Najafov et al., 2011, 2012), Akt_i VIII (Lindsley et al., 2005; Logie et al., 2007), and CGI1746 (Di Paolo et al., 2011) have been shown to be highly selective for

their respective targets in mammals, but their specificity in fish has not been directly examined. However, based on *in silico* analyses of the structural characteristics of their respective target proteins, it is very likely that GSK2334470, Akt_i VIII, and CGI1746 exert similar inhibitory activities and selectivities in fish as observed in mammals. Firstly, for the PDK1 inhibitor GSK2334470, its binding mode involves distinct pockets within the PDK1 G-loop, and a hydrogen bond network involving the catalytic Lys111, Glu130, and Thr222 at the floor of the binding pocket, as well as with Ser160 and Ala162 in the hinge region (Medina et al., 2011). These coordinating residues, as well as important residues forming the base of the lipophilic pocket at the base of the G-loop (Met134, Val143, and Leu159; Medina et al., 2011), are all conserved across vertebrate homologs of PDK1. Secondly, for Akt_i VIII, its specificity and inhibitory actions are dependent on its interaction with Trp80 within the kinase cleft (Green et al., 2008; Calleja et al., 2009). Importantly, the Akt_i VIII-coordinating residue Trp80 and the Trp80 interacting residues within the c-terminal hydrophobic motif (Phe469 and Phe472) are conserved in all vertebrate homologs of Akt1, Akt2, and Akt3 (Wu et al., 2010). Lastly, CGI1746 is known to stabilize BTK in its inactive conformation by sequestering Tyr551 (Di Paolo et al., 2011). Tyr551 and Tyr223 are both conserved in zebrafish homologs of BTK, as are CGI1746-coordinating residues found within the H3 pocket of the SH3 domain, including: Phe413, Asp521, Asn526, Leu542, and Val546 (Di Paolo et al., 2011). CGI1746 also interacts with the hinge region of BTK that is located between the N- and C-lobes of the kinase domain (formed by Thr474, Glu 475, and Met477) as well as through polar interactions with the catalytic lysine (Lys430; Di Paolo et al., 2011). These coordinating residues outside of the H3 pocket are also present in BTK sequences from

Xenopus laevis and zebrafish. However, it should be noted that non-mammalian vertebrates have a conservative substitution of the hinge residue Met477 with Leu477; although the M477L substitution does not appear to alter the overall geometry of the CGI1746 binding pocket when comparing the co-crystal structure of mammalian BTK and CGI1746 with homology models of the zebrafish BTK homolog.

5.3.2 Differences in the GnRH-selective involvement of PtdIns(3,4,5)P_3-dependent signalling within pituitary gonadotropes and somatotropes

PDK1, Akt, and BTK are common intracellular signalling elements downstream of PtdIns(3,4,5)P_3. Akt activation is tightly regulated by coordinate phosphorylation events at both Thr308 by PDK1 and Ser473 by TORC2 (Manning and Cantley, 2007). Interestingly, Akt phosphorylation studies focused exclusively on the regulation of Ser473, suggest that Akt-dependent signalling is modulated by mGnRH or GnRH1 analogs in a number of immortalized and oncogenic cell types (reviewed in Chapter 1). However, there are no reports examining the direct involvement of PDK1, Akt, and BTK in the control of pituitary hormone release. Results with selective inhibitors of PDK1, Akt, and BTK demonstrate unique differences in GnRHR-mediated signalling in pituitary gonadotropes and somatotropes. In particular, inhibition of PDK1, Akt, or BTK catalytic activity failed to alter GnRH2- or GnRH3-stimulated LH release responses. However, GnRH2-stimulated GH release was significantly reduced by treatment with the BTK inhibitor CGI1746, whereas stimulation of GH release by GnRH3 was actually potentiated by an allosteric inhibitor of Akt. These observations reveal, for the first time, ligand-dependent bias in the involvement of Akt- and BTK-dependent signalling

downstream of a GPCR. Furthermore, these results suggest that the biased activation of class I PI3K isoforms observed previously (Chapter 4) leads to selective involvement of downstream PtdIns(3,4,5)P_3-sensitive PH domain-containing effectors in goldfish pituitary somatotropes. Based on the inability of GSK2334470, Akt_i VIII, and CGI1746 to alter GnRH-elicited LH release, it would be tempting to infer that bias in the recruitment of PtdIns(3,4,5)P_3-sensitive effectors does not occurs in goldfish gonadotropes. However, such a conclusion is premature since other PtdIns(3,4,5)P_3-sensitive mechanisms have not been examined. In addition, due to the unique role of PIT-1 in antagonizing the membrane translocation of PtdIns(3,4,5)P_3-sensitive effectors, we cannot exclude the possibility that kinase-independent functions of PDK1, Akt, and BTK may also contribute to basal or GnRH-stimulated signalling in both goldfish pituitary gonadotropes and somatotropes. Nevertheless, results in this chapter reinforce the idea that cell type-specific differences exist in the roles of Akt- and BTK-dependent signalling in GnRH actions.

5.3.3 Integration of BTK-dependent signalling with known components of GnRH2-mediated signal transduction in goldfish pituitary somatotropes

The present results using CGI1746 suggest that BTK-dependent signalling is selectively activated by GnRH2 to stimulate GH secretion. In isolated goldfish somatotropes, GnRH2-stimulated increases in $[Ca^{2+}]_i$ are significantly reduced by pretreatment with broad-spectrum PI3K inhibitors (Chapter 3), indicating that PI3Ks are upstream effectors of Ca^{2+}-dependent signalling. In mammalian immune cells, BTK plays a central role in the control of PLCγ-mediated increased in $[Ca^{2+}]_i$ by phosphorylating

tyrosine residues that are important for regulating PLCγ catalytic activity (Takata and Kurosaki, 1996; Wantanabe et al., 2001; Guo et al., 2004). In addition, the PLCγ PH domain directly binds to PtdIns(3,4,5)P_3 (Falasca et al., 1998). Consequently, generation of PtdIns(3,4,5)P_3 by receptor-associated class I PI3K causes acute increases in Ins(1,4,5)P_3 production and intracellular Ca^{2+} release through membrane recruitment and activation of PLCγ (Rameh et al., 1998). Interestingly, GnRH2 actions on GH release are dependent on the hydrolytic activity of PLC, but GnRH2-stimulated GH release does not utilize Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores (Johnson and Chang, 2002). Thus, BTK-dependent activation of PLCγ likely participates in the activation of DAG-sensitive isoforms of PKC following GnRHR activation by GnRH2 in goldfish somatotropes. Interestingly, Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms also directly interact with the BTK PH domain and prevent BTK membrane translocation and activation (Yao et al., 1994; Kang et al., 2001). Thus PKC may also be involved in signal-specific modulation of the strength and duration of BTK-dependent signalling (Kang et al., 2001). Adding to this complexity, BTK can directly associate with PtdIns(4)P 5-kinases (PIP5Ks) and transport them to the plasma membrane to stimulate PtdIns(4,5)P_2 synthesis (Saito et al., 2003). This allows BTK to regulate local phosphoinositide turnover by stimulating the production of the common substrate required for both class I PI3Ks and PLCs (Saito et al., 2003; Carpenter, 2004). Understanding how the intricate relationship between PLC, PKC, and BTK contributes to biased signalling downstream of activated $G\alpha_q$-coupled receptors will be an important area of future research.

The involvement of BTK in GnRH2 actions on goldfish GH release also provides a conduit for cross-talk between GPCR- and tyrosine kinase-dependent signalling in

pituitary cells. In particular, phosphorylation of Tyr551 and subsequent autoactivation of membrane-associated BTK is controlled by SFKs, including c-Src (Rawlings et al., 1996; Mahajan et al., 1995; Afar et al., 1996). SFK-dependent signalling has been shown to control GnRHR-mediated actions in a number of mammalian cell types (Naor, 2009). Furthermore, GnRH2-stimulated GH release involves p110γ and full activation of BTK downstream of agonist-stimulated GPCRs is known to be dependent upon the catalytic activity of both p110γ and c-Src (Li et al., 1997). Whether and how SFKs contribute to ligand-selective signalling by GnRHRs should be examined in the future. However, membrane recruitment and activation of BTK can also be controlled through direct interactions with the $G\alpha_q$ class of G proteins (Bence et al., 1997) or Gβγ heterodimers (Tsukada et al., 1994; Lowry and Huang, 2002). Previous studies have demonstrated that GnRHRs couple predominantly to $G\alpha_{q/11}$ and results from Chapter 4 indicate that GnRH actions on hormone release are dependent upon signalling responses stimulated by Gβγ subunits. Therefore, it is possible that activated Gαβγ complexes could also directly contribute to BTK-dependent signalling downstream of GnRH2-stabilized GnRHRs in goldfish somatotropes.

5.3.4 Integration of Akt-dependent signalling with known components of GnRH3-mediated signal transduction in goldfish pituitary somatotropes

Results from this chapter reveal that Akt is actually a negative regulator of GnRH3-stimulated signalling in goldfish somatotropes. How activation of Akt negatively modulates GnRH3-induced, but not GnRH2-stimulated, GH release is not known but a possible mechanism can be postulated. The unique enhancement of GnRH3-stimulated

GH release following Akt inhibiton might involve an interaction between Akt- and Ca^{2+}-dependent signalling. One of the differences between GnRH2- and GnRH3-dependent signalling in somatotropes involves the selective use of Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores in GH release stimulated by GnRH3, but not GnRH2 (Johnson and Chang, 2002). In multiple cell types, Akt interacts with IP_3R subtypes and reduces their Ca^{2+} release activity via phosphorylation (Khan et al., 2006; Szado et al., 2008; Marchi et al., 2012). Inhibition of Akt might relieve inhibitory IP_3R phosphorylation to selectively potentiate GnRH3-stimulated GH release. Interestingly, atypical PKCζ, but not PKCα or PKCδ, can co-immunoprecipitate Akt and kinase-dead mutants of PKCζ result in global increases in intracellular phosphorylation of Akt, reducing Akt activity (Doornbos et al., 1999). PKCζ is expressed in goldfish pituitary cells (Klausen et al., 2005) and could potentially control Akt activity downstream of PLC activation. The involvement of PKCζ in GnRH signalling in goldfish pituitary cells is also quite intriguing considering that this isoform is directly activated by PtdIns(3,4,5)P_3 (Nakanishi et al., 1993). However, GnRH3 actions on LH release also involve Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores but the effects of Akt inhibition are selective for GnRH3 actions on GH and not LH release. The basis for this cell-type difference is at present unknown but may be due to selective uncoupling of Akt from class I PI3K-dependent signalling.

In contrast to the apparent role of Akt in regulating GnRH3 actions on GH secretion discussed above, results from Chapter 3 indicate that PI3K-dependent signalling likely contributed to GnRH3-dependent signal transduction at steps that are independent or downstream of increases in $[Ca^{2+}]_i$. Though somewhat of a paradox, the uncoupling of PI3K- and Akt-dependent signaling has been shown in several models of GPCR-

mediated signalling. In particular, signalling downstream of GPCRs coupled to $G\alpha_q$ can both positively and negatively regulate Akt signalling. Stimulation of class I PI3Ks by $G\beta\gamma$ heterodimers increases phosphorylation of Akt, whereas $G\alpha_q$ inhibits Akt activation downstream of $G\beta\gamma$-effector interactions (Bommakanti et al., 2000). $G\alpha_q$-coupled receptors can also utilize G protein-independent mechanisms to control Akt-dependent signalling (New et al., 2007; Defea, 2008). In particular, β-arrestins can form signalling complexes that recruit Akt and the protein phosphatases PP2A (Beaulieu et al., 2005, 2007, 2008) or PHLPP (Crotty et al., 2013). In both of these complexes, dephosphorylation of Akt negatively regulated Akt activity (Beaulieu et al., 2005; Crotty et al., 2013). Akt signalling can also be controlled upstream of arrestin scaffolds; Akt can physically interact with GRK2 and this interaction inhibits Akt activity (Liu et al., 2005). Furthermore, non-visual arrestins bind PtdIns(3,4,5)P_3 with high affinity, and treatment with PtdIns(3,4,5)P_3 can enhance arrestin interactions with GPCRs (Gaidarov et al., 1999). As a result, increases in the local effective PtdIns(3,4,5)P_3 concentration around the receptor by class I PI3Ks could stabilize GPCR-arrestin complexes that inactivate Akt. It is now well established that phosphorylation of GPCRs by GRKs facilitates the formation of arrestin signalling scaffolds, resulting in the uncoupling of receptors from G proteins (Ritter and Hall, 2009; Reiter et al., 2012; Irannejad and von Zastrow, 2014). Whether negative regulation of Akt activity contributes to GnRH3-selective signalling through interactions with GRKs and/or arrestins will be an important area for future research. However, regardless of what these future findings may reveal, it is quite clear that Akt serves a unique role as a convergence point for G protein-dependent and G protein-independent signalling downstream of GPCRs.

5.3.5 Novel roles for PtdIns(3,4,5)P_3-dependent signalling downstream of GnRHR activation

When coupled with the inhibitory actions of PIT-1, the observations that treatments with GSK2334470, Akt_i VIII, or GCI1746 do not affect LH release responses to GnRH application suggest the involvement of novel PtdIns(3,4,5)P_3-dependent regulators in the control of LH and GH release. Foremost, due to the known inhibitory actions of PIT-1 on the translocation and signalling by other important intracellular effectors, such as GRP1 and ARNO (Miao et al., 2012), it is possible that these PtdIns(3,4,5)P_3-sensitive targets are involved in GnRHR-mediated signalling. GRP1 and ARNO are members of the cytohesin family of Arf-GEFs (Jackson et al., 2000). Arfs belong to the Ras superfamily of small GTPases and are involved in fundamental biological processes, including cytoskeletal remodelling and exocytosis (Monier et al., 1998; Jackson et al., 2000; D'Souza-Schorey and Chavier, 2006). GRP1 and ARNO both regulate actin and membrane remodeling through activation of ARF1 and ARF6 (Radhakrishina et al., 1999; Klarlund et al., 1999; DiNitto et al., 2007). In particular, ARF6 is an important component of Ca^{2+}-regulated exocytosis in endocrine cell types, and contributes to the integrated control of hormone secretion in a number of model systems (D'Souza-Schorey and Chavier, 2006). It is likely that GRP1 and ARNO regulate Arf-dependent signalling to control basal and/or GnRH-stimulated hormone release responses.

5.3.6 Class I PI3Ks and PtdIns(3,4,5)P_3-dependent signal transduction in the control of basal LH and GH release

In addition to effects on GnRH-stimulated responses, results reveal novel roles for PDK1-, Akt-, and BTK-dependent signal transduction in the control of basal LH and GH release from goldfish pituitary cells. In goldfish, basal LH and GH release are not controlled by PKC, but instead require PKA- and cAMP-dependent signalling, as well as extracellular Ca^{2+} (Chang et al., 2000; Chapter 1). In this chapter, inhibition of PDK1 selectively increased basal GH, but not basal LH release responses. Since the actions of GSK2334470 are known to spare Akt activation (Najafov et al., 2011, 2012; also see Sections 1.7.2.4 and 5.2.2), it is likely that PDK1 catalytic activity contributes to basal GH release, at least in part, in an Akt-independent manner. The divergent effects of Akt_i VIII and GSK2334470 on basal LH release are also consistent with this hypothesis. This possibility is also supported by recent studies identifying a prominent role for Akt-independent PDK1 signalling downstream of oncogenic mutations in p110α (Vasudevan et al., 2009). In these systems, cells retain robust PDK1 membrane localization and exhibit a dependency on the PDK1 substrate SGK3 while Akt activation is suppressed (Vasudevan et al., 2009; Bruhn et al., 2013; Gasser et al., 2014). Thus, the Akt-independent actions of PDK1 on basal GH release could also involve SGK-mediated signalling. However, at this time, we cannot exclude the involvement of additional PDK1-regulated AGC family kinases or kinase-independent roles of PDK1.

Results from this chapter clearly demonstrate that Akt_i VIII elevates both basal LH and GH release, suggesting that Akt activation negatively regulates secretion of these hormones. Similar results have also been observed in insulin secreting β-cells, where

inhibition of Akt enhances newcomer granule fusions and the motility of intracellular insulin granules (Aoyagi et al., 2012). Interestingly, acute treatment with the p110α-selective inhibitor PIK-75 significantly increases the exocytic responses originating from newcomer granules during glucose-stimulated insulin secretion while fusion events arising from previously docked granules are decreased by a mechanism independent of $[Ca^{2+}]_i$ dynamics (Aoyagi et al., 2012). Taken together, these findings identify a role for class IA PI3K- and Akt-dependent signalling in the control of vesicular trafficking and fusion events governing basal secretion in representative endocrine cell types.

In goldfish, basal LH and GH release are not controlled by PKCs, but instead require PKA- and cAMP-dependent signalling (Chang et al., 2000). PKA does not possess a PDK1-sensitive residue within the T-loop (Pearce et al., 2010) but the C-terminal of PKA can directly interact with the kinase domain of PDK1 (Biondi et al., 2000). This interaction between PDK1 and PKA is thought to facilitate rapid phosphorylation of PKA at Thr197 by PDK1 leading to activation of the catalytic subunit of PKA (Cheng et al., 1998; Moore et al., 2002). Therefore, Akt and PKA are common substrates of PDK1 that both physically interact with PDK1 at the membrane. It is likely that PKA- and Akt-dependent signalling pathways communicate with one another to coordinate basal LH and GH release responses. While inhibiton of Akt results in a robust increase in both basal LH and GH release (this chapter), treatment with selective PKA inhibitors decreases basal LH and GH secretion (Chang et al., 2000). These results suggest opposing roles for cAMP/PKA- and Akt-dependent signalling. Interestingly, in mammalian systems, cAMP-dependent signalling inhibits Akt activation by reducing PtdIns(3,4,5)P_3 production and translocation of PDK1 to the plasma membrane (Kim et

al., 2001). Akt can also phosphorylate and activate PDE3B to reduce the intracellular concentrations of cAMP (Kitamura et al., 1999). Consequently, the inhibitory actions of cAMP-dependent PKA activation on Akt-dependent signalling may also feed forward by inactivating PDE-mediated hydrolysis of cAMP. In support of this, the broad-spectrum PI3K inhibitor wortmannin has been shown to increase insulin secretion by inhibiting PDE activity to increase cAMP content (Nunoi et al., 2000). Taken together, we hypothesize that the negative influence of Akt-dependent signalling can be relieved through tonic activation of PKA. It is possible that the catalytic activities of these effectors are tightly regulated in order to control basal hormone release responses.

Unlike manipulations of PDK1 and Akt, inhibition of BTK catalytic activity resulted in sustained reductions in basal LH and GH release, suggesting a positive role for BTK-dependent signalling in regulating the basal secretion of these hormones. Interestingly, the magnitude of changes in unstimulated LH and GH release following treatment with the BTK-selective inhibitor are similar to those observed using ATP-dependent inhibitors of class I PI3K catalytic activity, as well as allosteric modulators of Gβγ-dependent signaling (Chapter 4). These observations, when viewed together, strongly suggests that BTK-dependent signalling participates in the basal control of LH and GH release, as least in part, in a PtdIns(3,4,5)P_3-sensitive pathway that is independent from PDK1 and Akt. Furthermore, it is likely that BTK actions are dependent upon Gβγ-mediated activation of p110β and p110γ catalytic activity since inhibition of these two class IA PI3Ks similarly reduces basal LH and GH release (Chapter 4). In addition, as mentioned above, BTK can also be directly activated by Gβγ heterodimers (Tsukada et al., 1994; Lowry and Huang, 2002), although membrane

translocation of BTK would still be expected to be dependent on the production of PtdIns(3,4,5)P_3.

The divergent effects of PIT-1 and those of the selective inhibitors of PDK1-, Akt-, and BTK-dependent signalling on basal LH and GH secretion indicate that PtdIns(3,4,5)P_3-sensitive effectors have both stimulatory and inhibitory influences. Interestingly, previous results has revealed that while LY294002 and wortmannin acutely increase basal LH and GH release (Chapter 3) isoform-selective inhibitors of individual p110 catalytic subunits generally decrease basal hormone secretion (Chapter 4). Likewise, in pancreatic β-cells, broad-spectrum inhibitors of PI3K are known to enhance insulin secretion (Hagiwara et al., 1995; Zawalich and Zawalich, 2000; Eto et al., 2002), whereas isoform-selective inhibition of p110γ or class II PI3Ks results in the reduction of insulin secretion (MacDonald et al., 2004; Leibiger et al., 2010; Dominguez et al., 2010). Differential regulation of the PtdIns(3,4,5)P_3-sensitive signalling effectors described in this chapter provide the basis for these paradoxical observations using PI3K inhibitors, and again highlight the need for additional studies using isoform-selective inhibitors of the class II and class III PI3Ks discussed earlier in Chapters 3 and 4.

5.3.7 Summary

Class I PI3Ks are complex regulators of intracellular signal transduction. Overall, results from this thesis chapter add to the general understanding of functional selectivity in GPCR signal transduction networks by demonstrating, for the first time, the complexity of ligand-biased signalling downstream of class I PI3K catalytic activity. Results demonstrate that PtdIns(3,4,5)P_3-sensitive PH domain-containing signal

transduction components differentially participate in GnRH-elicited LH and GH secretion in a GnRH- and cell type-selective manner. In addition, important mechanistic differences in the control of basal and agonist-stimulated hormone release by PtdIns(3,4,5)P_3-dependent effectors are demonstrated. Overall, I hypothesize that biased activation of class I PI3Ks by GnRH variants results in unique PtdIns(3,4,5)P_3 signalling micorenvironments that facilitate the selective coupling of PH domain-containing components in response to activated GnRHRs. Given that the control of PtdIns(3,4,5)P_3 production by class I PI3Ks is both dynamic and subject to modulation by complex regulatory networks, class I PI3K signalling is an attractive intracellular platform for integrating the multifactorial regulation of gonadotrope and somatotrope activity. Future studies examining the long-term effects of PI3K-dependent signalling during the coordinate control of hormone production and secretion will provide further insights into the complexity of basal and agonist-stimulated regulation in goldfish and other vertebrate pituitary cells.

Figure 5.1 Schematic depicting the possible integration of class I PI3K-dependent signal transduction during GnRH2- and GnRH3-selective stimulation of acute hormone release from goldfish gonadotropes and somatotropes. In general, GnRH actions on LH and GH release do not require activation of the class IA p110α subunit, but instead depends on the ligand-biased activation of p110δ, p110β, and p110γ catalytic activity. However, all class I PI3Ks produce the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3); suggesting that GnRH-selective signalling responses require the differential recruitment of PtdIns(3,4,5)P_3-sensitive signalling effectors. In general, the presence of PtdIns(3,4,5)P_3-sensitive binding domains in a variety of signaling proteins mediates the complexity of signal transduction downstream of class I PI3Ks. Canonical intracellular effectors activated downstream of PtdIns(3,4,5)P_3 production include: phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK). The coordinate or selective activity of these effectors results in the regulation of class I PI3K-stimulated cellular responses. In this thesis Chapter, pharmacological mapping of canonical class I PI3K-dependent signalling during acute basal and GnRH-stimulated hormone release was achieved using selective PDK1 (GSK2334470; 3 μM), Akt (Akt_i VIII; allosteric; 10 μM), and BTK (CGI1746; 1 μM) small molecule inhibitors. Additionally, in order to potentially identify non-canonical signalling effectors activated downstream of class I PI3Ks, we utilized a antagonist of PtdIns (3,4,5)P_3-PH domain interactions called PIT-1 (100 μM).

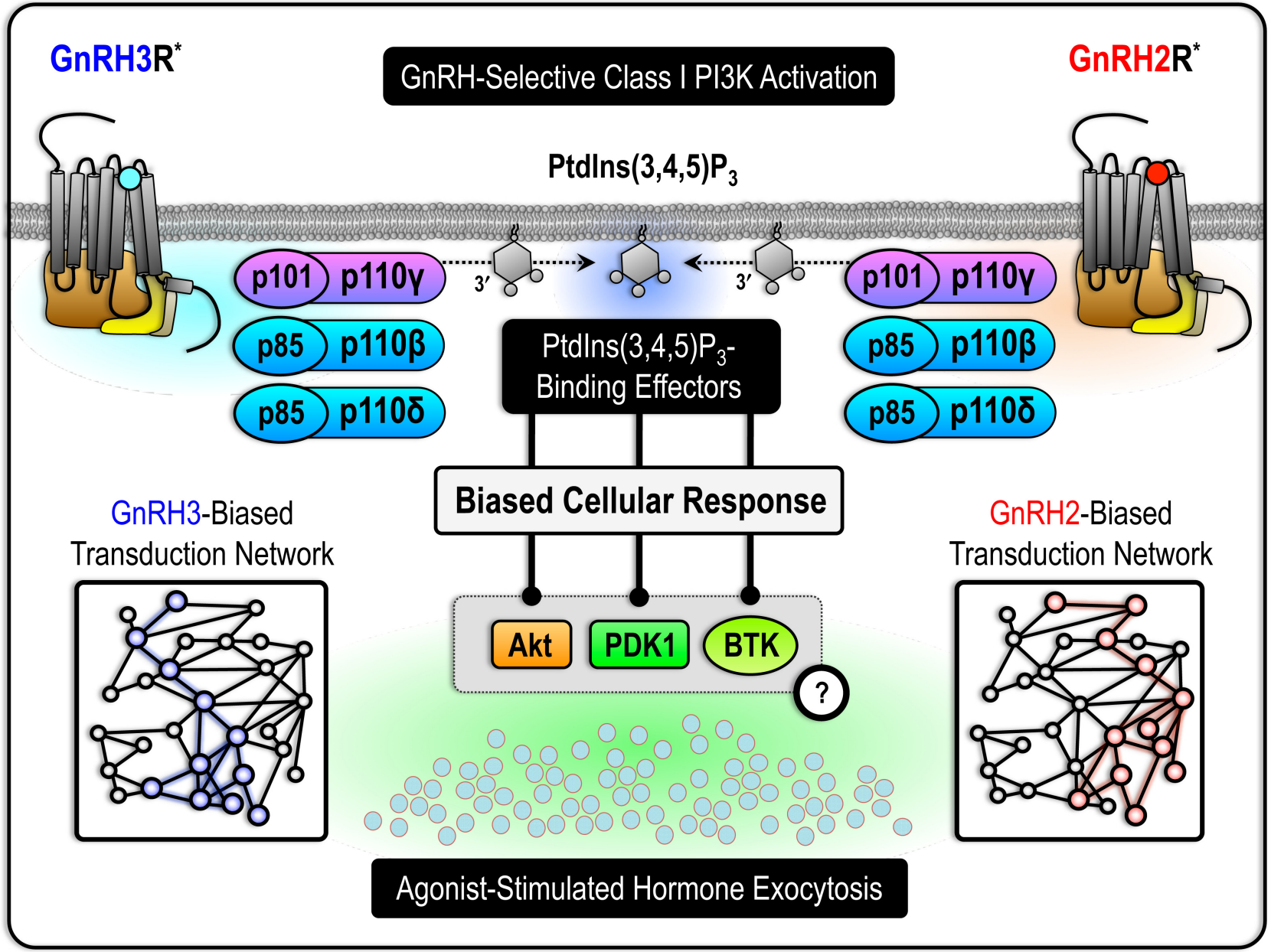
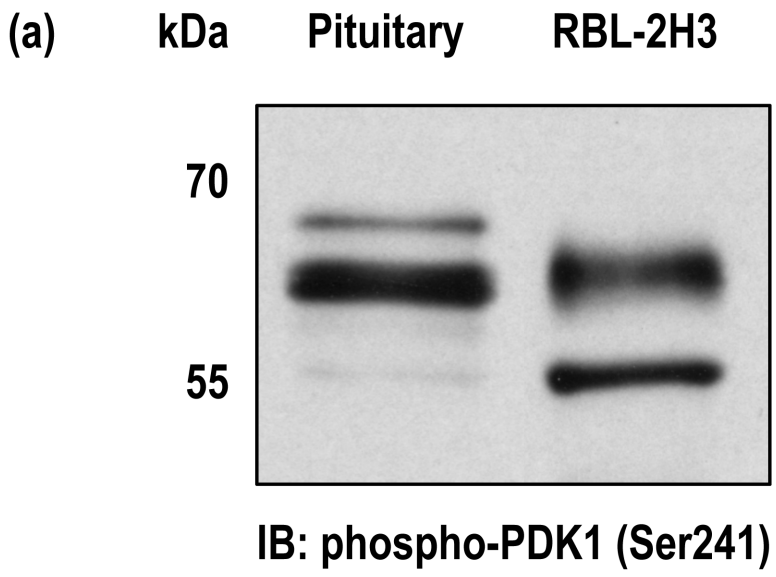

GnRH3R*
GnRH-Selective Class I PI3K Activation
GnRH2R*
PtdIns(3,4,5)P3
p101
p110γ
p85
p110β
p85
p110δ
3′
3′
p101
p110γ
p85
p110β
p85
p110δ
PtdIns(3,4,5)P3-
Binding Effectors
Biased Cellular Response
GnRH3-Biased
Transduction Network
GnRH2-Biased
Transduction Network
Akt
PDK1
BTK
?
Agonist-Stimulated Hormone Exocytosis

Figure 5.2 Expression of catalytically active PDK1 in primary goldfish pituitary cell cultures under unstimulated conditions following 2 hrs in static culture. Representative immunoblots (from one of three independent experiments) of whole cell lysates isolated from mixed goldfish pituitary cell cultures using primary antibodies recognizing (a) PDK1 phosphorylated at residue Ser241, and (b) the same membrane probed with a PDK1 antibody (expected molecular mass of between 58 and 68 kDa). Positive controls for immunoblotting were performed using whole cell lysates from the RBL-2H3 rat basophilic leukaemia mast cell line, known to express constitutively active PDK1 phosphorylated at the Ser241 autoregulatory residue.


(a)
kDa
Pituitary
RBL-2H3
70
55
IB: phospho-PDK1 (Ser241)

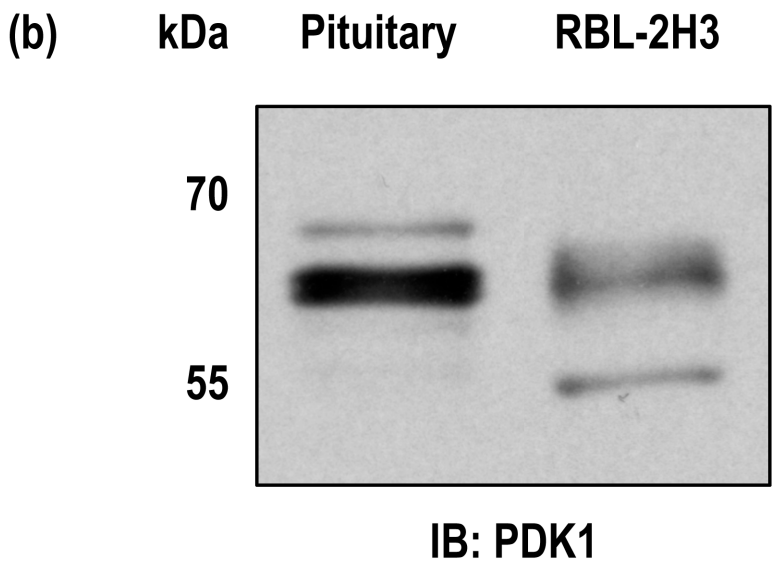
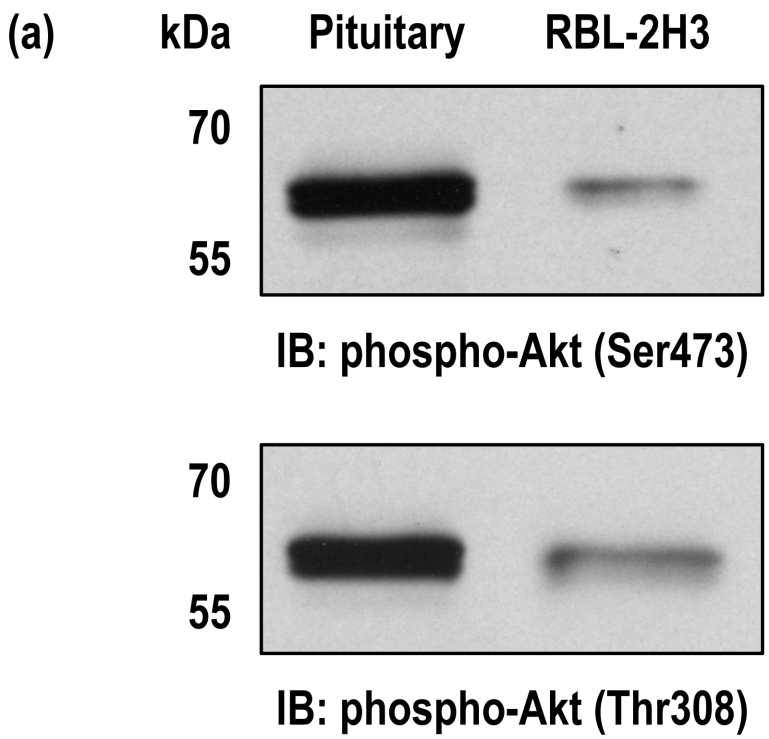

(b)
kDa
Pituitary
RBL-2H3
70
55
IB: PDK1

Figure 5.3 Expression of phosphorylated (Ser473 and Thr308) and total Akt in primary goldfish pituitary cell cultures under unstimulated conditions following 2 hrs in static culture. Representative immunoblots (from one of three independent experiments) of whole cell lysates isolated from mixed goldfish pituitary cell cultures using primary antibodies recognizing (a) Akt phosphorylated at either residue Ser473 (top) or Thr308 (bottom), and (b) the same membrane probed with a pan-Akt antibody. All of the Akt antibodies used cross-react with conserved sequences within Akt1, Akt2, and Akt3 (molecular mass is expected to be ~ 60 kDa). Positive controls for Akt immunoblotting were performed using whole cell lystates isolated from a rat basophilic leukaemia cell line (RBL-2H3).


(a)
kDa
Pituitary
RBL-2H3
70
55
IB: phospho-Akt (Ser473)
70
55
IB: phospho-Akt (Thr308)

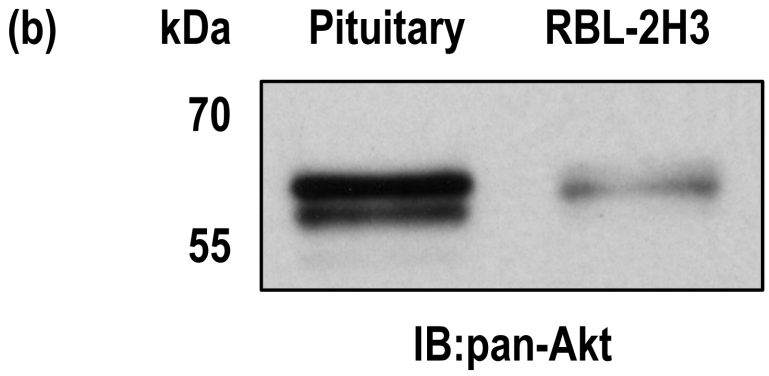

(b)
kDa
Pituitary
RBL-2H3
70
55
IB:pan-Akt

Figure 5.4 Expression of immunoreactive (a) Akt1, (b) Akt2, and (b) Akt3 isoforms in primary goldfish pituitary cell cultures. Immunoblots of whole cell lysates isolated from mixed goldfish pituitary cell cultures using isoform-selective primary antibodies raised against human (a) Akt1 (PKBα; expected molecular mass of 60 kDa), (b) Akt2 (PKBβ; expected molecular mass of 60 kDa), and (c) Akt3 (PKBγ; expected molecular mass of 60 kDa). Positive controls for immunoblotting were performed using whole cell lysates from the RBL-2H3 rat basophilic leukaemia mast cell line. Importantly, the RBL-2H3 cell line is known to express Akt1 and Akt2, but not Akt3.

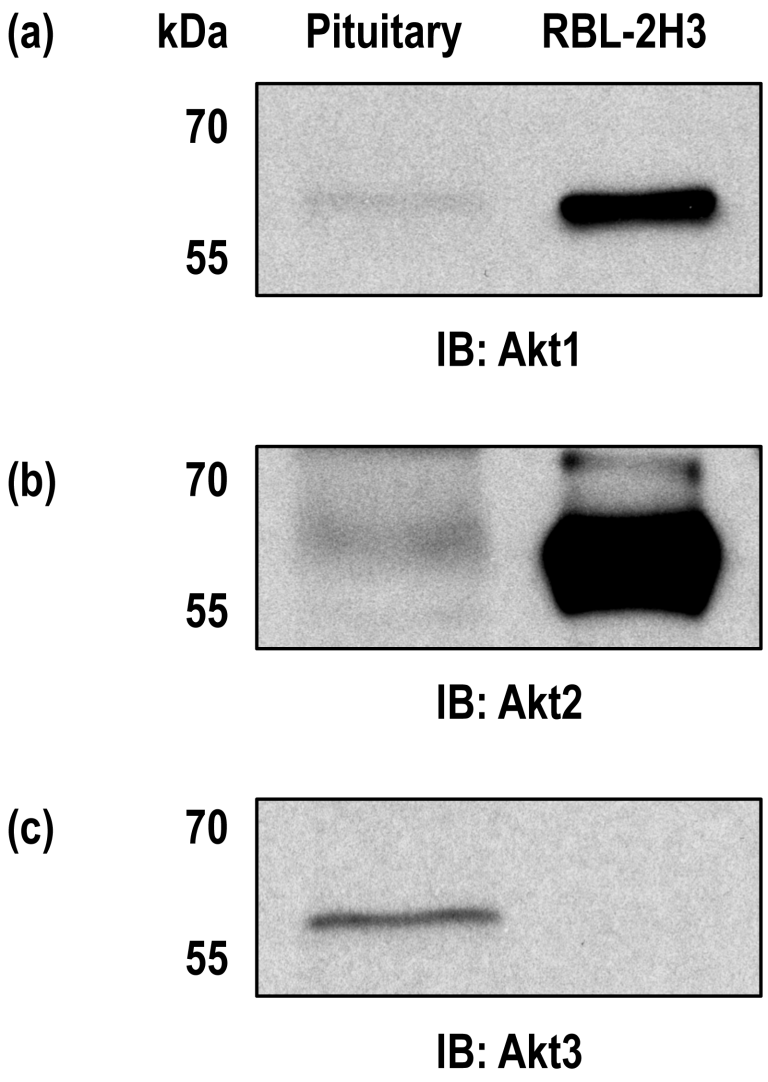
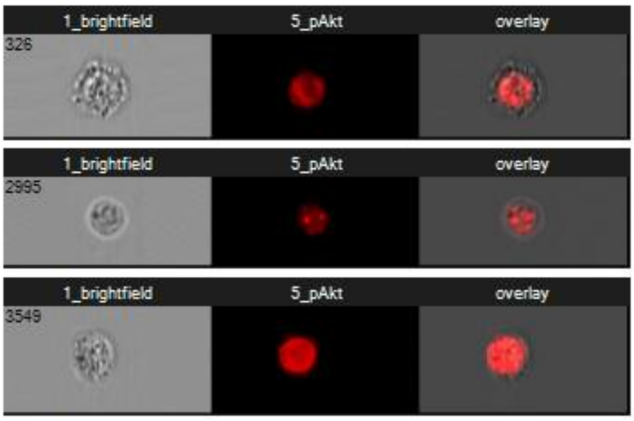

(a)
kDa
Pituitary
RBL-2H3
70
55
IB: Akt1
(b)
70
55
IB: Akt2
(c)
70
55
IB: Akt3

Figure 5.5 Expression of phosphorylated (Ser473) and total Akt in morphologically identified pituitary gonadotropes using imaging flow cytometry. (a) Individual goldfish gonadotropes were identified based on their unique morphological characteristics (Van Goor et al., 1994) and their size as well as scatter properties. (Chang et al., 2014). Representative bright field images and the relative phospho-Akt (Ser473) staining (red colour; channel 5) are shown. Fluorescently conjugated phospho-Akt (Ser473; D9E XP™; Alexa Fluor® 647 Conjugate; 1:50) primary antibodies were used. (b) Pre-absorption of the phospho-Akt (Ser473) monoclonal antibody with the phospho-Akt (Ser473) blocking peptide (Cell Signaling Technologies) abolishes immunostaining in morphologically identified goldfish pituitary gonadotropes. (c) Subsets of cells were also stained with a pan-Akt antibody (1:100; green colour; channel 2) and the nuclear counter-stain Hoechst 33342 (red colour; channel 5). Representative images are shown. Primary pan-Akt antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200).

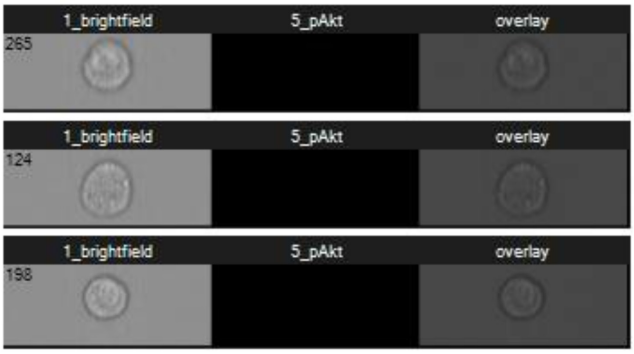
Morphologically Identified Pituitary Gonadotropes

(a) IF: phospho-Akt (Ser473)



− phospho-Akt (Ser473) peptide

(b) IF: phospho-Akt (Ser473)



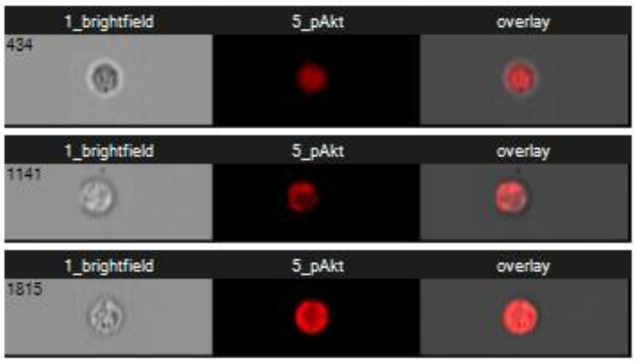
+ phospho-Akt (Ser473) peptide

(c) IF: pan-Akt

Figure 5.6 Expression of phosphorylated (Ser473) and total Akt in morphologically identified pituitary somatotropes using imaging flow cytometry. (a) Individual goldfish somatotropes were identified based on their unique morphological characteristics (Van Goor et al., 1994) and their size as well as scatter properties. (Chang et al., 2014). Representative bright field images and the relative phospho-Akt (Ser473) staining (red colour; channel 5) are shown. Fluorescently conjugated phospho-Akt (Ser473; D9E XP™; Alexa Fluor® 647 Conjugate) primary antibodies were used. (b) Pre-absorption of the phospho-Akt (Ser473; 1:50) monoclonal antibody with the phospho-Akt (Ser473) blocking peptide (Cell Signaling Technologies) abolishes immunostaining in morphologically identified goldfish pituitary somatotropes. (c) Subsets of cells were also stained with a pan-Akt antibody (1:100; green colour; channel 2) and the nuclear counter-stain Hoechst 33342 (red colour; channel 5). Representative images are shown. Primary pan-Akt antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200).

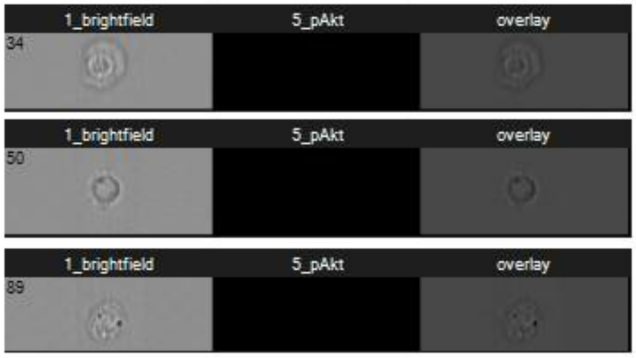
Morphologically Identified Pituitary Somatotropes

(a) IF: phospho-Akt (Ser473)



− phospho-Akt (Ser473) peptide

(b) IF: phospho-Akt (Ser473)

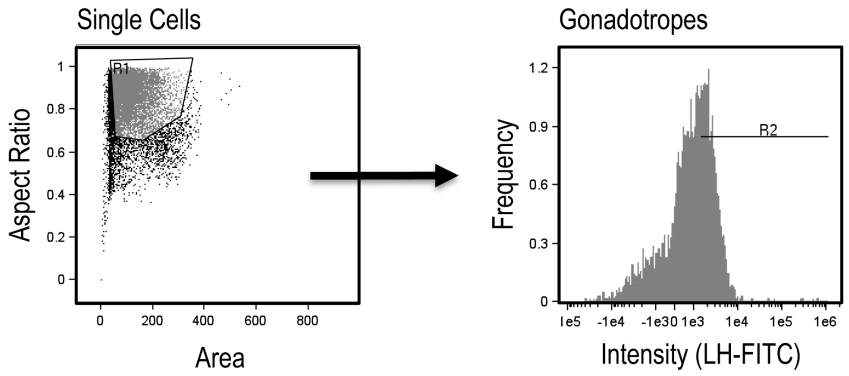


+ phospho-Akt (Ser473) peptide

(c) IF: pan-Akt

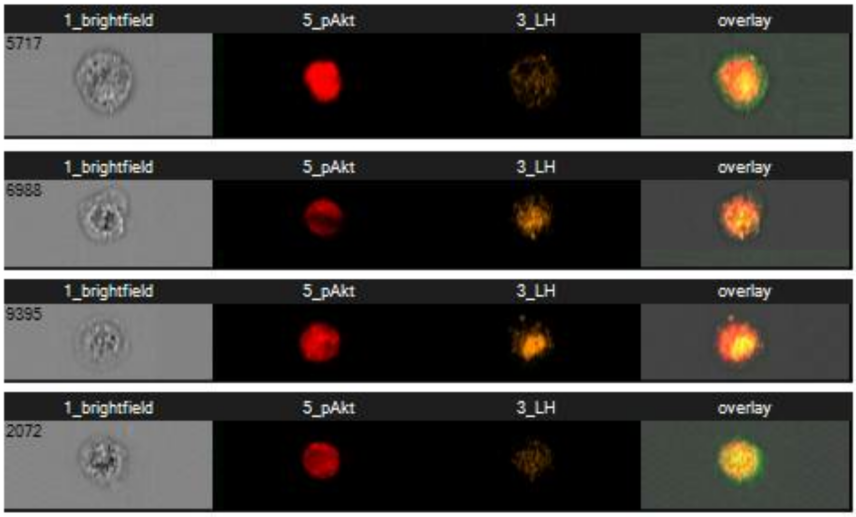
Figure 5.7 Double-labeling of phosphorylated Akt (Ser473) and luteinizing hormone (LH) within mixed pituitary cell populations using imaging flow cytometry. (a) Single cells were first identified based on their area, aspect ratio, and imaging focus (left) as well as the intensity of anti-LH staining (right); this population of LH-positive cells was used for all subsequent analyses. (b) Using the collected images, goldfish gonadotropes were identified by staining positively with LH antiserum (rabbit anti carp cGTH-378, 1:6000; Cook et al., 1991) and possessing morphological characteristics of identified gonadotropes (Van Goor et al., 1994). Primary LH antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200), while phospho-Akt staining required fluorescently conjugated phospho-Akt (Ser473; D9E XP™; Alexa Fluor® 647 Conjugate; 1:50) primary antibodies. Representative bright field images and relative phospho-Akt (Ser473; red colour; channel 5) and LH (orange colour; channel 3) co-staining are shown. (c) Subsets of cells were also stained with the secondary antibody alone (green colour; channel 2) and the Hoechst 33342 nuclear counter-stain (red colour; channel 5). Representative images are shown.

(a)



Pituitary Gonadotropes

(b)



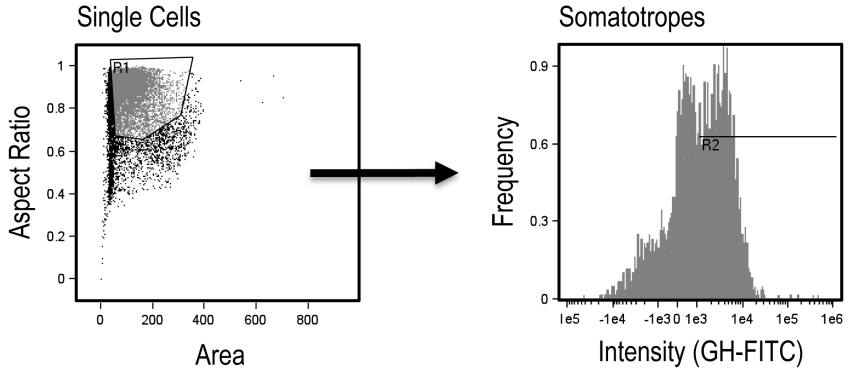
IF: phospho-Akt (Ser473) and LH

(c)

Secondary Antibody Alone

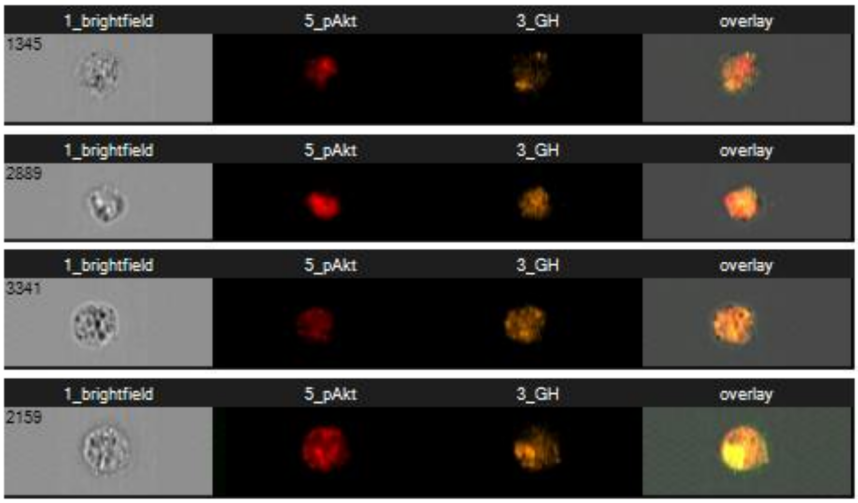
Figure 5.8 Double-labeling of phosphorylated Akt (Ser473) and growth hormone (GH) within mixed pituitary cell populations using imaging flow cytometry. (a) Single cells were first identified based on their area, aspect ratio, and imaging focus (left) as well as the intensity of anti-GH staining (right); this population of GH-positive cells was used for all subsequent analyses. (b) Using the collected images, goldfish somatotropes were identified by staining positively with GH antiserum (rabbit anti-carp kcGH R#1 primary antibody, 1:3000; Cook et al., 1991) and possessing morphological characteristics of identified somatotropes (Van Goor et al., 1994). Primary GH antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200), while phospho-Akt staining required fluorescently conjugated phospho-Akt (Ser473; D9E XP™; Alexa Fluor® 647 Conjugate; 1:50) primary antibodies. Representative bright field images and relative phospho-Akt (Ser473; red colour; channel 5) and GH (orange colour; channel 3) co-staining are shown. (c) Subsets of cells were also stained with the secondary antibody alone (green colour; channel 2) and the Hoechst 33342 nuclear counter-stain (red colour; channel 5). Representative images are shown.

(a)



Pituitary Somatotropes

(b)



IF: phospho-Akt (Ser473) and GH

(c)

Secondary Antibody Alone

Figure 5.9 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^{-}; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^{-}$; 3 μM; c,d), Akt (Akt_i VIII; Akt^{-}; 10 μM; e,f), and BTK (CGI1746; BTK^{-}; 1 μM; g,h) on basal and GnRH2-stimulated LH release responses. For each panel, LH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified LH release responses are shown on the right (b,d,f,h). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 2.62 ± 0.39 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in February to August (PIT-1, completed in February-March; GSK2334470, completed in February-April; Akt_i VIII, completed in July-August; CGI1746, completed in February) are shown. The gray horizontal bar indicates the duration of the inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$).

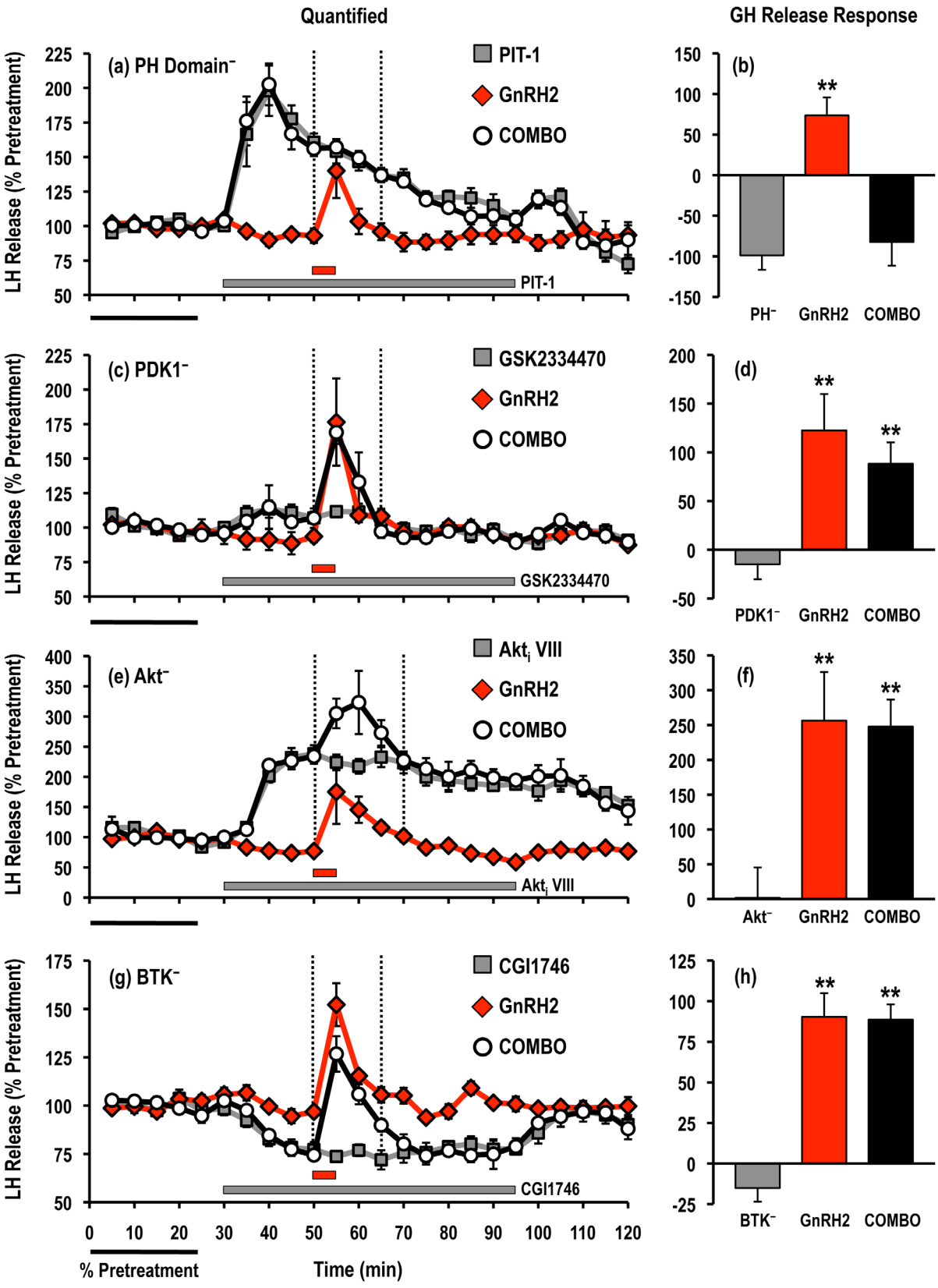

Quantified
GH Release Response
(a) PH Domain⁻
PIT-1
GnRH2
COMBO
LH Release (% Pretreatment)
(b)
PH⁻ GnRH2 COMBO
(c) PDK1⁻
GSK2334470
(d)
PDK1⁻ GnRH2 COMBO
(e) Akt⁻
Akt$_i$ VIII
(f)
Akt⁻ GnRH2 COMBO
(g) BTK⁻
CGI1746
(h)
BTK⁻ GnRH2 COMBO
% Pretreatment
Time (min)

Figure 5.10 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^{-}; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^{-}$; 3 μM; c,d), Akt (Akt_i VIII; Akt^{-}; 10 μM; e,f), and BTK (CGI1746; BTK^{-}; 1 μM; g,h) on basal and GnRH3-stimulated LH release responses. For each panel, LH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified LH release responses are shown on the right (b,d,f,h). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 2.54 ± 0.45 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in February to August (PIT-1, completed in February-March; GSK2334470, completed in February-April; Akt_i VIII, completed in July-August; CGI1746, completed in February) are shown. The gray horizontal bar indicates the duration of the inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$).

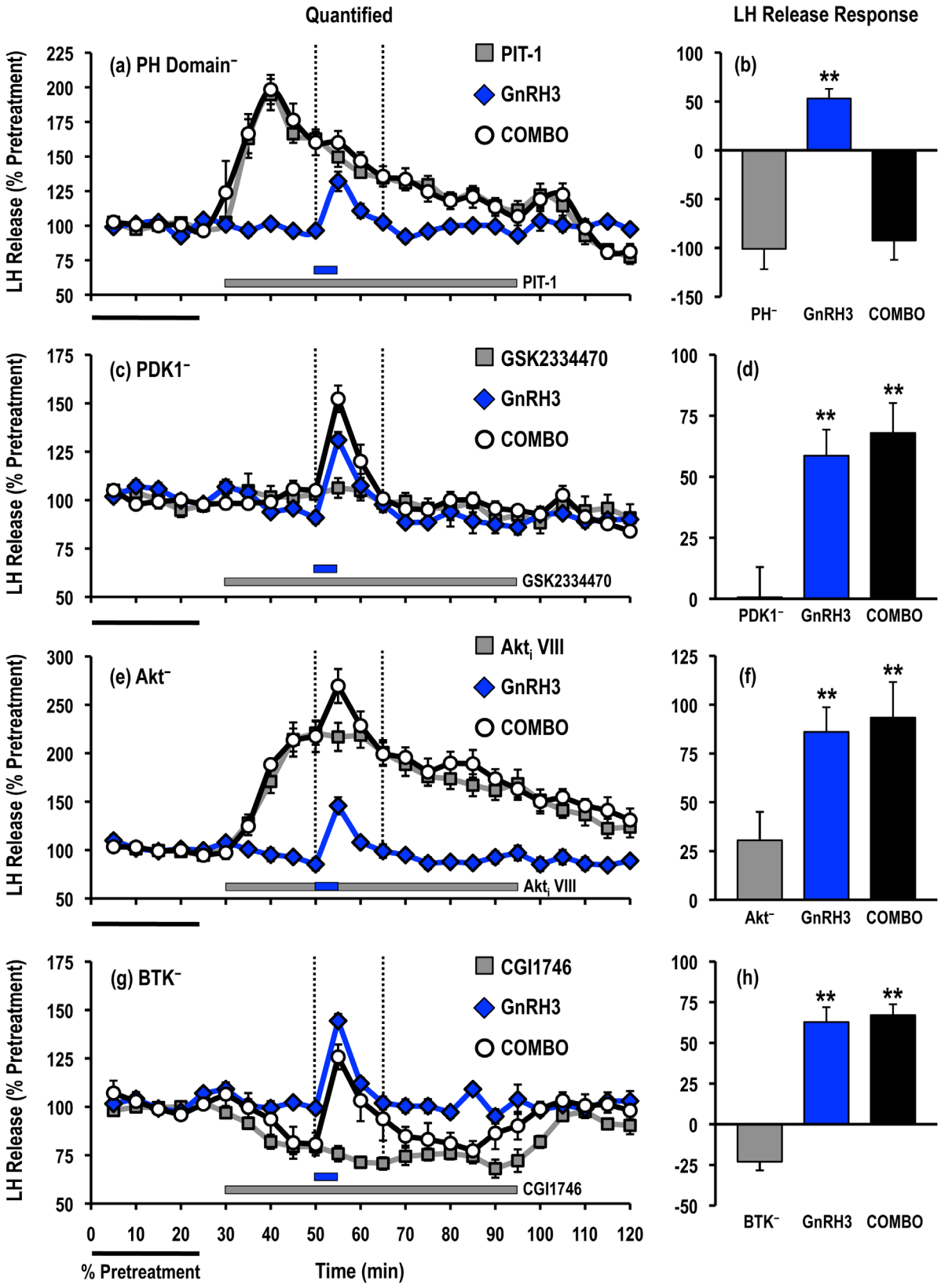

Quantified
LH Release Response
(a) PH Domain⁻
PIT-1
GnRH3
COMBO
PIT-1
(b)
PH⁻ GnRH3 COMBO
(c) PDK1⁻
GSK2334470
GnRH3
COMBO
GSK2334470
(d)
PDK1⁻ GnRH3 COMBO
(e) Akt⁻
Akt$_i$ VIII
GnRH3
COMBO
Akt$_i$ VIII
(f)
Akt⁻ GnRH3 COMBO
(g) BTK⁻
CGI1746
GnRH3
COMBO
CGI1746
(h)
BTK⁻ GnRH3 COMBO
LH Release (% Pretreatment)
% Pretreatment
Time (min)

Figure 5.11 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^{-}; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^{-}$; 3 μM; c,d), Akt (Akt_i VIII; Akt^{-}; 10 μM; e,f), and BTK (CGI1746; BTK^{-}; 1 μM; g,h) on basal and GnRH2-stimulated GH release responses. For each panel, GH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified GH release responses are shown on the right (b,d,f,h). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 5.79 ± 1.39 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in February to August (PIT-1, completed in February-March; GSK2334470, completed in February-April; Akt_i VIII, completed in July-August; CGI1746, completed in February) are shown. The gray horizontal bar indicates the duration of the inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$).

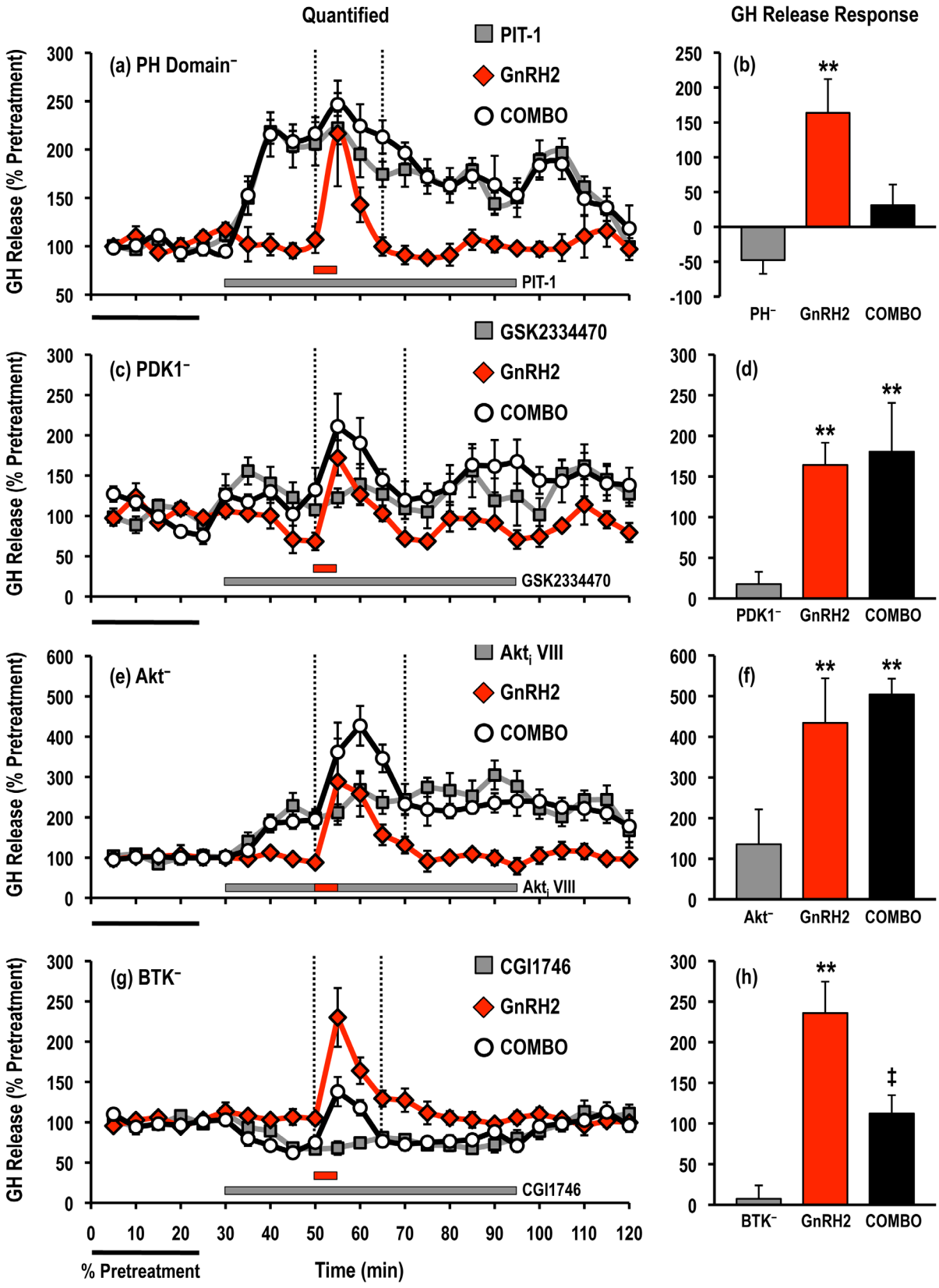

Quantified
GH Release Response
(a) PH Domain⁻
PIT-1
GnRH2
COMBO
(b)
(c) PDK1⁻
GSK2334470
(d)
(e) Akt⁻
Akt$_i$ VIII
(f)
(g) BTK⁻
CGI1746
(h)
GH Release (% Pretreatment)
% Pretreatment
Time (min)
PH⁻
PDK1⁻
Akt⁻
BTK⁻

Figure 5.12 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^{-}; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^{-}$; 3 μM; c,d), Akt (Akt_i VIII; Akt^{-}; 10 μM; e,f), and BTK (CGI1746; BTK^{-}; 1 μM; g,h) on basal and GnRH3-stimulated GH release responses. For each panel, GH release profiles are shown on the left (a,c,e,g; gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified GH release responses are shown on the right (b,d,f,h). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 4.69 ± 0.77 ng/ml; n = 8, from four independent cell preparations). Pooled responses from experiments with pituitary cells prepared from goldfish in February to August (PIT-1, completed in February-March; GSK2334470, completed in February-April; Akt_i VIII, completed in July-August; CGI1746, completed in February) are shown. The gray horizontal bar indicates the duration of the inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Tukey's HSD multiple comparisons; $p < 0.05$).

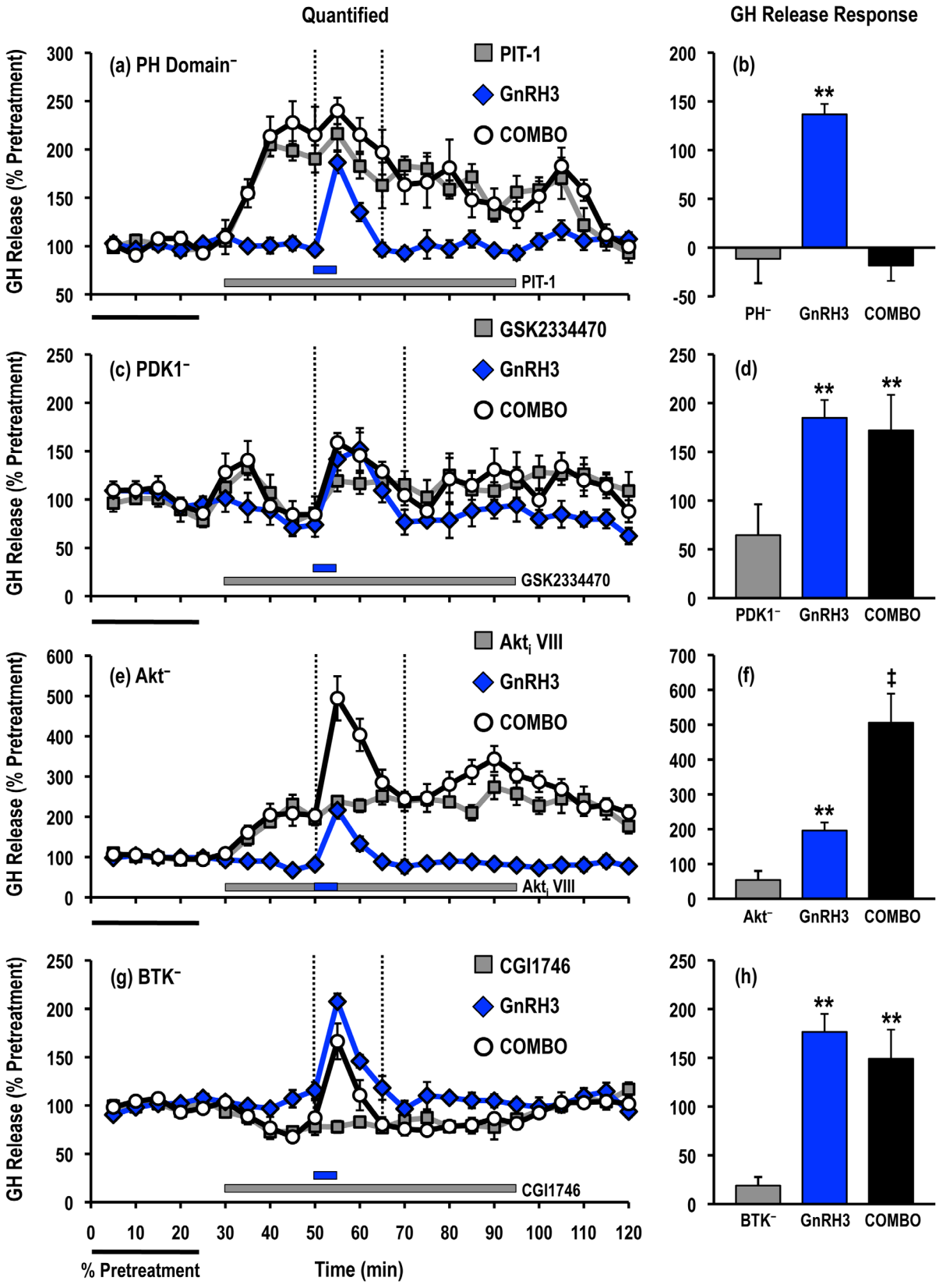

Quantified
GH Release Response
(a) PH Domain⁻
PIT-1
GnRH3
COMBO
PIT-1
(b)
PH⁻
GnRH3
COMBO
(c) PDK1⁻
GSK2334470
GnRH3
COMBO
GSK2334470
(d)
PDK1⁻
GnRH3
COMBO
(e) Akt⁻
Akt$_i$ VIII
GnRH3
COMBO
Akt$_i$ VIII
(f)
Akt⁻
GnRH3
COMBO
(g) BTK⁻
CGI1746
GnRH3
COMBO
CGI1746
(h)
BTK⁻
GnRH3
COMBO
GH Release (% Pretreatment)
% Pretreatment
Time (min)

Figure 5.13 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^{-}; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^{-}$; 3 μM; c,d), Akt (Akt_i VIII; Akt^{-}; 10 μM; e,f), and BTK (CGI1746; BTK^{-}; 1 μM; g,h) on basal LH release. LH release profiles are shown in the top panel (a; orange solid square, PIT-1 alone; red solid diamond, GSK2334470 alone; green solid circle, Akt_i VIII alone; blue solid diamond, CGI1746 alone) and quantified responses are shown on the bottom (b; mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (coloured bars; orange, PIT-1 alone; red, GSK2334470 alone; green, Akt_i VIII alone; blue, CGI1746 alone) was quantified as the average % pretreatment value over the entire duration of the inhibitor treatment (30-95 min; the grey horizontal bar; $n = 16$, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$).

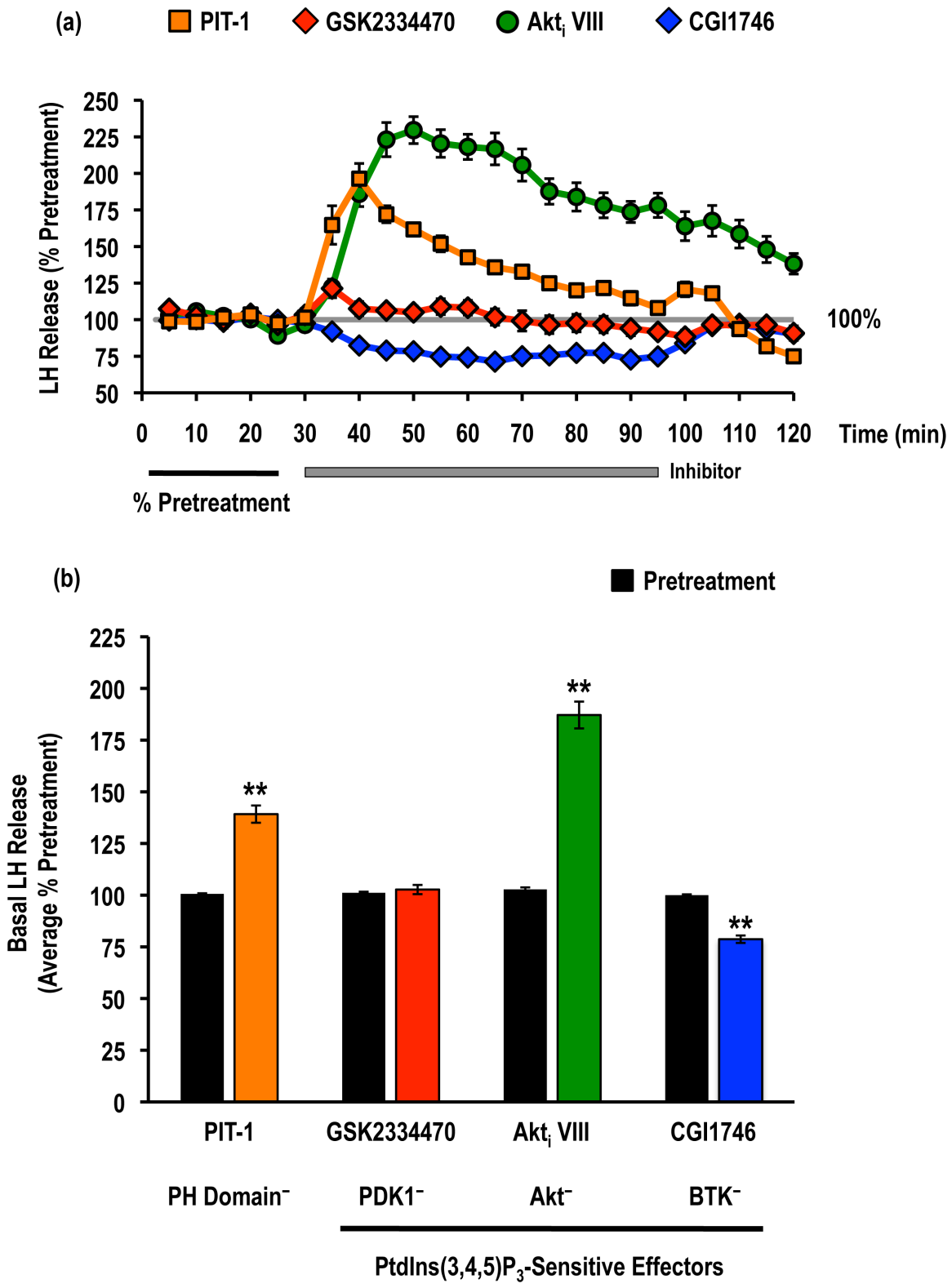

(a)
PIT-1
GSK2334470
Akt$_i$ VIII
CGI1746
LH Release (% Pretreatment)
250
225
200
175
150
125
100
75
50
100%
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
Inhibitor
% Pretreatment
(b)
Pretreatment
Basal LH Release
(Average % Pretreatment)
225
200
175
150
125
100
75
50
25
0
**
**
**
PIT-1
GSK2334470
Akt$_i$ VIII
CGI1746
PH Domain$^-$
PDK1$^-$
Akt$^-$
BTK$^-$
PtdIns(3,4,5)P$_3$-Sensitive Effectors

Figure 5.14 Effects of selective small molecule inhibitors of PtdIns(3,4,5)P_3-sensitive PH domain interactions (PIT-1; PH^-; 100 μM; a,b), PDK1 (GSK2334470; $PDK1^-$; 3 μM; c,d), Akt (Akt_i VIII; Akt^-; 10 μM; e,f), and BTK (CGI1746; BTK^-; 1 μM; g,h) on basal GH release. GH release profiles are shown in the top panel (a; orange solid square, PIT-1 alone; red solid diamond, GSK2334470 alone; green solid circle, Akt_i VIII alone; blue solid diamond, CGI1746 alone) and quantified responses are shown on the bottom (b; mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (coloured bars; orange, PIT-1 alone; red, GSK2334470 alone; green, Akt_i VIII alone; blue, CGI1746 alone) was quantified as the average % pretreatment value over the entire duration of the inhibitor treatment (30-95 min; the grey horizontal bar; $n = 16$, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$).

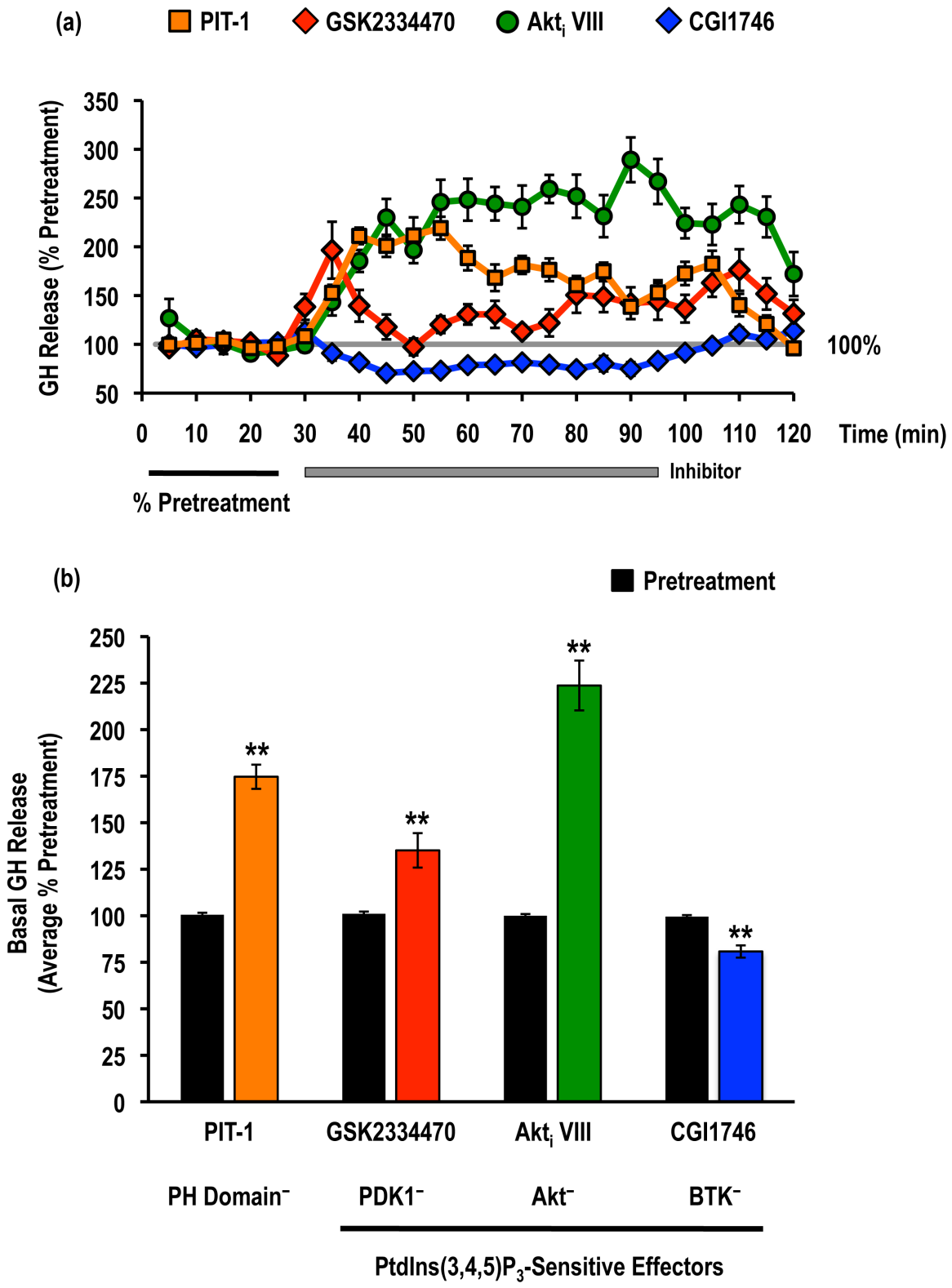
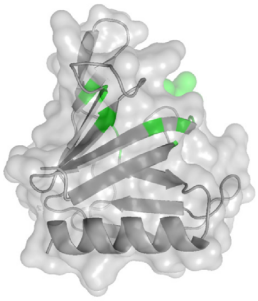

(a)
PIT-1
GSK2334470
Akt$_i$ VIII
CGI1746
GH Release (% Pretreatment)
350
300
250
200
150
100
50
100%
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
Inhibitor
% Pretreatment
(b)
Pretreatment
Basal GH Release
(Average % Pretreatment)
250
225
200
175
150
125
100
75
50
25
0
**
**
**
**
PIT-1
GSK2334470
Akt$_i$ VIII
CGI1746
PH Domain$^-$
PDK1$^-$
Akt$^-$
BTK$^-$
PtdIns(3,4,5)P$_3$-Sensitive Effectors

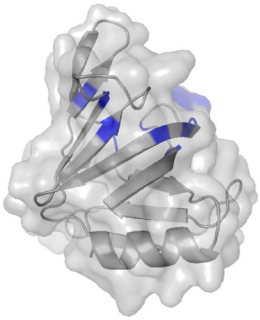
Figure 5.15 Mapping of the PtdIns(3,4,5)P_3-binding surface within the PH domains of the zebrafish homologs of GRP1, ARNO, PDK1, Akt, and BTK. PtdIns(3,4,5)P_3 is the major lipid product of agonist-stimulated class I PI3Ks and a small subclass of pleckstrin homology (PH) domains, including those found within general receptor for phosphoinositides 1 (GRP1), ADP-ribosylation factor (Arf) nucleotide-binding-site opener (ARNO), phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (Akt), and Bruton's tyrosine kinase (BTK) recognize PtdIns(3,4,5)P_3 with remarkable specificity and high affinity. Homology models of the PH domain regions within the zebrafish homologs of GRP1 (a; green), ARNO (b; blue), PDK1 (c; red), Akt (d; yellow), and BTK (e; orange) are shown on the left (prepared using PyMol); whereas, the overall sequence similarities of each PH domain, as compared to the aligned human sequences, are provided on the right. Residues forming the PtdIns(3,4,5)P_3 interaction surface were identified (coloured residues; left) using the structural features identified in the National Center for Biotechnology Information Conserved Domain Database (NCBI CCD). Homology models are oriented with the membrane-binding surface facing upwards. For each of the PH domains analyzed, the PtdIns(3,4,5)P_3 interaction surface was surface accessible and 100% conserved across the human and zebrafish sequences.

(a)



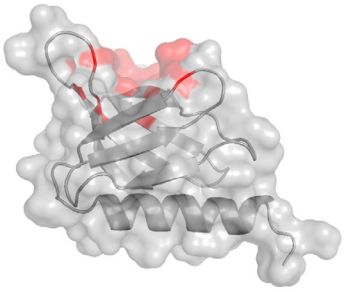
Zebrafish GRP1 PH Domain
94.1% Identity / 97.5% Positives
100% PtdIns(3,4,5)P_3-Binding Residues
UniProtKB ID: E7F1N2

(b)



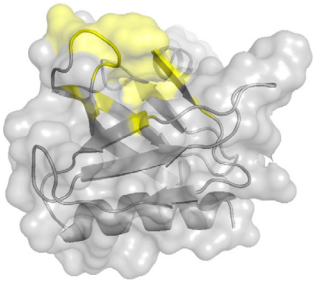
Zebrafish ARNO PH Domain
95.8% Identity / 99.2% Positives
100% PtdIns(3,4,5)P_3-Binding Residues
UniProtKB ID: F1QDR1

(c)



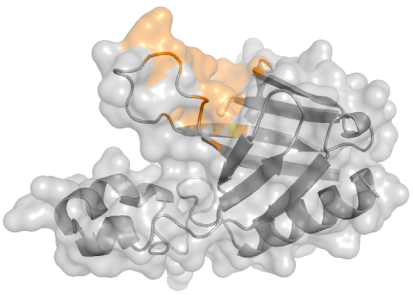
Zebrafish PDK1 PH Domain
92.4% Identity / 94.6% Positives
100% PtdIns(3,4,5)P_3-Binding Residues
UniProtKB ID: Q6NZV1

(d)



Zebrafish Akt1 PH Domain
93.2% Identity / 97.1% Positives
100% PtdIns(3,4,5)P_3-Binding Residues
UniProtKB ID: M4MD44

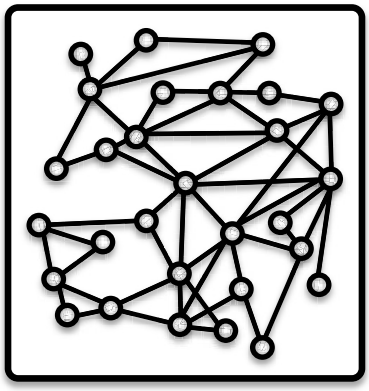
(e)



Zebrafish BTK PH Domain
54.6% Identity / 79.2% Positives
100% PtdIns(3,4,5)P_3-Binding Residues
UniProtKB ID: E7F4S0

Chapter Six:

PI3K-dependent signalling selectively regulates long-term effects on basal, as well as GnRH-stimulated, hormone secretion and cellular availability



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

6.1 Introduction

In Chapters 3, 4, and 5 of this thesis, we have outlined a novel role for PI3K-dependent signalling in the control of acute LH and GH release responses to both endogenous isoforms of GnRH found in goldfish. Furthermore, we have demonstrated that GnRHRs selectively utilize the class I PI3Ks and downstream PtdIns(3,4,5)P_3-sensitive effectors in mediating the differential intracellular responses induced by GnRH2 or GnRH3. However, whether PI3K-dependent signalling participates in regulating long-term hormone release and production in pituitary cells has not been tested in any model system. The objectives of this Chapter are to examine the involvement of PI3K-dependent signalling in long-term basal and GnRH-stimulated hormone release, cellular hormone content, and total hormone availability. Primary cultures of dispersed goldfish pituitary cells were used to enable the study of direct pituitary cell actions of GnRH over 2, 6, 12, and 24 hrs. However, unlike column perifusion, the use of a static incubation protocol preserves autocrine and paracrine communication within the cell culture during prolonged exposures. Overall, our results indicate that PI3K-dependent signalling differentially modulates long-term basal and GnRH-stimulated hormone release, as well as total hormone availability, in a time-, cell type-, and GnRH-selective manner.

6.2 Results

6.2.1 Static incubation with LY294002 reduces phosphorylation of Akt, but not PDK1, in goldfish pituitary fragments

In order to further validate that LY294002 is an effective inhibitor of PI3K-dependent signalling in the goldfish pituitary over prolonged exposures, we examined the

ability of LY294002 (10 μM) to affect the phosphorylation of PDK1 (Ser241) and Akt (Ser473) in goldfish pituitary fragments. PDK1 and Akt are both classical PI3K-dependent signal transduction targets that are known to interact with one another at the plasma membrane upon binding to PtdIns(3,4,5)P_3 (reviewed in Chapter 1). Interestingly, phosphorylation of Akt is extremely sensitive to pan-PI3K inhibitors including LY294002, and phosphorylation of Akt at Ser473, in particular, has become a common index of PI3K-dependent signalling (Manning and Cantley, 2007). Conversely, PDK1 is constitutively active in eukaryotic cells following autophosphorylation of Ser241 (Alessi et al., 1997). However, phosphorylation of PDK1 at Ser241 does not appear to be directly correlated with PtdIns(3,4,5)P_3 production, in general, and is not sensitive to treatment with LY294002 (Vasudevan et al., 2009).

As anticipated, treatment with LY294002 (10 μM) significantly reduced phosphorylation of Akt (Ser473) in excised goldfish pituitary fragments under basal conditions, as well as in the presence of GnRH3 (100 nM) or GnRH2 (100 nM), in 2 hr static incubations (Figure 6.1). However, unlike in dispersed pituitary cells (Chapter 5), Akt immunoblotting revealed three distinct bands in goldfish pituitary tissue samples of which the central band is both of the appropriate size (~60 kDa) and corresponded with the Akt immunoreactive band observed in RBL-2H3 cells (data not shown). Pre-incubation of the primary antibody with the competing antigenic peptide removed all three immunoreactive bands (data not shown). Alternatively, in preliminary experiments, phosphorylation of PDK1 (Ser241) did not appear to be drastically altered by 2 hr treatments of LY294002 alone, GnRHs alone, or combinations of either endogenous GnRH with LY294002 (Figure 6.2).

6.2.2 Involvement of PI3K-dependent signalling during prolonged actions on hormone release, cell content, and total availability

For use in long-term cell cultures, we chose to use the pan-specific PI3K inhibitor LY294002 and not wortmannin, due primarily to the known actions of wortmannin on sustained agonist-induced release responses (Nakanishi et al., 1995). The prolonged effects of wortmannin are due in large part to the inhibition of type III PI4Ks, and as a result of the irreversible covalent association of wortmannin with PI3K superfamily catalytic subunits (discussed in Section 3.2). I rationalized that the use of the reversible pan-PI3K inhibitor LY294002 rather than wortmannin would limit any compounding influences from type III PI4K perturbation and might also minimize compensatory changes in the signalling network architecture that tend to follow prolonged exposures to an irreversible inhibitor (Knight and Shokat, 2005; Michel and Seifert, 2015; Fabbro, 2015). LY294002 was used at a concentration of 10 μM, which was effective in inhibiting acute GnRH actions on LH and GH release (Chapter 3). Additional information regarding the reported target selectivity of LY294002 at this dosage are provided in Table 3.1.

LH and GH release, as well as cellular hormonal content, were examined following treatments with either LY294002 alone, GnRH alone, GnRH + LY294002 or vehicle (control) for 2, 6, 12, and 24 hrs in static incubation. Total hormone availability (sum of released and cellular content) was calculated as an index of hormone production during the incubation time period. Importantly, the primary GH antibody used in the radioimmunoassay has been shown to detect a single immunoreactive GH protein band

that corresponds to mature GH, and not GH fragments (Chapter 2; Zhou et al., 2004) while the LH antibody does not cross react with monomeric GtH-α, FSH-β, LH-β subunits (Chapter 2; Yang et al., 2010), Analysis of the results with two-way ANOVA indicated that significant time and treatment effects, as well as interactions between time and treatment, were present for all the LH and GH parameters monitored ($p<0.001$ in all cases).

6.2.3 LY294002 effects on GnRH2 and GnRH3 actions on long-term LH release, cell content, and total available LH in static incubation

LH release was generally higher in GnRH2 (100 nM) alone or GnRH3 (100 nM) alone treatment groups and significant increases relative to untreated controls were observed at 2, 6, 12 and 24 hrs for GnRH3, and 6 and 24 hrs for GnRH2 (Figure 6.3). LY294002 (10 μM) alone did not significantly affect basal LH release (Figure 6.3). Co-treatment with LY294002 significantly reduced GnRH3-stimulated LH release at 12 and 24 hrs, as well as GnRH2-stimulated increase at 6 and 24 hrs (Figure 6.3). Interestingly at 2 and 6 hrs, the LH release responses to GnRH3 were even further elevated in the presence of LY294002 (responses to the combination of GnRH3 + LY294002 were significantly greater than those elicited by GnRH3 alone; Figure 6.3).

Total cellular LH content was not affected by treatments at 2 hrs but significant elevations were seen at 6 hrs with LY294002 alone and GnRH2 alone treatments, as well as with the GnRH3 alone treatment at 6, 12, and 24 hrs (Figure 6.3). On the other hand, LY294002 significantly reduced GnRH actions on LH content, with the exception of the GnRH3-induced response at 6 hrs (Figure 6.3).

Significant elevations in the calculated total available LH relative to controls were seen with LY294002 alone treatments at 6 hrs (Figure 6.3). GnRH3 alone and GnRH2 alone treatments significantly increased total LH availability at 6, 12, and 24 hrs, and at 6 and 24 hrs, respectively (Figure 6.3). Responses to GnRH3 at 12 and 24 hrs, and the 6 hrs response to GnRH2 were significantly reduced in the presence of LY294002 (Figure 6.3). Surprisingly, while neither GnRH3 alone nor LY294002 alone treatment affected total available LH at 2 hrs, the combination of GnRH3 + LY294002 significantly increased total LH (Figure 6.3).

6.2.4 LY294002 effects on GnRH2 and GnRH3 actions on long-term GH release, cell content, and total available GH in static incubation

Treatment with GnRH3 (100 nM) alone significantly increased GH release relative to untreated controls at 6, 12 and 24 hrs in static incubation while GnRH2 (100 nM) alone treatment was also effective at 2, 6 and 24 hrs (Figure 6.4). Treatment with LY294002 (10 μM) alone likewise increased GH release at 2 and 6 hrs (Figure 6.4). Co-application of LY294002 reduced the GH release response to GnRH3 at 12 hrs and that to GnRH2 at 6 hrs, but further elevated the responses to GnRH3 and GnRH2 at 24 and 12 hrs, respectively (Figure 6.4). While neither GnRH2 nor LY294002 alone treatments altered GH release at 12 hrs, the combination treatment of GnRH2 + LY294002 caused a significant increase in GH secretion (Figure 6.4).

In general, treatments with LY294002, GnRH2, or GnRH3 alone either had no effect or significantly reduced cellular GH contents relative to untreated controls at all time points with the exception of GnRH2 alone treatments at 24 hrs where a significant

increase was observed (Figure 6.4). The GnRH2-elicited increase in cellular GH content at 24 hrs and the decrease in 2 hrs were significantly attenuated in the presence of LY294002 (Figure 6.4).

Significant suppression of the calculated total available GH relative to controls were observed with LY294002 alone treatment at 12 and 24 hrs, GnRH3 alone treatment at 2 and 24 hrs, and GnRH2 alone treatment at 2 hrs (Figure 6.4). On the other hand, significant increases were seen with LY294002 alone treatment at 6 hrs and GnRH2 alone treatment at 24 hrs (Figure 6.4). GnRH2-induced changes in total GH available were significantly attenuated in the presence of LY294002 (Figure 6.4).

6.3 Discussion

Results presented in this chapter not only add to the information on the involvement of PI3K-dependent signalling in LH and GH secretion, but also provides insight into the participation of PI3Ks in the long-term regulation of gonadotrope and somatotrope functions. Furthermore, these results add support for the previous findings demonstrating the differential involvement of PI3K-dependent signalling in the control of acute GnRH2- and GnRH3-stimulated LH and GH release responses measured in column perifusion, which were obtained in part through the use of LY294002 (Chapter 3). More importantly, results from the present study suggest that PI3K-dependent signalling mediates long-term actions on hormone release and cellular content in a time-, pituitary cell type-, and GnRH-specific manner.

6.3.1 Involvement of PI3K-dependent signalling in long-term GnRH actions on LH and GH release

GnRH2 and GnRH3 stimulated LH and GH release with different temporal characteristics in both gonadotropes and somatotropes in static incubation studies in this Chapter, confirming the presence of GnRH isoform-, cell type-, and time-dependent differences in prolonged GnRH actions on LH and GH secretion in goldfish. Complementing previous findings on acute GnRH-stimulated LH and GH release (Chapter 3), the present results provide information on the participation of PI3K-dependent signalling on long-term LH and GH secretion. The ability of LY294002 to attenuate GnRH3-elicited LH and GH release at 12 and 24 hrs, as well as GnRH2-stimulated increases in GH at 6 hrs and LH secretion at 6 and 24 hrs, indicates that PI3K signalling also mediates long-term GnRH stimulation of pituitary hormone secretion. However, LY294002 did not reduce the LH release responses to GnRH3 at 6 hrs or GH release responses to GnRH3 at 2 and 6 hrs, suggesting the presence of temporal differences in the involvement of PI3K-dependent signalling during GnRH-stimulated hormone release.

6.3.2 Involvement of PI3K-dependent signalling in GnRH actions on long-term cellular hormone content and total availability

In goldfish, previous work has shown that both endogenous GnRHs stimulate GtH-α, FSH-β, LH-β, as well as GH mRNA levels after 12 hrs of static treatment in primary cultures of dispersed pituitary cells (Klausen et al., 2002). However, these known time-dependent effects with regards to GnRH-stimulated increases in hormone mRNA

are dissociated temporally from changes in cellular LH and GH contents and total hormone availability in goldfish pituitary cell cultures observed in the present study (Tables 6.1 and 6.2). Results from these static incubation studies also support the conclusion that temporal and isoform differences in the influences of GnRH2 and GnRH3 on LH and GH cellular content, as well as total availability exist. In general, while both GnRH2 and GnRH3 are effective in elevating LH cellular content and total LH availability at some time points during the 24 hr incubation period, GnRH2 and GnRH3 treatments are largely inhibitory to cellular GH content and only GnRH2 is able to exert increases in total GH availability at a late, 24 hr time point (Tables 6.1 and 6.2).

PI3Ks are central to the cellular control of mRNA synthesis and also have a well-established role in the control of protein translation in many systems (Vanhaesebroeck et al., 2001; Engelman et al., 2006). However, to the best of our knowledge, only two studies have previously examined the involvement of PI3Ks in GnRH actions on hormone gene expression in pituitary cells. Briefly, treatment with LY294002 or wortmannin did not inhibit GnRH analog (des-Gly10,[D-Ala6]-GnRH)-stimulated increases in GTH subunit promotor activites in immortalized LβT2 and αT3-1 gonadotrope cell lines (Mutiara et al., 2008; Kanasaki et al., 2008). Likewise, static incubations with LY294002 did not alter GnRH-stimulated LHβ-subunit immunoreactivity in LβT2 gonadotropes (Liu et al., 2005). Results from these reports suggest that PI3K-dependent signalling does not mediate GnRH actions on GTH gene expression and synthesis. However, whether holomeric LH protein production is affected by inhibition of PI3K-dependent signalling has not been examined.

The present study represents the first report demonstrating the involvement of PI3Ks during GnRH actions on the control of pituitary cellular hormone content and total protein availability in any vertebrate system, as well as in an untransformed, primary pituitary cell culture. In terms of LH cellular content and total LH availability, treatment with LY294002 reduced GnRH3-elicited increases at 12 and 24 hrs but not at 6 hrs. Likewise, LY294002 reversed the GnRH2-induced elevation of cellular LH content and total LH availabity at 6 hrs, but not the GnRH2 influence on total LH at 24 hrs (Table 6.1; Figure 6.3). In contrast, LY294002 had no effect on GnRH3-induced suppression of GH cell content and total availability but was able to attenuate both the suppressive (2 hrs) and stimulatory (24 hrs) influence of GnRH2 on these parameters (Table 6.2; Figure 6.4). These observations indicate that PI3K-dependent signalling plays a role in mediating long-term GnRH effects on gonadotrope and somatotrope hormone protein availability (and hormone protein production by implication) in an GnRH-isoform selective and cell type-specific manner. These findings clearly differ from those described above for immortalized mammalian gonadotropes. Whether these differences represent species variation and/or the experimental models used, including the potential differences between immortalized cells and primary cell cultures, and/or the parameters monitored is unclear at present. On the other hand, some of the time-dependent effects of GnRH2 and GnRH3 on cellular hormone content and total availability were not attenuated by LY294002; for example, the GnRH3-inuced suppression of GH cellular hormone content and total availability, and the GnRH3- and GnRH2-induced elevations in total LH availability at 6 and 24 hrs, respectively. Thus, not all long-term GnRH actions on hormone production in goldfish pituitary cells may be mediated by the

catalytic activity of PI3Ks. Nevertheless, future studies examining the actions of pan-specific and isoform-selective PI3K inhibitors on GnRH-stimulated changes in GTH-α, FSH-β, LH-β, and GH mRNA levels in primary goldfish pituitary cells will add to our understanding of the signal transduction mechanisms responsible for the coordinate control of hormone protein and mRNA expression in pituitary cells.

6.3.3 Understanding the complexity of PI3K-dependent signalling during the regulation of basal LH and GH release and total availability

The participation of PI3K-dependent signalling in the control of gonadotrope and somatotrope functions under basal conditions is also revealed by the results of the present study. LY294002 treatment reduced phosphorylation of the classical PI3K signal transduction target Akt (Ser473) under basal condition; however, it does not affect the phosphorylation of PDK1 (Ser241), which is known not to be an intracellular target for LY294002-mediated inhibition (Vasudevan et al., 2009). On the hand, phosphorylated p85-type class IA PI3K regulatory subunits (Tyr458; Chapter 4), PDK1 (Ser241; Chapter 5), and Akt (Thr308 and Ser473; Chapter 5) are present in goldfish pituitary tissue extracts under unstimulated conditions. Thus, it is likely that PI3K-dependent signalling is active in goldfish pituitary cells even in basal conditions, *in vitro*. Treatment with LY294002 alone did not affect long-term basal LH release, but significantly increased basal GH release at 2 and 6 hrs (Table 6.1; Table 6.2). Interestingly, pan-PI3K inhibitors wortmannin and LY294002 had the tendency to transiently increase basal LH and GH release in column perifusion experiments (Chapter 3). These observations suggest that PI3K-dependent signalling exerts negative influences in the control of basal LH and GH

release in a time- and cell type-dependent manner. On the surface, this conclusion appears at odds with the proposed stimulatory role of PI3K in mediating GnRH induction of hormone secretion. However, inhibition of class I PI3K isoforms consistently and significantly reduced the average basal hormone release, although in an isoform-selective manner (Chapter 4). Taken together, these results demonstrate an uncoupling of the cellular responses to pan- and class I-selective inhibitors, and suggest that class II or III PI3Ks may also be negative regulators of basal hormone release. Interestingly, several studies have demonstrated that basal secretion and agonist-stimulated LH and GH release are regulated differently in goldfish pituitary cells (Chang et al., 2000; Johnson et al., 2002; Johnson et al., 2003; Pemberton et al., 2013, 2014); thus, such dichotomy in the influences of PI3Ks on hormone secretion likely reflects the complexity of intracellular signalling elements involved in regulating hormone exocytosis.

In addition to a role in controlling basal hormone release, results suggest that PI3K-dependent signalling pathways have stimulatory influences on long-term GH production while having inhibitory influences on LH and GH production over a shorter time frame (Tables 6.1 and 6.2; Figures 6.3 and 6.4). LY294002 elevated total LH and GH availability at 6 hrs but suppressed total GH availability and cellular LH and GH contents at longer treatment durations (LH: 12 hrs; GH: 12 and 24 hrs). The mechanisms and cellular targets by which PI3K-dependent signalling components exerts their effects in the control of basal hormone release and hormone production in goldfish gonadotropes and somatotropes are unknown and requires future examination.

Paradoxically, while LY294002 did not alter basal LH release at 2 and 6 hrs when applied alone, co-treatment with LY294002 further elevated the LH release response to

GnRH3 at these times, and the combination of GnRH3 and LY294002 significantly increased total LH availability at 2 hrs, a time when each of the drugs alone had no effect (Table 6.1; Figure 6.3). In the case of somatotropes, LY294002 did not alter basal GH secretion at 12 and 24 hrs when applied alone, but its presence further enhanced the GH release response to GnRH3 at 24 hrs and in combination with GnRH2 at 12 hrs (Table 6.2; Figure 6.4). Ostensibly, these observations are not consistent with the proposed participation of PI3K-dependent signalling in mediating some of the long-term effects of GnRH3 and GnRH2 as discussed above. On the other hand, prolonged exposures to GnRH result in agonist-mediated desensitization of the GnRH response in goldfish pituitary fragments (Habibi, 1991a, 1991b). GnRH-induced desensitization is thought to be due in part to a loss of GnRHR content, but changes in post-receptor mechanisms, including uncoupling of the GnRHR from intracellular effectors, are also likely to be involved (Habibi, 1991a, 1991b). Interestingly, PI3K-dependent signalling is known to be involved in GPCR endocytosis and the catalytic activity of class I PI3Ks are required for the internalization of β-adrenergic receptors, a classical model of class A GPCR function (Naga Prasad et al., 2002, 2005; Sato et al., 2003). Though entirely speculative, prolonged inhibition of PI3K-dependent signalling may affect GnRHR desensitization allowing for increased GnRH actions on hormone release at different static incubation time points in a GnRH-isoform selective manner as observed in the present study.

6.3.4 Akt isoforms in goldfish pituitary tissues

In the present study as well as in Chapters 4 and 5, the primary antibodies used for both the phosphorylated Akt (Ser473) and pan-Akt immunoblotting cross-react with

highly conserved sequences within Akt1, Akt2, and Akt3. Interestingly, data from these chapters also demonstrate that in both pituitary tissue fragments and dispersed cells, the antibodies produced against phosphorylated Akt (Ser473) as well as pan-Akt produced multiple immunoreactive bands. Likewise, a previous study in goldfish had demonstrated a single band for phosphorylayted Akt immunoreactivty (Ser473; Sigma-Aldrich, St. Louis, MO, USA) using retinal extracts, although immunoblots of the total Akt immunoreactivity were not provided (Koriyama et al., 2007). Consistent with our present observations, pan-Akt immunoblotting in rainbow trout hepatocytes also produced multiple immunoreactive bands (Reindl et al., 2011; Bergan et al., 2013). Since goldfish exhibit polyploidy and pre-absorption of the pan-Akt antibody with the antigenic peptide removed all Akt immunoreactive bands, it is possible that additional Akt isoforms exists in goldfish. In support of this, immunoblotting for Akt1, Akt2, and Akt3 presented in Chapter 5 suggests that multiple Akt isoforms are present in goldfish pituitary cells. Interestingly, the immunoblots presented in this chapter utilize protein extracts from goldfish pituitary fragments, which also contain hypothalamic neuronal terminals (Chang et al., 1990; Yu et al., 1991), and clearly show 3 immunoreactive pan-Akt bands; whereas only two immunoreactive pan-Akt bands are consistently observed in lysates prepared from dispersed cells (Chapters 4 and 5). These discrepancies might reflect tissue- or cell-specific protein modifications to the Akt isoforms that result in changes in their electrophoretic mobility. Regardless, whether individual Akt isoforms exert selective intracellular actions is largely uncharacterized, although recent evidence from mammals suggests that functional selectivity downstream of Akt1, Akt2, and Akt3 could be important for the integrated control of cellular responses (Gonzalez and McGraw,

2009a,b; Clark and Toker, 2014; Toker and Marmiroli, 2014). Taken together, these data presented in Chapters 4, 5, and 6 strongly suggest that multiple Akt isoforms are present in the goldfish pituitary and are subject to PI3K-dependent phosphorylation.

6.3.5 Summary

Overall, this is the first study to examine the role of PI3K-dependent signalling in the regulation of basal and GnRH-stimulated total LH and GH protein availability in primary cultures of pituitary cells. Results demonstrate that temporal differences in the involvement of PI3K catalytic activites differentially regulate basal and GnRH-induced actions on long-term hormone release and availability. These findings add to the known differential actions of PI3Ks in the control of acute LH and GH release responses reported previously, and indicate that PI3K-dependent signalling differentially modulates basal and GnRH-stimulated hormone release in a time-, cell type-, and GnRH-selective manner. Interestingly, information from cancer cell models has extensively outlined the complex intracellular feedback networks that control PI3K-dependent signalling (Carracedo and Pandolfi, 2008), making PI3Ks an attractive target for integrating the multifactorial control of endocrine cell signalling (Hirsch et al., 2007). In particular, activation of the highly conserved lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a common cellular mechanism for directly attenuating class I PI3K signalling by reducing cellular concentrations of PtdIns(3,4,5)P_3 (Carracedo and Pandolfi, 2008). Given that the neuroendocrine regulation of gonadotrope and somatotrope functions in the goldfish is multifactorial in nature, with multiple stimulatory and inhibitory factors directly controlling each cell type (Chang et al., 2009,

2012), how PI3K-dependent signalling participates in the effects of other neuroendocrine regulators needs to be examined in the future.

Table 6.1 Summary of time-dependent GnRH- and LY294002-mediated effects on goldfish pituitary LH release, cell content, and total availability in comparison to GnRH-stimulated changes in GTH subunit mRNA expression previously shown in Klausen et al., 2002[1].

Treatment	Duration (hrs)	LH release	LH cell content	Total LH availability	GTHα mRNA[1]	LHβ mRNA[1]
LY294002	**2**	↔	↔	↔	NT	NT
	6	↔	↑	↑	NT	NT
	12	↔	↓	↔	NT	NT
	24	↔	↔	↔	NT	NT
GnRH2	**2**	↔	↔	↔	NT	NT
	6	↑°	↑°	↑°	↔	↔
	12	↔	↔	↔	↑	↑
	24	↑°	↔	↑	↔	↔
GnRH3	**2**	↑*	↔	↔#	NT	NT
	6	↑*	↑	↑	↓	↓
	12	↑°	↑°	↑°	↑	↑
	24	↑°	↑°	↑°	↔	↓

↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control; *, GnRH effect significantly enhanced by LY294002; °, GnRH effect significantly attenuated by LY294002; #, response significantly greater than untreated controls when in the presence of LY294002; NT, not tested.

Table 6.2 Summary of time-dependent GnRH- and LY294002-mediated effects on goldfish pituitary GH release, cell content, and total availability in comparison to GnRH-stimulated changes in GH mRNA expression previously shown in Klausen et al., 2002[1].

Treatment	Duration (hrs)	GH release	GH cell content	Total GH availability	GH mRNA[1]
LY294002	**2**	↑	↔	↔	NT
	6	↑	↔	↑	NT
	12	↔	↓	↓	NT
	24	↔	↓	↓	NT
GnRH2	**2**	↑ *	↓ °	↓ °	NT
	6	↑ °	↓	↔	↔
	12	↔ #	↔	↔	↑
	24	↑	↑ °	↑ °	↔
GnRH3	**2**	↔ #	↓	↓	NT
	6	↑	↓	↔	↔
	12	↑ °	↓	↔	↑
	24	↑ *	↓	↓	↔

↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control; *, GnRH effect significantly enhanced by LY294002; °, GnRH effect significantly attenuated by LY294002; #, response significantly greater then untreated controls when in the presence of LY294002; NT, not tested.

Figure 6.1 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on the expression of immunoreactive phosphorylated Akt (Ser473) in goldfish pituitary fragments following 2 hrs in static incubation under basal and GnRH2- or GnRH3-stimulated (100 nM) conditions. (a) Representative immunoblots of a goldfish pituitary tissue lysate probed with a primary antibody that recognizes Akt phosphorylated at residue Ser473, and immunoblotting of the same membrane with a pan-Akt antibody. (b) Quantified ratios of phosphorylated Akt (Ser473) to total immunoreactive pan-Akt in treatments groups (black, untreated; gray, LY294002 alone; blue, GnRH3 alone; dark blue, GnRH3 + LY294002; red, GnRH2 alone; dark red, GnRH2 + LY294002), expressed as a percentage of unstimulated controls are presented (mean ± SEM, $n = 3$; with goldfish in April, a time of year when the gonads are at a sexually matured/pre-spawning stage). For both the phosphorylated Akt (Ser473) and pan-Akt immunoblotting experiments, the antibodies used cross-react with conserved sequences within Akt1, Akt2, and Akt3 (molecular mass is expected to be ~ 60 kDa). Treatments that are significantly different from one another are identified by astericks (**; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$). For information about the specificity of LY294002, refer to Table 3.1.

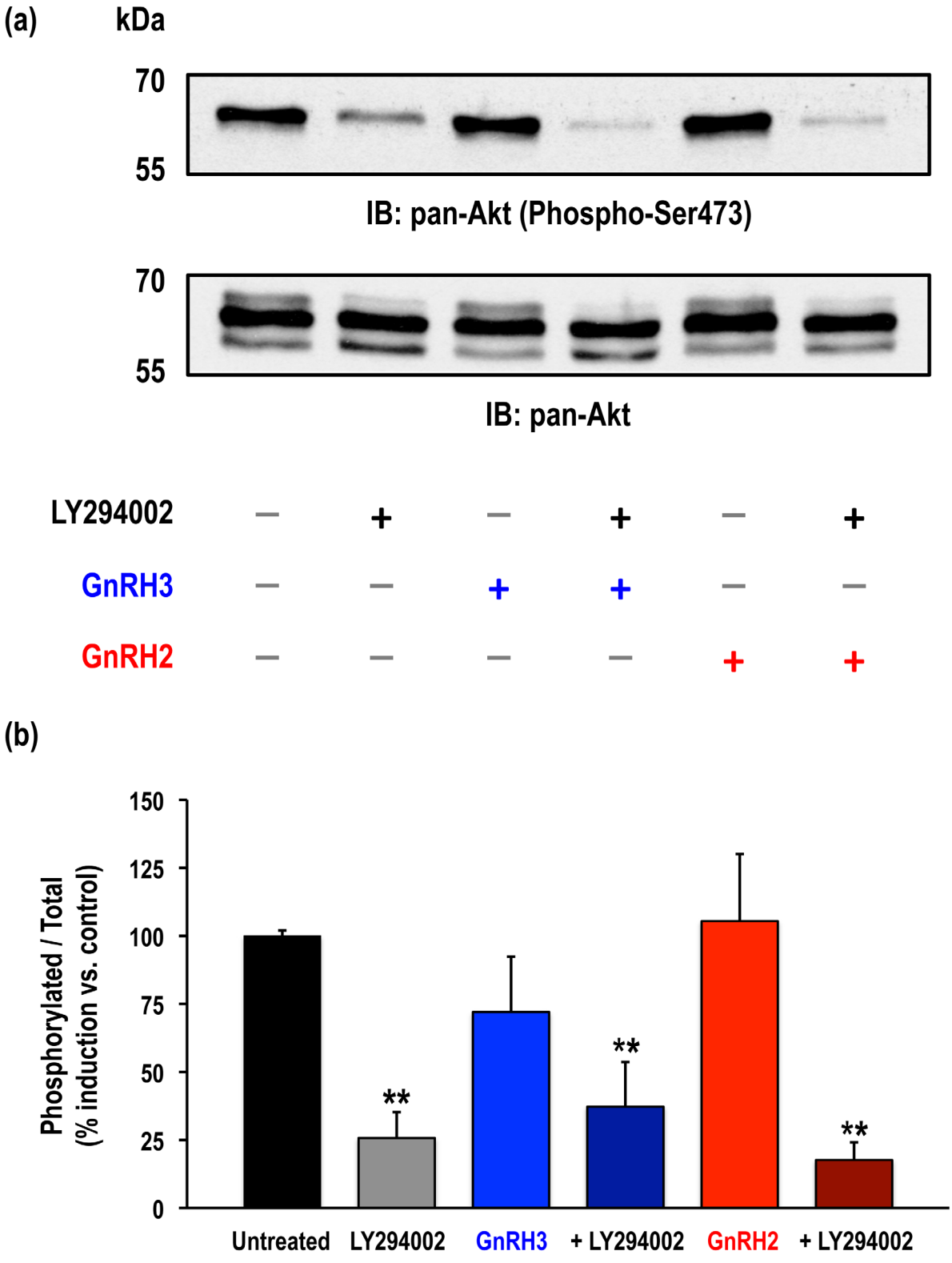

(a)
kDa
70
55
IB: pan-Akt (Phospho-Ser473)
70
55
IB: pan-Akt
LY294002 – + – + – +
GnRH3 – – + + – –
GnRH2 – – – – + +
(b)
Phosphorylated / Total
(% induction vs. control)
150
125
100
75
50
25
0
**
**
**
Untreated
LY294002
GnRH3
+ LY294002
GnRH2
+ LY294002

Figure 6.2 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on the expression of immunoreactive phosphorylated PDK1 (Ser241) in goldfish pituitary fragments following 2 hrs in static incubation under basal and GnRH2- or GnRH3-stimulated (100 nM) conditions. (a) Immunoblots of a goldfish pituitary tissue lysate probed with a primary antibody that recognizes PDK1 phosphorylated at residue Ser241, and immunoblotting of the same membrane with a PDK1 antibody (expected molecular mass of between 58 and 68 kDa). (b) Quantified ratios of phosphorylated PDK1 (Ser241) to total immunoreactive PDK1 in treatments groups (black, untreated; gray, LY294002 alone; blue, GnRH3 alone; dark blue, GnRH3 + LY294002; red, GnRH2 alone; dark red, GnRH2 + LY294002), expressed as a percentage of unstimulated controls are presented using goldfish in April, a time of year when the gonads are at a sexually matured/pre-spawning stage. For information about the specificity of LY294002, refer to Table 3.1.

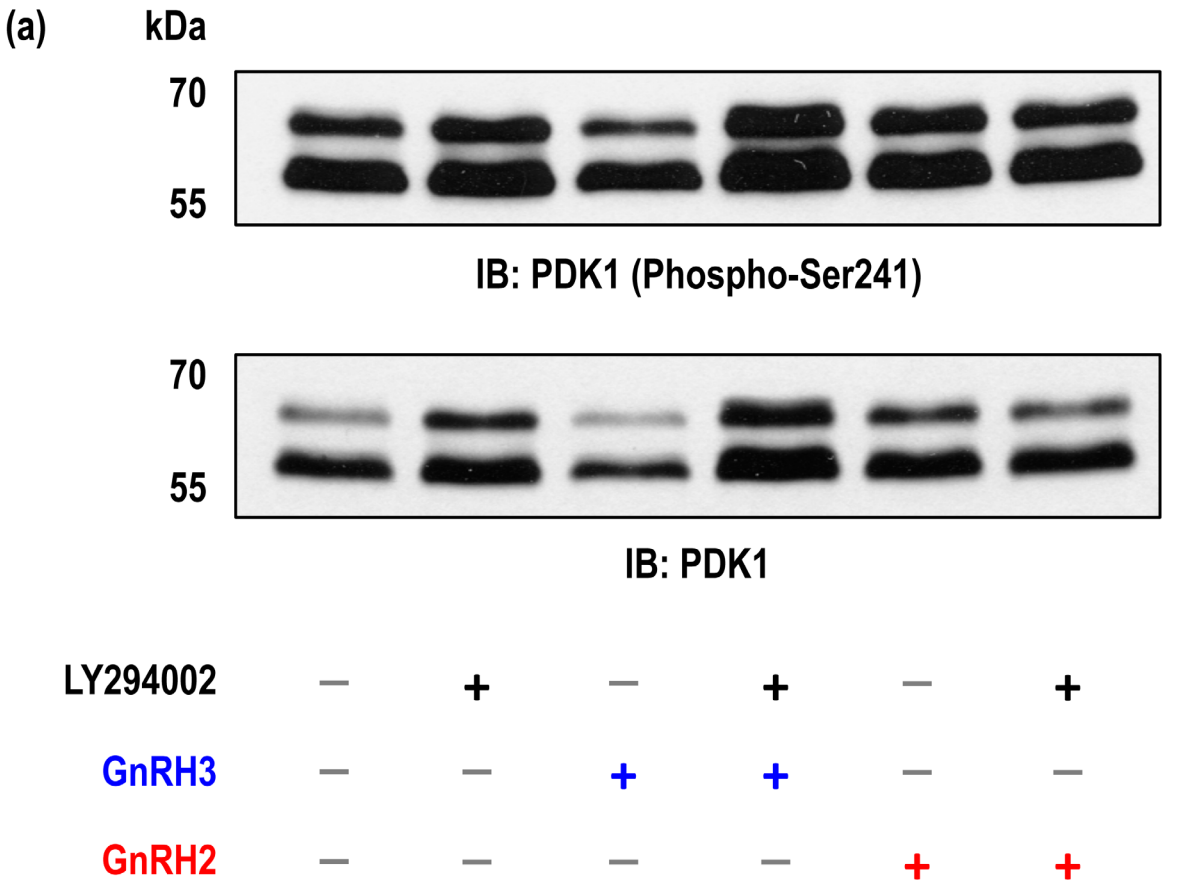

(a)
kDa
70
55
IB: PDK1 (Phospho-Ser241)
70
55
IB: PDK1
LY294002 – + – + – +
GnRH3 – – + + – –
GnRH2 – – – – + +
(b)
Phosphorylated / Total
(% induction vs. control)
150
125
100
75
50
25
0
Untreated
LY294002
GnRH3
+ LY294002
GnRH2
+ LY294002

Figure 6.3 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on basal, GnRH2-, and GnRH3-stimulated LH release and total availability over 2, 6, 12 and 24 hrs in static incubation. Treatment groups are identified by consecutive Arabic numerals, respectively: (1; black) untreated, (2; gray) LY294002 (10 μM) alone, (3; blue) GnRH3 (100 nM) alone, (4; dark blue) GnRH3 + LY294002, (5; red) GnRH2 (100 nM) alone, and (6; dark red) GnRH2 + LY294002. Pooled results are shown with each treatment having been replicated three times using independently prepared dispersed pituitary cells (4×10^6 cells/treatment) from goldfish at early gonadal recrudescence (October to December) and are expressed as the total μg LH present within the sample (mean ± SEM, n = 3). The amount of LH released into the media during treatment (LH release) and the cellular LH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay and expressed as the total μg LH present within the sample. The sum of the LH release and cellular LH content values in each matched treatment sample was calculated as an index of total LH available (total LH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Different letters of the alphabet identify treatment responses that are significantly different from one another (ANOVA followed by Fisher's LSD multiple comparisons where applicable; $P < 0.05$). For information about the specificity of LY294002, refer to Table 3.1.

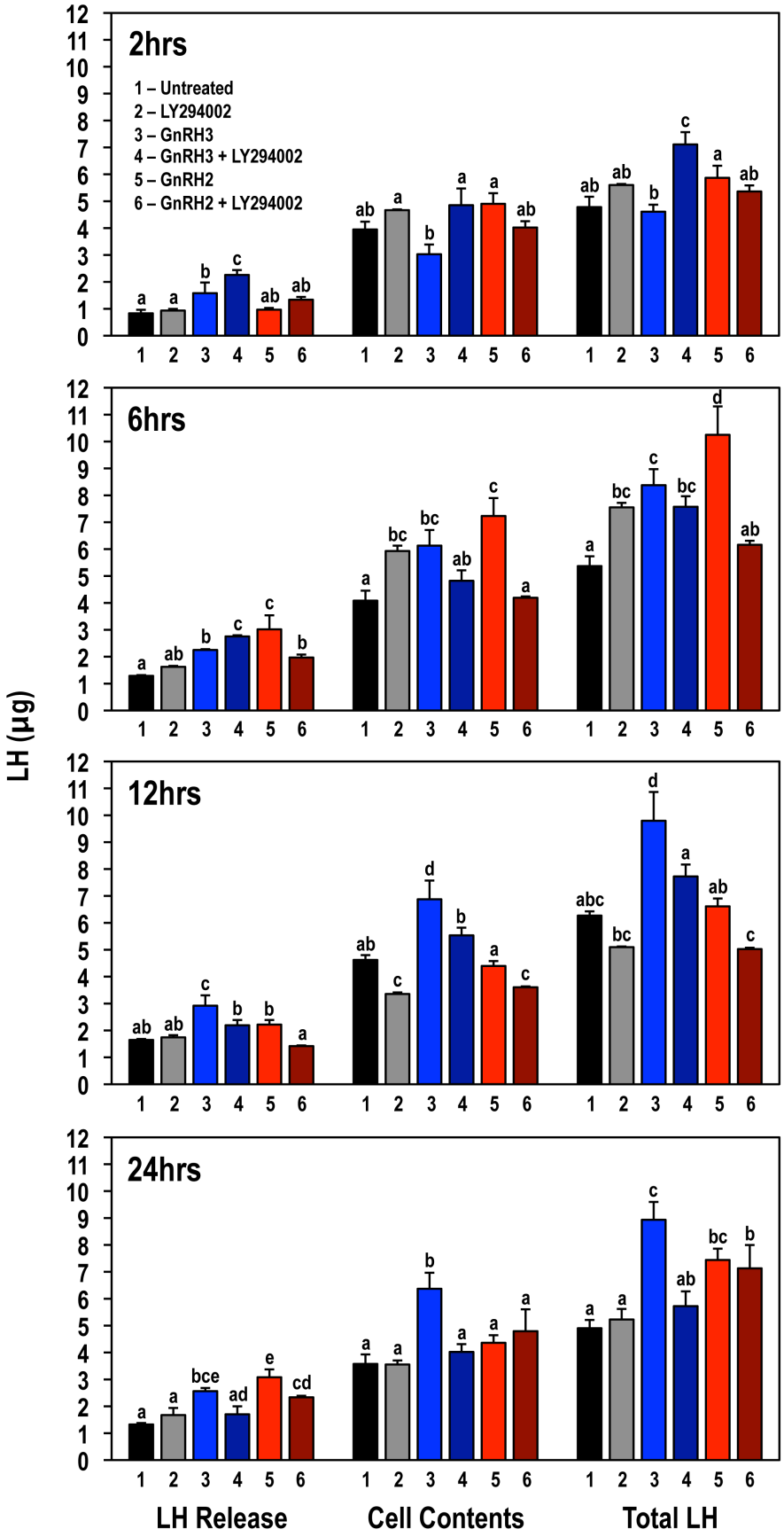
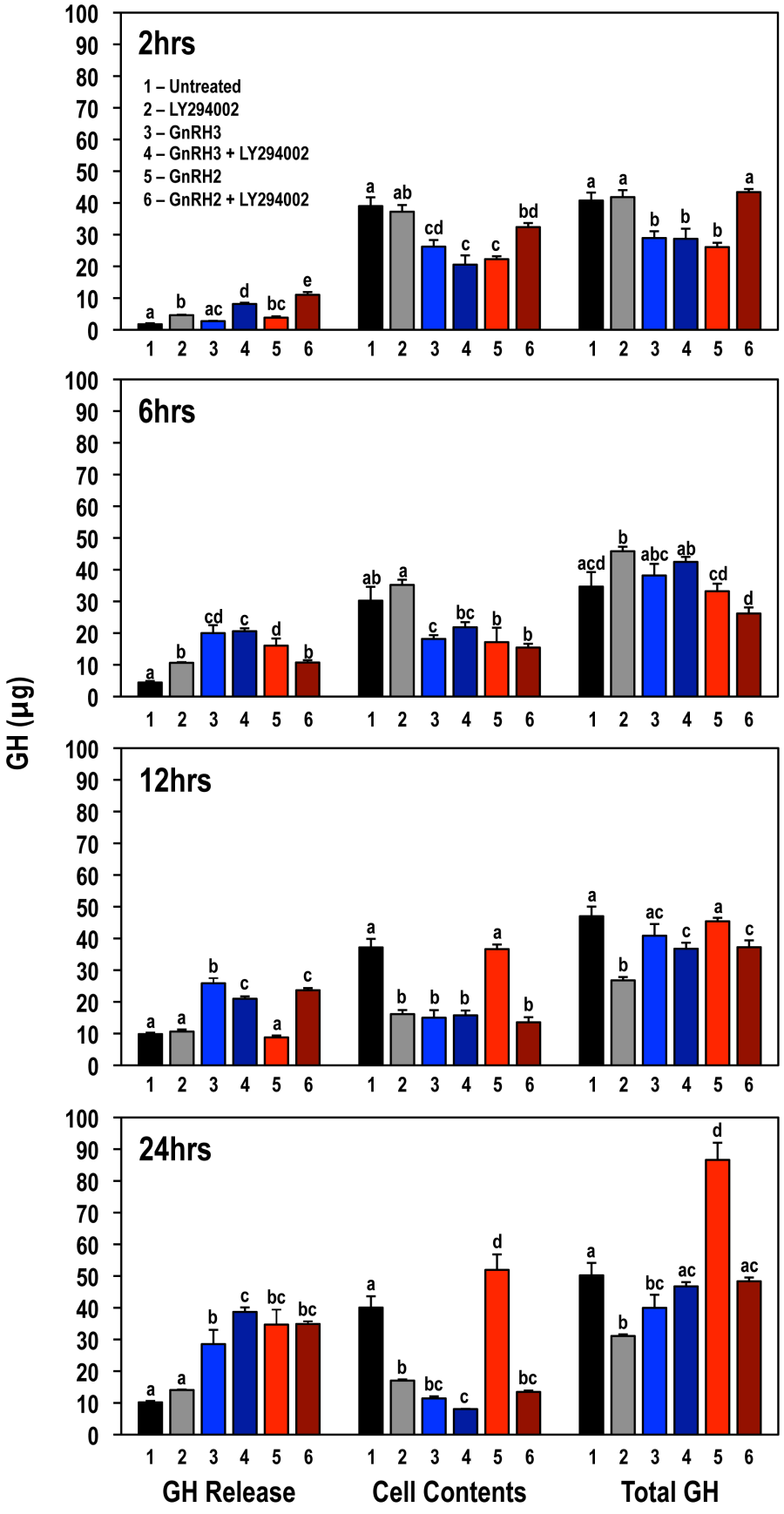
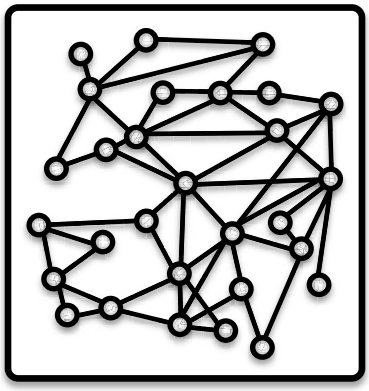

2hrs
1 – Untreated
2 – LY294002
3 – GnRH3
4 – GnRH3 + LY294002
5 – GnRH2
6 – GnRH2 + LY294002
6hrs
12hrs
24hrs
LH (µg)
LH Release
Cell Contents
Total LH

Figure 6.4 Effects of the pan-PI3K inhibitor LY294002 (10 μM) on basal, GnRH2-, and GnRH3-stimulated GH release and total availability over 2, 6, 12 and 24 hrs in static incubation. Treatment groups are identified by consecutive Arabic numerals, respectively: (1; black) untreated, (2; gray) LY294002 (10 μM) alone, (3; blue) GnRH3 (100 nM) alone, (4; dark blue) GnRH3 + LY294002, (5; red) GnRH2 (100 nM) alone, and (6; dark red) GnRH2 + LY294002. Pooled results are shown with each treatment having been replicated three times using independently prepared dispersed pituitary cells (4×10^6 cells/treatment) from goldfish at early gonadal recrudescence (October to December) and are expressed as the total μg GH present within the sample (mean ± SEM, n = 3). The amount of GH released into the media during treatment (GH release) and the cellular GH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay and expressed as the total μg GH present within the sample. The sum of the GH release and cellular GH content values in each matched treatment sample was calculated as an index of total GH available (total GH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Different letters of the alphabet identify treatment responses that are significantly different from one another (ANOVA followed by Fisher's LSD multiple comparisons where applicable; $P < 0.05$). For information about the specificity of LY294002, refer to Table 3.1.


2hrs
1 – Untreated
2 – LY294002
3 – GnRH3
4 – GnRH3 + LY294002
5 – GnRH2
6 – GnRH2 + LY294002
6hrs
12hrs
24hrs
GH (µg)
GH Release
Cell Contents
Total GH

Chapter Seven:

Acute and long-term involvement of Raf-MEK-ERK signalling during basal and GnRH-stimulated actions on hormone release and availability



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

7.1 Introduction

Results from this thesis have outlined a novel role for PI3K-dependent signalling in the control of acute and long-term goldfish pituitary cell LH and GH secretion, as well as in the maintenance of total hormone availability (Chapters 3, 4, 5, and 6). Furthermore, PI3Ks are shown to differentially integrate with other GnRH-sensitive intracellular effectors, including PKCs and Ca^{2+} (Chapter 3), in the control of acute hormone release. However, studies using oncogenic cell models have recently outlined an important role for cross-talk between PI3K-dependent signalling pathways and isoforms of MAPK (Mendoza et al., 2011; Aksamitiene et al., 2012).

Interestingly, GnRHR activation also recruits numerous isoforms of MAPK in a variety of mammalian cell types and MAPKs participate in a number of canonical GnRH-stimulated actions, such as the regulation of pituitary hormone gene expression, gonadal steroidogenesis, and tumor cell apoptosis (Dobkin-Bekman et al., 2006; Naor, 2009; Naor and Huhtaniemi, 2013). MAPK-dependent signalling is highly conserved in eukaryotes and is organized as linear three-kinase cascades involving MAPK activation by a MAPK kinase (MAPKK or MEK), which in turn is activated by a MAPK kinase kinase (MAPKKK or MEK kinase; Schaeffer and Weber, 1999). Seven MEK homologues (MEK1, MEK2, MKK4, MEK5, MKK6, and MKK7) and four families of MAPK (extracellular signal-regulated kinase (ERK)1/2, ERK5, cJun N-terminal kinase (JNK), and p38 MAPK) have been identified across eukaryotes (Schaeffer and Weber, 1999). Among these MAPK cascades, activation of ERK1 (p44 MAPK) and ERK2 (p42 MAPK) has been demonstrated to mediate GnRH actions in immortalized gonadotropes cell lines (Dobkin-Bekman et al., 2006). In general, ERK1 and ERK2 are activated by

dual phosphorylation of conserved Thr and Tyr residues within their activation segment by the related MEK1 and MEK2 kinases, respectively; although both MEK1/2 and ERK1/2 have been shown to be functionally redundant (Boulton et al., 1991; Crews and Erikson, 1992; Crews et al., 1992; Favata et al., 1998). Additionally, Raf family kinases activate MEK1 and MEK2 by phosphorylation of their activation segment (at Ser218 and Ser22 in MEK1; Kyriakis et al., 1992), thereby playing the part of the MAPKKK and forming the canonical Raf-MEK-ERK signalling module (Lavoie and Therrien, 2015).

In terms of goldfish pituitary cells, both GnRH2 and GnRH3 can stimulate phosphorylation of ERK1/2, and MEK1/2-dependent signalling mediates acute LH release responses to both GnRHs (Klausen et al., 2008; Chang et al., 2009). Furthermore, GnRH3- and GnRH2-induced increases in GTH-α, LH-β, and GH mRNA expression are also MEK1/2-dependent (Klausen et al., 2005a, 2008). Interestingly, treatment with the PKC-activating phorbol ester TPA induces MEK1/2-dependent phosphorylation of ERK1/2 in mixed populations of goldfish pituitary cells but GnRH actions on GTH subunit and GH mRNA expression are not mediated by novel or conventional PKCs (Klausen et al., 2008). Whether and how MEK1/2-dependent signalling interacts with PKC-dependent signalling to modulate LH and GH release is unknown. In fact, the role of MEK1/2-dependent signalling in the neuroendocrine control of hormone secretion in primary pituitary cells is not well examined and the involvement of the Raf-MEK-ERK cassette in GnRH actions on GH release has not been investigated in any pituitary cell system.

The objectives of this chapter were to examine the involvement of MEK1/2-dependent signalling in acute and prolonged GnRH actions on goldfish gonadotrope and

somatotrope functions, as well as the potential interactions of Raf-MEK-ERK with PKC- and PI3K-dependent signalling in the control of LH and GH release. As in previous chapters, acute hormone release responses were measured using cell column perifusion while long-term effects were examined with static incubation experiments. Overall, the involvement of Raf-MEK-ERK signalling was largely examined by monitoring the effects of selective MEK1/2 inhibitors on basal, GnRH-induced, and PKC activator-elicited hormone secretion, as well as by monitoring the levels of phosphorylated ERK1/2 (Thr202/Tyr204). As a first investigation into the possible interactions between PI3Ks and the Raf-MEK-ERK cassette, the influence of treatments with pan-PI3K and class I PI3K isoform-specific inhibitors on ERK1/2 phosphorylation (Thr202/Tyr204) was quantified.

7.2 Results

7.2.1 ERK1/2 immunoreactivity is present in goldfish pituitary cell extracts

In a previous study with goldfish mixed pituitary cell cultures, both GnRH3 and GnRH2 could stimulate MEK1/2-dependent phosphorylation of ERK1/2 at 30 min (Klausen et al., 2008). In this study, immunoblotting with a primary antibody raised against human ERK1/2 revealed two distinct immunoreactive bands between 35 and 55 kDa in goldfish pituitary cell lysates that corresponded with those observed in mammalian RBL-2H3 cell lysates (known to be 42 and 44 kDa in size; positive controls; Figure 7.1). Likewise, protein extracts from unstimulated goldfish pituitary cells also reacted positively with antibodies directed against phosphorylated ERK1/2 (Thr202/Tyr204; Figure 7.1). In agreement with previous studies in mammals,

unstimulated RBL-2H3 cells did not posses immunoreactive phosphorylated ERK1/2 (Cortes et al., 2014), whereas goldfish pituitary cell lysates expressed phosphorylated ERK1/2 under basal conditions (Figure 7.1). Pre-absorption with an antigenic peptide reduced the ability of the phosphorylated ERK1/2 (Thr202/Tyr204) antibody to label morphologically identified pituitary gonadotropes (Figure 7.2) and somatotropes (Figure 7.3) within mixed pituitary cell populations. Identified pituitary gonadotropes and somatotropes could also be immunostained using ERK1/2 primary antibodies (Figures 7.2c and 7.3c). Furthermore, co-staining of phosphorylated ERK1/2 (Thr202/Tyr204) within LH-expressing gonadotopes (Figure 7.4) and GH-expressing somatotropes (Figure 7.5) was visualized using an imaging flow cytometry technique under unstimulated conditions; indicating that both of these pituitary cell types express activated ERK1/2.

7.2.2 Effects of MEK1/2 inhibitors on acute and long-term hormone actions in primary pituitary cell cultures.

In order to examine the involvement of MEK1/2-dependent signalling in basal and GnRH-stimulated actions in primary cultures of dispersed goldfish pituitary cells, we utilized the selective MEK1/2 inhibitors U0126 and PD098059. Notably, perifusion experiments using U0126 in combination with GnRHs were done using a final concentration of 50 nM; targeting the reported IC_{50} for MEK1 (IC_{50} = 72 nM; Favata et al., 1998) and MEK2 (IC_{50} = 58 nM; Favata et al., 1998) isoforms. Since previous studies on the involvement of ERK1/2 activation in acute GnRH-induced LH secretion utilized the MEK1/2 inhibitor PD098059, this inhibitor was also used in acute GH release experiments with GnRH. At the 100 μM concentration used, PD098059 likely also

affected both MEK1 (IC_{50} = ~10 μM; Alessi et al., 1995) and MEK2 (IC_{50} = 50 μM; Alessi et al., 1995). Similar to U0126, PD098059 does not significantly inhibit other serine/threonine kinase or lipid kinases, including the MAPK homologs JNK and p38 MAPK as well as PKA, PKCα, and PI3Ks (Alessi et al., 1995; Dudley et al., 1995). However, prolonged GnRH exposures and experiments using potent PKC activators used a U0126 working concentration of 10 μM. Notably, this concentration has been demonstrated to potently and selectively block both MEK1 and MEK2, as well as the functional responses mediated by MEK1/2-dependent signalling in numerous cell culture systems (Bain et al., 2007), but does not affect closely-related protein kinases, including PKC, JNK, ERK, and p38 MAPK (Favata et al., 1998; Bain et al., 2007). In agreement with these studies in mammals, in 2 hr static cultures, treatment with U0126 (10 μM) alone significantly decreases phosphorylation of ERK1/2 (Thr202/Tyr204; Figure 7.6). These data strongly suggests that U0126 is an effective tool for examining MEK1/2-dependent signalling in goldfish pituitary cell cultures.

7.2.2.1 Effects of MEK1/2 inhibitors on acute GnRH-stimulated GH release

The involvement of MEK1/2-dependent signalling in GnRH stimulation of acute GH release was examined in a column perifusion system. Treatments with maximally effective concentrations of GnRH2 (100 nM; Chang et al., 1990) or GnRH3 (100 nM; Chang et al., 1990) significantly increased GH secretion (Figures 7.7 and 7.8). Treatments with either U0126 (50 nM) or PD098059 (100 μM) significantly reduced GnRH3- (Figure 7.8), but not the GnRH2-induced, GH release responses (Figure 7.7).

7.2.2.2 U0126 differentially affects acute LH and GH release responses to synthetic PKC activators

Whether activation of MEK1/2 occurs downstream of PKC-dependent signalling was investigated by examining the effect of U0126 on the LH and GH release responses to the novel and conventional PKC activators TPA and DiC8 in column perifusion. Since both MEK1/2 inhibitors showed similar effects on GnRH-induced LH (Klausen et al., 2008; Chang et al., 2009) and GH release (Section 7.2.2.1), subsequent experiments using PKC activators and prolonged GnRH treatments in static culture used only U0126; due primarily to its higher affinity and selectivity for both MEK1 and MEK2 isoforms (Alessi et al., 1995; Favata et al., 1998; Bain et al., 2007). Furthermore, based on the effects observed on phosphorylation of ERK1/2 (Thr202/Tyr204; Section 7.2.2), in these and subsequent experiments U0126 was used at 10 μM. As expected, the previously demonstrated maximally stimulatory concentration of TPA (100 nM; Chang et al., 1991) and DiC8 (100 μM; Chang et al., 1991) elicited LH and GH release from perifused goldfish pituitary cells (Figures 7.9 and 7.10). Pretreatment with U0126 (10 μM) significantly reduced net GH (Figure 7.10), but not net LH, (Figure 7.9) release responses to both PKC activators. Interestingly, the kinetics of the GH release response to TPA and DiC8 were differentially affected by U0126. The magnitude of the peak response (maximal increase) to DiC8, but not that to TPA, was attenuated by U0126; the peak response to DiC8 was approximately halved in the presence of U0126 (Figure 7.10; 516 ± 43 and 256 ± 41 % pretreatment in the absence and presence of U0126, respectively; $p < 0.05$; paired Student's *t*-test).

7.2.2.3 U0126 differentially affects acute basal LH and GH release responses

The effects of U0126 on basal LH and GH secretion in column perifusion were also analysed using pooled data from inhibitor alone columns. Treatment with U0126 (10 μM) significantly reduced the average basal LH release by 14% (Figure 7.11a). Interestingly, in terms of GH release, the presence of U0126 (10 μM) caused a transient increase in basal release; however, when averaged over the course of the entire U0126 treatment, the average GH release was significantly reduced by roughly 10% (Figure 7.11b).

7.2.2.4 Involvement of MEK1/2-dependent signalling in the control of long-term basal and GnRH-stimulated hormone release, cell content, and total hormone availability

The amount of LH released into the media was generally higher in GnRH3 (100 nM) alone and GnRH2 (100 nM) alone groups and significant increases relative to untreated controls were observed at 6, 12 and 24 hrs for GnRH3, as well as 6 and 24 hrs for GnRH2 (Figure 7.12). Treatment with U0126 (10 μM) alone did not significantly affect basal LH release and in its presence, GnRH2 and GnRH3 failed to significantly elevate LH release relative to untreated controls at any of the time points examined (Figure 7.12).

Cellular LH content was not affected by treatments at 6 and 24 hrs but significant elevations were seen at 2 hrs with the combinational GnRH plus U0126 treatments, and at 12 hrs with the GnRH3 alone treatment (Figure 7.12). Treatments had no effect on the calculated total amount of LH available at 24 hrs and U0126 alone also did not

significantly alter total LH availability at other time points examined relative to untreated controls (Figure 7.12). However, significant elevations in total available LH relative to controls were seen with the combinational GnRH plus U0126 treatments at 2 hrs, GnRH2 alone treatment at 6 hrs, and GnRH3 alone treatment at 12 hrs (Figure 7.12).

Treatment with GnRH (100 nM) generally resulted in greater GH release and the increases were significant relative to untreated controls at 6, 12 and 24 hrs in static incubation (Figure 7.13). Likewise, GnRH2 (100 nM) significantly elevated GH secretion above untreated controls at 2, 6 and 24 hrs (Figure 7.13). Treatment with U0126 (10 μM) alone tended to increase GH secretion and significant differences were observed at 2 and 12 hrs. The presence of U0126 reduced the GH release response to GnRH3 at 6, 12 and 24 hrs to levels not different from U0126 alone; however, U0126 did not affect the GH release response elicited by GnRH2 at 6 hrs (Figure 7.13). Paradoxically at 2 hrs, U0126 reduced GnRH2-induced GH release to a level not significantly different from controls and U0126 alone while the combined U0126 and GnRH3 treatment resulted in a further elevation in GH release relative to that to U0126 alone (Figure 7.13).

In general, all treatments significantly reduced cellular GH contents relative to untreated controls at all time points with the exceptions of the combined GnRH2 and U0126 treatment at 6 hrs, and U0126 alone and GnRH2 alone treatments at 12 hrs (Figure 7.13). When the total available GH (sum of released plus remaining cell content) was calculated, no significant differences were observed between treatment groups at 12 hrs but treatments generally resulted in lower total available GH at other time points (Figure 7.13). Significant reductions relative to controls were observed at 2 hrs with all

treatment groups and at 6 and 24 hrs with U0126 alone and the combination of GnRH3 and U0126 (Figure 7.13).

7.2.3 Effects of pan-PI3K and isoform-selective class I PI3K inhibitors on ERK1/2 phosphorylation

In order to examine the relationship between PI3Ks and Raf-MEK-ERK signalling in goldfish pituitary cells, the ability of broad-spectrum and isoform-selective PI3K inhibitors to alter the phosphorylation of ERK1/2 (Thr202/Tyr204) was measured as an index of PI3K-dependent ERK1/2 activation. Results show that treatments with the pan-PI3K inhibitor LY294002 (10 μM) or isoform-selective class I PI3K inhibitors (refer to Table 4.1 for the experimental concentrations used) do not alter basal phosphorylation of ERK1/2 (Thr202/Tyr204) in 2 hr static incubations (Figures 7.14).

7.3 Discussion

This is one of a small number of studies examining the involvement of Raf-MEK-ERK signalling in the control of hormone release from primary pituitary cells. Results in this chapter confirm previous findings that ERK1/2 is constitutively active in goldfish pituitary gonadotropes and somatotropes (Klausen et al., 2008). However, in contrast with the previous study which shows the presence of three immunoreactive phosphorylated (Tyr204) or total ERK1/2 bands (Klausen et al., 2008), the data from the present study showed only two immunoreactive phosphorylated ERK1/2 (Thr202/Tyr204) bands, whereas two strongly and one weakly immunoreactive total ERK1/2 bands were observed. These discrepancies may be due to differences in the

properties of the phosphorylated and total ERK1/2 antibodies used between the present and the previous studies. More importantly, results in this thesis chapter suggest that differential participation of the Raf-MEK-ERK transduction cascade during GnRH2 and GnRH3 actions, possibly through selective integration with PKC-dependent signalling, contributes to the integrated control of LH and GH secretion. Furthermore, dissociation between the influences of Raf-MEK-ERK signalling on total hormone protein availability was observed in relationship to previous results on hormone subunit mRNA expression.

7.3.1 MEK1/2-dependent signalling is differentially involved in GnRH- and PKC-mediated stimulation of LH and GH release

Relatively little is known regarding the role of MEK1/2-dependent signalling in the control of GnRH-stimulated hormone secretion as compared with studies examining GnRH actions on gene expression. Previous work on goldfish pituitary cells have indicated that GnRH2 and GnRH3 can activate ERK1/2 through MEK1/2-dependent signal transduction and that acute LH release induced by both GnRHs is mediated by MEK1/2-dependent signal transduction (Klausen et al., 2008; Chang et al., 2009). LH release responses to long-term treatments with GnRH2 (6 and 24 hrs) and GnRH3 (12 and 24 hrs) are similarly reduced by the selective MEK1/2 inhibitor U0126 to control levels in the present study. Although mGnRH-stimulated LH release is reduced by U0126 in primary cultures of ovine pituitary cells (Yang et al., 2005), LH secretory responses to pulsatile mGnRH applications are not significantly altered by PD098059 in rats (Haisenleder et al., 1998); suggesting that the involvement of MEK1/2-dependent

signalling in GnRH effects on LH secretion may be species- and/or treatment protocol-dependent.

Although PD098059 did not alter basal or GH-releasing hormone-stimulated GH secretion in human pituitary adenomas (Lania et al., 2003), the involvement of MEK1/2-dependent signalling in GnRH-induced GH release has not been studied previously. Results from the present study demonstrate that MEK1/2-dependent signalling is differentially involved in GnRH actions on GH release in goldfish. Application of two selective inhibitors of MEK1/2, U0126 and PD098059, decreased GnRH3-elicited, but not GnRH2-stimulated, acute GH release from perifused goldfish pituitary cells. U0126 similarly reduced the long-term GnRH3-induced GH release at 6, 12, and 24 hrs of static incubation; however, GnRH2-induced GH secretion was also attenuated by treatment with U0126 at 2 and 24 hrs. These observations indicate Raf-MEK-ERK involvement in GnRH stimulation of GH release differs between acute and long-term secretion, being selective for GnRH3 in short-term stimulation protocols but the isoform-selectivity is not seen in long-term GnRH actions.

In goldfish gonadotropes and somatotropes, GnRH stimulation of hormone release is dependent on PKC-dependent signal transduction. Interestingly, mGnRH-stimulated ERK1/2 activation in LβT2 and αT3-1 gonadotropes is PKC-dependent (Benard et al., 2001) and a PKC activator also induces ERK1/2 phosphorylation in primary cultures of goldfish (Klausen et al., 2008), as well as in tilapia (Gur et al., 2001) pituitary cells. These observations suggest that PKCs may participate in GnRH actions in goldfish LH and GH secretion through MEK1/2-dependent activation of ERK1/2. Although treatment with U0126 reduced the GH responses to DiC8 and TPA from

perifused goldfish pituitary cells, PKC activator-induced LH release was not affected. However, DiC8 and TPA selectively activate the conventional (α, βI, βII, γ) and novel (δ, ε, θ, η/L), but not atypical (ζ, ι/λ), PKC isoforms (Newton et al., 2001) and goldfish pituitary cells contain immunoreactive PKCs belonging to all three PKC families (i.e., PKCα, δ, θ, and ζ; Klausen et al., 2005a). These results indicate that conventional and novel PKCs likely do not participate in GnRHR-mediated activation of MEK1/2-dependent signalling in terms of acute LH release but may be involved in GnRH stimulation of GH secretion. However, the atypical PKCζ isoform has been shown to activate ERK1/2 in squamous carcinoma cells (Valkova et al., 2010) and translocation of PKCζ to the nucleus is stimulated by mGnRH in αT3-1 gonadotropes (Kratzmeier et al., 1996). Since immunoreactive PKCζ is present in dispersed goldfish pituitary cell extracts, whether PKCζ plays a role in the activation of Raf-MEK-ERK signalling in GnRH actions on acute LH and GH release is currently unknown. In addition, whether PKCs, in general, play a role in MEK1/2-dependent regulation of long-term GnRH actions remains to be investigated.

Interestingly, U0126 decreased basal LH and GH release in column perifusion studies while it did not alter basal LH release but tended to elevate basal GH release in static incubation experiments, and significantly so at 2 and 12 hrs. These observations suggest that the Raf-MEK-ERK signalling cascade has a unique regulatory influence on unstimulated GH release over the long-term. It is known that basal secretion and stimulated hormone release may be regulated differently (Chang et al., 2000; Johnson et al., 2002, 2003). However, given that MEK1/2-dependent signalling is likely involved in mediating GnRH2 and GnRH3 stimulation of long-term GH release, these results on the

effects of U0126 on basal GH secretion indicate the actions of signal transduction elements in the control of basal and agonist-regulated hormone secretion are complex.

7.3.2 Involvement of MEK1/2-dependent signalling in GnRH actions on long-term cellular LH and GH content, and total availability

Although GnRH effects on LH and GH mRNA expression are well known (Naor, 2009), relatively little is known regarding GnRH actions on LH and GH protein production. In an attempt to assess GnRH actions on LH and GH protein production, we measured cellular LH and GH contents and calculated the total LH and GH available (sum of cellular content and amount released) over 2, 6, 12, and 24 hrs in static incubation studies with the caveat that the influence of protein degradation on these measurements is likely consistent across treatments. Interestingly, cellular GH contents are generally decreased by GnRH and/or U0126 treatments while LH contents are not (Tables 7.1 and 7.2). These data suggest that hormone protein synthesis tends not to keep up with hormone secretion in goldfish somatotropes, but not gonadotropes, under these testing conditions; in addition, the activity of the Raf-MEK-ERK cassette may modulate basal GH, but not LH, protein availability.

Previous experiments have shown that static incubation with GnRH2 and GnRH3 increase GTHα and LHβ mRNA levels at 12 hrs (Klausen et al., 2002) and that MEK1/2-dependent signalling mediates these GnRH effects (Klausen et al., 2005a, 2008). In the present study, GnRH2 and GnRH3 increased total LH availability at 6 and 12 hrs, respectively, suggesting that LH protein synthesis was elevated at these time points (Table 7.1). The ability of U0126 to reduce the elevation of total LH availability by

GnRH2 and GnRH3 to levels not different from controls indicates MEK1/2 catalytic activity participates in the regulation of LH protein synthesis. Likewise, treatment with MEK1/2 inhibitors reduces GnRH-induced increases in LHβ protein-positive cells (Liu et al., 2002) and stimulation of a cap-dependent translational reporter in LβT2 gonadotropes (Nguyen et al., 2004). Taken together, these results suggest that MEK1/2-dependent signalling participates in the ability of GnRH2 and GnRH3 to enhance LH mRNA translation and protein synthesis in goldfish, as well as mammalian, gonadotropes. Interestingly, GnRH3 also reduces the mRNA levels of GTHα at 6 hrs and LHβ at 6 and 24 hrs in a previous study with goldfish pituitary cells (Klausen et al., 2002). However, neither treatment with U0126 alone, GnRH3 alone, nor the combination of GnRH3 and U0126 lead to suppression of total LH availablity at any time point in the present study (Table 7.1), raising the possibility that the ability of GnRH3 to reduce LHβ mRNA levels is not mediated by MEK1/2-dependent signalling. On the other hand, total GH availability was unaffected by the GnRH3 at 6, 12 and 24 hrs, and GnRH2 at 2 and 12 hrs; furthermore, this was even reduced by GnRH2 and GnRH3 at 2 hrs.

Interestingly, data presented in Chapter 6 demonstrated that GnRH2 significantly elevated total GH protein availability at 24 hrs and a tendency for an increase was also seen in this Chapter, although differences were not significant. These results indicate that, perhaps with the exception of GnRH2 actions at 24 hrs, GH protein production is not stimulated by the two GnRHs and may even be inhibited at an early time point. This is in contrast with GH mRNA expression measurements where both GnRH2 and GnRH3 increased GH mRNA levels at 12 hrs and no effects were detected at 6 and 24 hrs (Klausen et al., 2002; Table 7.2). Furthermore, while both the GnRH2- and GnRH3-

elicited increases in GH mRNA levels are reduced by MEK1/2 inhibition (Klausen et al., 2005a), this is not reflected in the present results on total GH protein availability (Table 7.2). No significant differences were generally seen between GnRH alone and the combined GnRH plus U0126 treatment groups with the exception of GnRH2 at 6 hr where the presence of the combination treatment increased total GH availability relative to GnRH2 alone. When taken together with the findings that U0126 treatment alone reduced total GH availability at 2, 6 and 24 hrs; these observations suggest that MEK1/2-dependent signalling is required for maintenance of basal GH protein synthesis, as well as GnRH2 and GnRH3 stimulation of GH mRNA expression.

7.3.3 The Raf-MEK-ERK cascade and differential GnRH2 and GnRH3-stimulated signalling in goldfish gonadotropes and somatotropes

Results from the present study supports the idea that Raf-MEK-ERK signalling is differentially involved in GnRH2 and GnRH3 actions in goldfish gonadotropes and somatotropes in a time-, GnRH isoform-, and cell type-specific manner (Figures 7.15 and 7.16). How GnRH2 and GnRH3 activate Raf-MEK-ERK in goldfish pituitary cells has not been extensively studied but several possibilities exist. For example, first, evidence from mammalian systems suggests that changes in intracellular Ca^{2+} levels may play a role (Dobkin-Bekman et al., 2006; Naor, 2009). GnRH2 and GnRH3 are known to elicit different Ca^{2+} response kinetics and to utilize different intracellular Ca^{2+} stores and Ca^{2+} buffering systems in goldfish gonadotropes, as well as in somatotropes (Chang et al., 2009, 2012). Interestingly, mGnRH-agonist activation of Raf-MEK-ERK signalling has been shown to interact with Ca^{2+}-signalling machinery in primary rat pituitary cell

cultures, as well as αT3-1 and LβT2 gonadotropes (Mulvaney et al., 1999; Mulvaney and Roberson, 2000; Yokoi et al., 2000). How activated Raf-MEK-ERK integrates with Ca^{2+}-dependent signalling to control GnRH2 and GnRH3 actions on both gonadotropes and somatotropes in goldfish will be an important focus for future research.

Second, although PKCs do not participate in the MEK1/2-sensitive GnRH stimulation of LH and GH mRNA production, TPA activates phorphorylation of ERK1/2 in mixed populations of goldfish pituitary cells (Klausen et al., 2008) and the acute GH release response to TPA and DiC8 are reduced by MEK1/2 inhibition. This suggests that conventional and novel PKC isoforms may be upstream of MEK1/2 activation in terms of GnRH-stimulated GH release. Furthermore, as discussed in Section 7.3.1, the possibility that the atypical PKC isoform PKCζ may participate in the recruitment of MEK1/2-dependent signalling in GnRH action on LH or GH release cannot be excluded. Thus, differential involvement of PKC isoforms is a possible avenue by which GnRH-, cell type-, and function-specific utilization of MEK1/2-dependent signalling may be manifested in goldfish pituitary cells.

Third, GnRH activation of MEK1/2-dependent signalling has been shown to involve receptor transactivation in several cell types (Shah et al., 2006). Consequently, whether GnRHR-mediated transactivation of RTKs or direct G protein-mediated activation of Raf-MEK-ERK signalling occurs to control the differential actions of GnRH2 and GnRH3 in these cells is a possibility that needs to be investigated in the future. Interestingly, GPCRs are thought to indirectly activate the Raf-MEK-ERK cassette through signalling downstream of heterotrimeric G proteins, including Gβγ subunits (Smrcka et al., 2008; Khan et al., 2013). Results in Chapter 4 demonstrate that

Gβγ-dependent signalling contributes to GnRH2 and GnRH3 actions on hormone release in both gonadotropes and somatotropes. Though likely, this possibility needs to be confirmed in future studies examining the effects of inhibitors of Gβγ subunits on GnRH-induced ERK1/2 phosphorylation.

7.3.4 Integration of PI3K-dependent and Raf-MEK-ERK signalling in goldfish gonadotropes and somatotropes

PI3K-dependent and Raf-MEK-ERK signalling are both central regulators of cellular metabolism, proliferation, survival, and differentiation. Not surprisingly, PI3K-controlled signalling networks and Raf-MEK-ERK commonly communicate through intricate cross-talk, as well as compensation pathways, to coordinately control cell functions (Mendoza et al., 2011; Aksamitiene et al., 2012). In the present thesis chapter, I explored the architecture of the Raf-MEK-ERK signalling network present within goldfish pituitary cells using pan- and isoform-selective inhibitors of PI3Ks. Results demonstrate that under basal conditions, PI3K-dependent signalling is not a major upstream regulator of ERK1/2 activation. However, these findings do not address whether PI3Ks contribute to ERK1/2 activation during agonist-stimulation. As discussed above (Section 7.3.4), MEK1/2-dependent signalling was downstream of novel and conventional PKCs in the control of acute GH release responses. Although, whether PKCs in part mediate the downstream actions of PI3Ks on GH release from goldfish pituitary cells has not been investigated, and PI3K-dependent signalling can regulate PKCs through membrane recruitment of PDK1 in many mammalian systems (Mora et al., 2004; Pearce et al., 2010). On the other hand, inhibition of PDK1 does not inhibit GnRH-

stimulated acute LH or GH release (Chapter 5). Taken together, these results suggest that PI3Ks are unlikely to signal upstream of novel or conventional PKCs and Raf-MEK-ERK during acute GnRH3-stimulated GH release. However in addition to PKCs, class I PI3Ks are also known to be able to activate Raf-MEK-ERK signalling through recruitment of the PtdIns(3,4,5)P_3-sensitive Rac-GEF P-Rex-1 (PtdIns(3,4,5)P_3-dependent Rac exchange factor 1) in breast cancer cells (Welch et al., 2002; Ebi et al., 2013; Dillon et al., 2014). Whether agonist-selective integration of PI3Ks with Raf-MEK-ERK signalling, and PtdIns(3,4,5)P_3-dependent activation through P-Rex1 in particular, occurs in goldfish pituitary cells under the influence of GnRH remains to be investigated.

Outside of acting upstream or downstream of one another, several lines of evidence suggest that cross-talk between PI3K-dependent signalling and the Raf-MEK-ERK pathway may contribute to GnRH selectivity, particularly in somatotropes. Inhibition of Akt-dependent signalling selectively enhances GnRH3-, but not GnRH2-stimulated GH release responses (Chapter 5). Akt inhibits the activation of MEK1/2 and ERK1/2 by phosphorylating inhibitory sites in the N-termius of the upstream serine/threonine Raf kinases (Zimmerman and Moelling, 1999; Rommel et al., 1999; Guan et al., 2000). Intriguingly, the level of cross-talk between Akt- and Raf-dependent transduction can be dynamically regulated by the agonist-type as well as the intracellular signal intensity and duration to selectively control cellular responses (Moelling et al., 2002). Alternatively, PKCs activate Raf by direct phosphorylation (Kolch et al., 1993; Marias et al., 1998), suggesting that the PKC-mediated activation of Raf-MEK-ERK signalling may be enhanced by selective inhibition of Akt and the removal of inhibitory signals to Raf. The convergence of PKC- and Akt-dependent signalling on Raf activation

might help to explain the selective role played by Akt in the enhancement of GnRH3-stimulated acute GH release from goldfish somatotropes observed in Chapter 5. However, in goldfish gonadotropes, these signalling interactions are unlikely to control the acute actions of either GnRH considering that GnRH-stimulated hormone release was insensitive to Akt inhibition, and the LH release responses stimulated by PKC activators were not altered by inhibition of MEK1/2. Though speculative, the integrated control of Raf-mediated signalling likely represents an important signal transduction node that may contribute to GnRH-selective actions, or to GnRH interactions with other neuroendocrine factors, in goldfish gonadotropes and somatotropes.

7.3.5 Summary

Results presented in this Chapter indicate that although PI3K-dependent signalling, including downstream components PDK1 and Akt, as well as Raf-MEK-ERK are constitutively active in goldfish pituitary cells, PI3Ks are not a major regulator of basal Raf-MEK-ERK activity in unstimulated goldfish pituitary cells. Just as importantly, this is the first study to examine the involvement of MEK1/2-dependent signalling in GnRH-induced GH secretion in any species; as well as one of a few studies to examine the time course of GnRH effects on, and possible role of Raf-MEK-ERK in, the regulation of total LH and GH protein availability. The discontinuities observed between the temporal changes in hormone mRNA and protein levels following GnRH treatment and/or MEK1/2 inhibition indicates that GH and LH gene expression and protein synthesis are uncoupled in goldfish, a phenomenon that has been shown in other systems (Nguyen et al., 2004; Dobkin-Bekman et al., 2006). Results also indicate that Raf-MEK-

ERK signalling participates in the regulation of basal GH, but not basal LH, protein availability, as well as differentially mediates GnRH2-, GnRH3-, and PKC-mediated stimulation of LH and GH release. Interestingly, while GnRH2 and GnRH3 are both effective in increasing GTHα, LHβ, and GH mRNA expression, these GnRHs only elevate total LH availability and are generally ineffective at increasing GH protein availability; suggesting that while GnRH can increase both LH gene transcription and translation, they selectively elevate GH gene transcription but not necessarily translation. By implication, neuroendocrine regulators other than GnRH likely play an important stimulatory role in the production of GH protein. Overall, the present results clearly demonstrate that temporal differences in the recruitment of Raf-MEK-ERK signalling regulate hormone release and hormone availability/production in a time-, pituitary cell type-, and GnRH-isoform-specific manner.

Table 7.1 Summary of time-dependent GnRH- and U0126-mediated effects on goldfish pituitary LH release, cell content, and total availability in comparison to GnRH-stimulated changes in GTH subunit mRNA expression previously shown in Klausen et al., 2002[1].

Treatment	Duration (hrs)	LH release	LH cell content	Total LH availability	GTHα mRNA[1]	LHβ mRNA[1]
U0126	**2**	↔	↔	↔	NT	NT
	6	↔	↔	↔	NT	NT
	12	↔	↔	↔	NT	NT
	24	↔	↔	↔	NT	NT
GnRH2	**2**	↔	↔ #	↔ #	NT	NT
	6	↑ °	↔	↑ °	↔	↔
	12	↔	↔	↔	↑ •	↑ •
	24	↑ °	↔	↔	↔	↔
GnRH3	**2**	↔	↔ #	↔ #	NT	NT
	6	↑	↔	↔	↓	↓
	12	↑ °	↑ °	↑ °	↑ •	↑ •
	24	↑ °	↔	↔	↔	↓

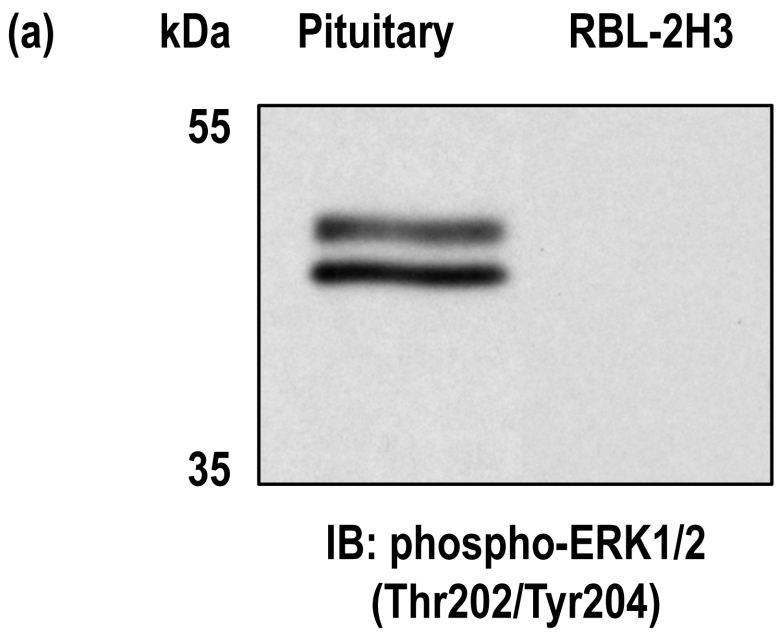
↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control; *, GnRH effect significantly enhanced by U0126; °, GnRH effect significantly attenuated by U0126; #, response significantly greater than untreated controls when in the presence of U0126; NT, not tested; • significantly reduced by another MEK1/2 inhibitor PD98059.

Table 7.2 Summary of time-dependent GnRH- and U0126-mediated effects on goldfish pituitary GH release, cell content, and total availability in comparison to GnRH-stimulated changes in GH mRNA expression previously shown in Klausen et al., 2002[1].

Treatment	Duration (hrs)	GH release	GH cell content	Total GH availability	GH mRNA[1]
U0126	**2**	↑	↓	↓	NT
	6	↔	↓	↓	NT
	12	↑	↔	↔	NT
	24	↔	↓	↓	NT
GnRH2	**2**	↑ *	↓	↓	NT
	6	↑	↓	↔	↔
	12	↔ #	↔	↔	↑ •
	24	↑ °	↓	↔	↔
GnRH3	**2**	↔ #	↓	↓	NT
	6	↑ °	↓	↔	↔
	12	↑ °	↓	↔	↑ •
	24	↑ °	↓	↔	↔

↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control; *, GnRH effect significantly enhanced by U0126; °, GnRH effect significantly attenuated by U0126; #, response significantly greater then untreated controls when in the presence of U0126; NT, not tested; • significantly reduced by another MEK1/2 inhibitor PD98059.

Figure 7.1 Expression of phosphorylated (Thr202/Tyr204) and total ERK1/2 in primary goldfish pituitary cell cultures under unstimulated conditions following 2 hrs in static culture. Representative immunoblots (from one of three independent experiments) of whole cell lysates isolated from mixed goldfish pituitary cell cultures using primary antibodies recognizing (a) ERK1 and ERK2 phosphorylated at two conserved residues in the activation segment (Thr202 and Tyr204), and (b) the same membrane probed with an ERK1/2 antibody (expected molecular mass of 42 and 44 kDa). Positive controls for immunoblotting were performed using whole cell lysates from the RBL-2H3 rat basophilic leukaemia mast cell line. Importantly, the RBL-2H3 cell line is known to express low (undetectable) levels of phosphorylated ERK1/2 (Thr202/Tyr204) under unstimulated conditions.


(a)
kDa
Pituitary
RBL-2H3
55
35
IB: phospho-ERK1/2
(Thr202/Tyr204)

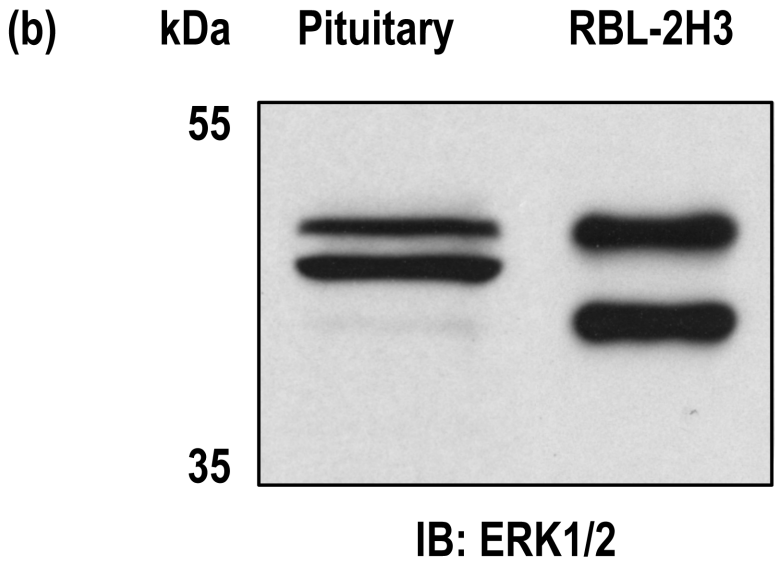
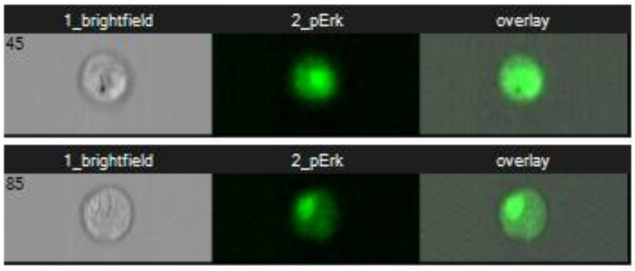

(b)
kDa
Pituitary
RBL-2H3
55
35
IB: ERK1/2

Figure 7.2 Expression of phosphorylated (Thr202/Tyr204) and total ERK1/2 in morphologically identified pituitary gonadotropes using imaging flow cytometry. (a) Individual goldfish gonadotropes were identified based on their unique morphological characteristics (Van Goor et al., 1994) and their size as well as scatter properties. (Chang et al., 2014). Representative bright field images and the relative phospho-ERK1/2 (Thr202/Tyr204) staining (green colour; channel 2) are shown. Fluorescently conjugated phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™; Alexa Fluor® 488 Conjugate; 1:50) primary antibodies were used. (b) Pre-absorption of the phospho-ERK1/2 (Thr202/ Tyr204) monoclonal antibody with the phospho-ERK1/2 (Thr202/Tyr204) blocking peptide (Cell Signaling Technologies) reduces immunostaining in morphologically identified goldfish pituitary gonadotropes. (c) Subsets of cells were also stained with a ERK1/2 antibody (1:100; green colour; channel 2) and the nuclear counter-stain Hoechst 33342 (red colour; channel 5). Representative images are shown. Primary pan-ERK1/2 antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200).

Morphologically Identified Pituitary Gonadotropes

(a) IF: phospho-ERK1/2 (Thr202/Tyr204)



− phospho-ERK1/2 (Thr202/Tyr204) peptide

(b) IF: phospho-ERK1/2 (Thr202/Tyr204)

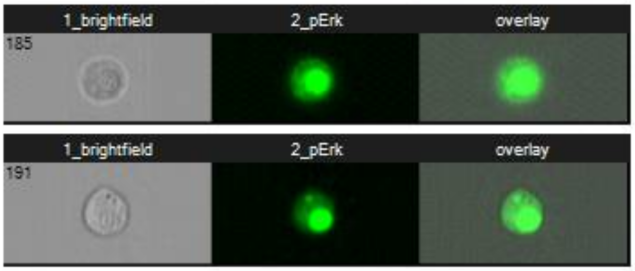
+ phospho-ERK1/2 (Thr202/Tyr204) peptide

(c) IF: ERK1/2

Figure 7.3 Expression of phosphorylated (Thr202/Tyr204) and total ERK1/2 in morphologically identified pituitary somatotropes using imaging flow cytometry. (a) Individual goldfish somatotropes were identified based on their unique morphological characteristics (Van Goor et al., 1994) and their size as well as scatter properties. (Chang et al., 2014). Representative bright field images and the relative phospho-ERK1/2 (Thr202/Tyr204) staining (green colour; channel 2) are shown. Fluorescently conjugated phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™; Alexa Fluor® 488 Conjugate; 1:50) primary antibodies were used. (b) Pre-absorption of the phospho-ERK1/2 (Thr202/ Tyr204) monoclonal antibody with the phospho-ERK1/2 (Thr202/Tyr204) blocking peptide (Cell Signaling Technologies) reduces immunostaining in morphologically identified goldfish pituitary somatotropes. (c) Subsets of cells were also stained with a ERK1/2 antibody (1:100; green colour; channel 2) and the nuclear counter-stain Hoechst 33342 (red colour; channel 5). Representative images are shown. Primary pan-ERK1/2 antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200).

Morphologically Identified Pituitary Somatotropes

(a) IF: phospho-ERK1/2 (Thr202/Tyr204)



− phospho-ERK1/2 (Thr202/Tyr204) peptide

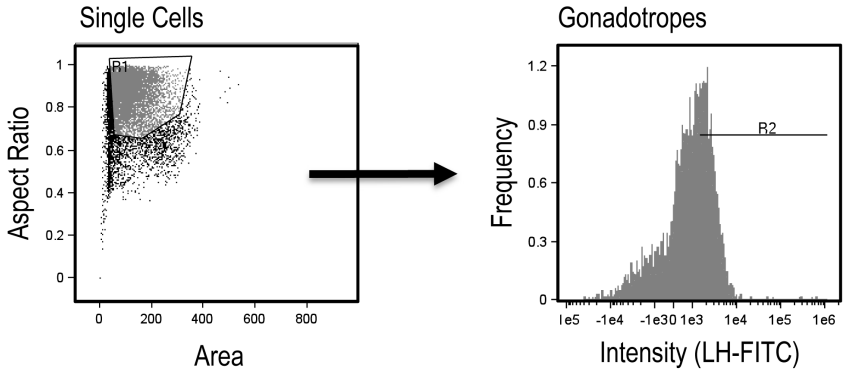
(b) IF: phospho-ERK1/2 (Thr202/Tyr204)

+ phospho-ERK1/2 (Thr202/Tyr204) peptide

(c) IF: ERK1/2

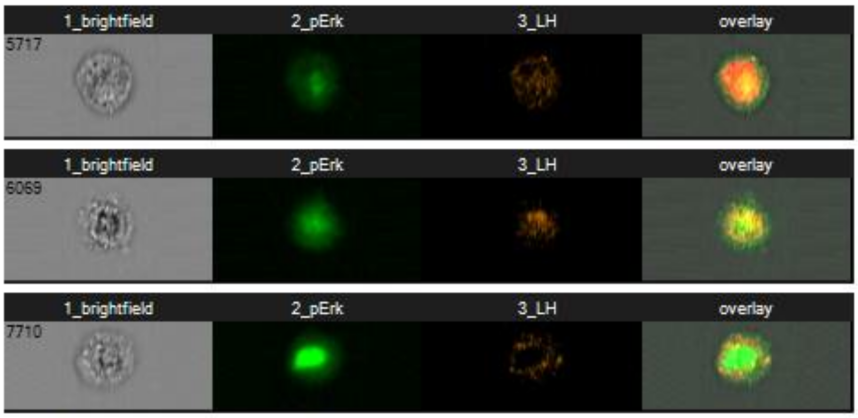
Figure 7.4 Double-labeling of phosphorylated ERK1/2 (Thr202/Tyr204) and luteinizing hormone (LH) within mixed pituitary cell populations using imaging flow cytometry. (a) Single cells were first identified based on their area, aspect ratio, and imaging focus (left) as well as the intensity of anti-LH staining (right); this population of LH-positive cells was used for all subsequent analyses. (b) Using the collected images, goldfish gonadotropes were identified by staining positively with LH antiserum (rabbit anti carp cGTH-378, 1:6000; Cook et al., 1991) and possessing morphological characteristics of identified gonadotropes (Van Goor et al., 1994). Primary LH antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to R-phycoerythrin (PE; 1:200), while phospho-ERK1/2 staining required fluorescently conjugated phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™; Alexa Fluor® 488 Conjugate; 1:50) primary antibodies. Representative bright field images and relative phospho-ERK1/2 (Thr202/ Tyr204; green colour; channel 2) and LH (orange colour; channel 3) co-staining are shown.

(a)



Pituitary Gonadotropes

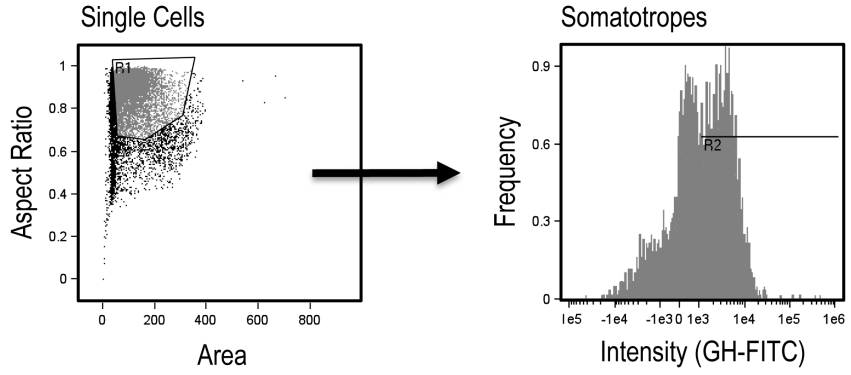
(b)



IF: phospho-ERK1/2 (Thr202/Tyr204) and LH

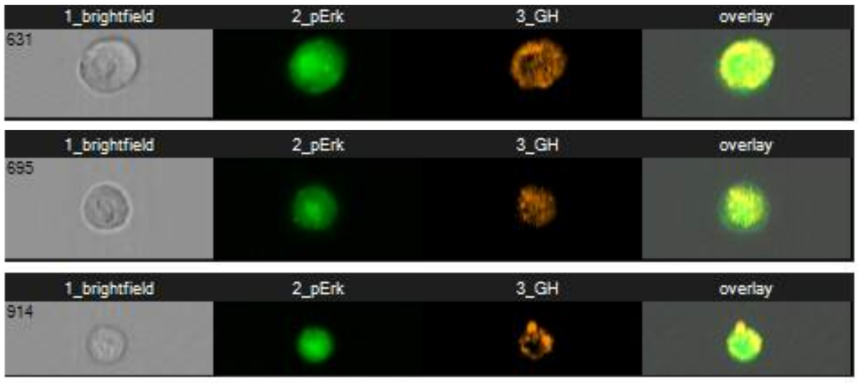
Figure 7.5 Double-labeling of phosphorylated ERK1/2 (Thr202/Tyr204) and growth hormone (GH) within mixed pituitary cell populations using imaging flow cytometry. (a) Single cells were first identified based on their area, aspect ratio, and imaging focus (left) as well as the intensity of anti-GH staining (right); this population of GH-positive cells was used for all subsequent analyses. (b) Using the collected images, goldfish somatotropes were identified by staining positively with GH antiserum (rabbit anti-carp kcGH R#1 primary antibody, 1:3000; Cook et al., 1991) and possessing morphological characteristics of identified somatotropes (Van Goor et al., 1994). Primary GH antibody staining was visualized using goat anti-rabbit IgG secondary antibodies conjugated to R-phycoerythrin (PE; 1:200), while phospho-ERK1/2 staining required fluorescently conjugated phospho-ERK1/2 (Thr202/Tyr204; D13.14.4E XP™; Alexa Fluor® 488 Conjugate; 1:50) primary antibodies. Representative bright field images and relative phospho-ERK1/2 (Thr202/ Tyr204; green colour; channel 2) and GH (orange colour; channel 3) co-staining are shown.

(a)



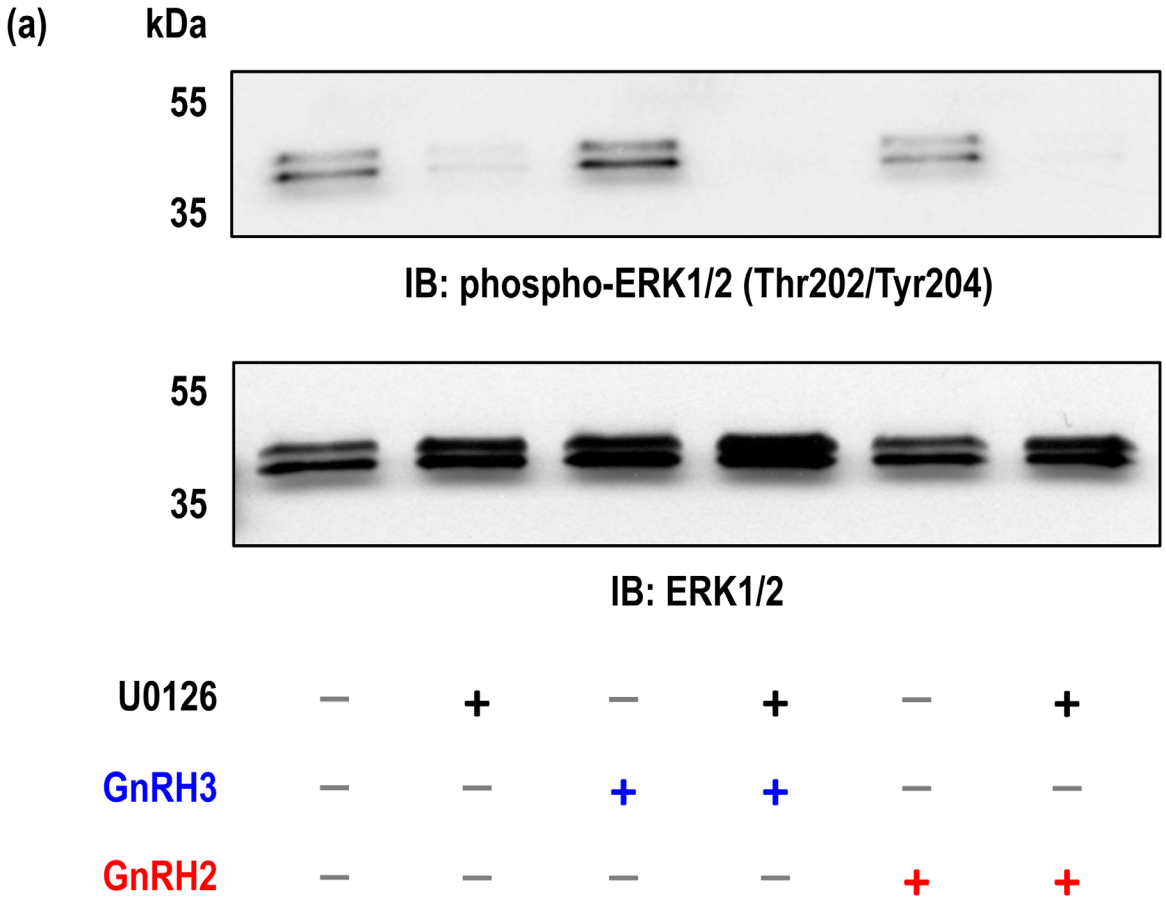
Pituitary Somatotropes

(b)



IF: phospho-ERK1/2 (Thr202/Tyr204) and GH

Figure 7.6 Effects of the selective MEK1/2 inhibitor U0126 (10 μM) on the expression of immunoreactive phosphorylated ERK1/2 (Thr202/Tyr204) in goldfish pituitary fragments following 2 hrs in static incubation under basal and GnRH2- or GnRH3-stimulated (100 nM) conditions. (a) Immunoblots of a goldfish pituitary tissue lysate probed with a primary antibody that recognizes ERK1 and ERK2 phosphorylated at two conserved residues in the activation segment (Thr202 and Tyr204), and the same membrane probed with an ERK1/2 antibody (expected molecular mass of 42 and 44 kDa). (b) Quantified ratios of phosphorylated ERK1/2 (Thr202/Tyr204) to total immunoreactive ERK1/2 in treatments groups (black, untreated; gray, U0126 alone; blue, GnRH3 alone; dark blue, GnRH3 + U0126; red, GnRH2 alone; dark red, GnRH2 + U0126), expressed as a percentage of unstimulated controls are presented using goldfish in April, a time of year when the gonads are at a sexually matured/pre-spawning stage.


(a)
kDa
55
35
IB: phospho-ERK1/2 (Thr202/Tyr204)
55
35
IB: ERK1/2
U0126 – + – + – +
GnRH3 – – + + – –
GnRH2 – – – – + +

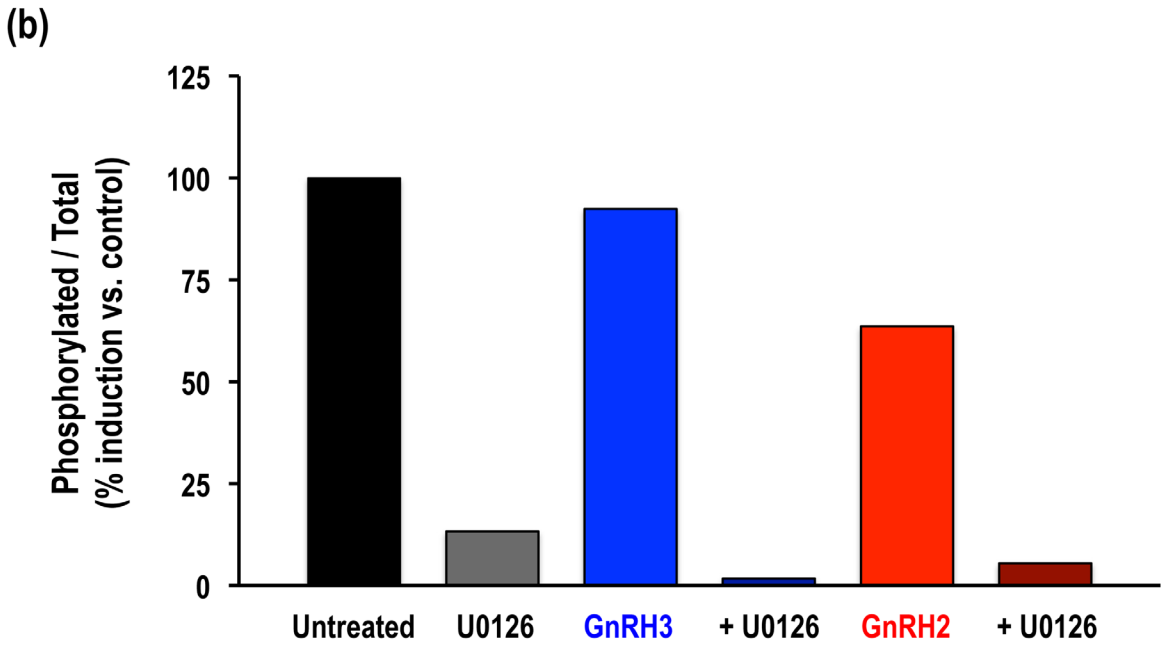

(b)
Phosphorylated / Total
(% induction vs. control)
125
100
75
50
25
0
Untreated
U0126
GnRH3
+ U0126
GnRH2
+ U0126

Figure 7.7 Effects of the selective MEK1/2 inhibitors U0126 (50 nM; a,b) and PD98059 (100 μM; c,d) on basal and GnRH2 (100 nM) induced GH release responses. For each panel, GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; red solid diamond, GnRH2 alone; open circle, GnRH2 + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; MEK1/2^{-} denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 50.4 ± 4.1 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish at early gonadal recrudescence (October to January). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the red horizontal bar represents the 5-min GnRH2 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

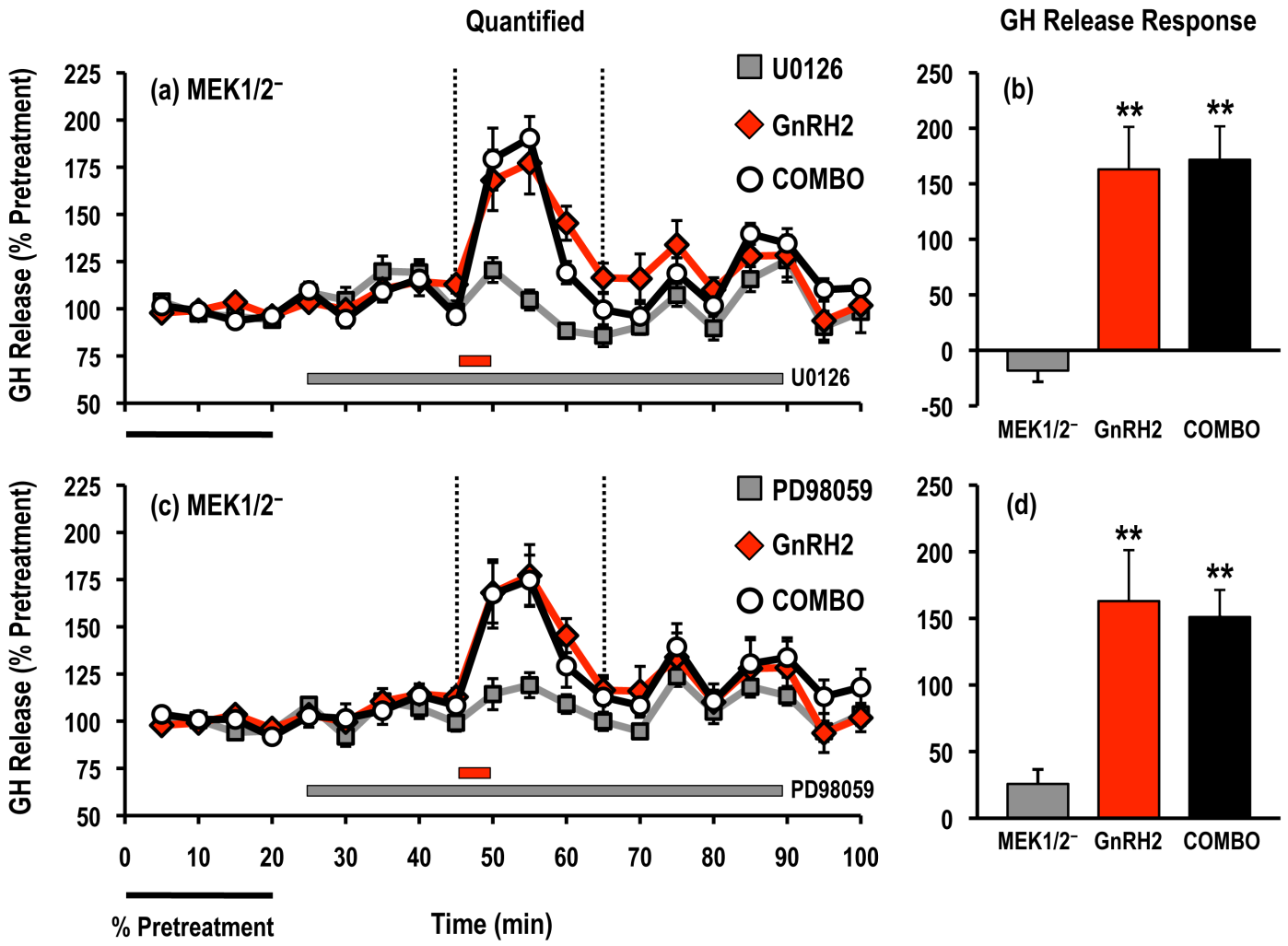

Quantified
GH Release Response
(a) MEK1/2⁻
U0126
GnRH2
COMBO
U0126
GH Release (% Pretreatment)
(b)
MEK1/2⁻ GnRH2 COMBO
(c) MEK1/2⁻
PD98059
GnRH2
COMBO
PD98059
(d)
% Pretreatment
Time (min)

Figure 7.8 Effects of the selective MEK1/2 inhibitors U0126 (50 nM; a,b) and PD98059 (100 μM; c,d) on basal and GnRH3 (100 nM) induced GH release responses. For each panel, GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; blue solid diamond, GnRH3 alone; open circle, GnRH3 + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; MEK1/2^{-} denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 50.4 ± 4.1 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish at early gonadal recrudescence (October to January). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the blue horizontal bar represents the 5-min GnRH3 (100 nM) exposure. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

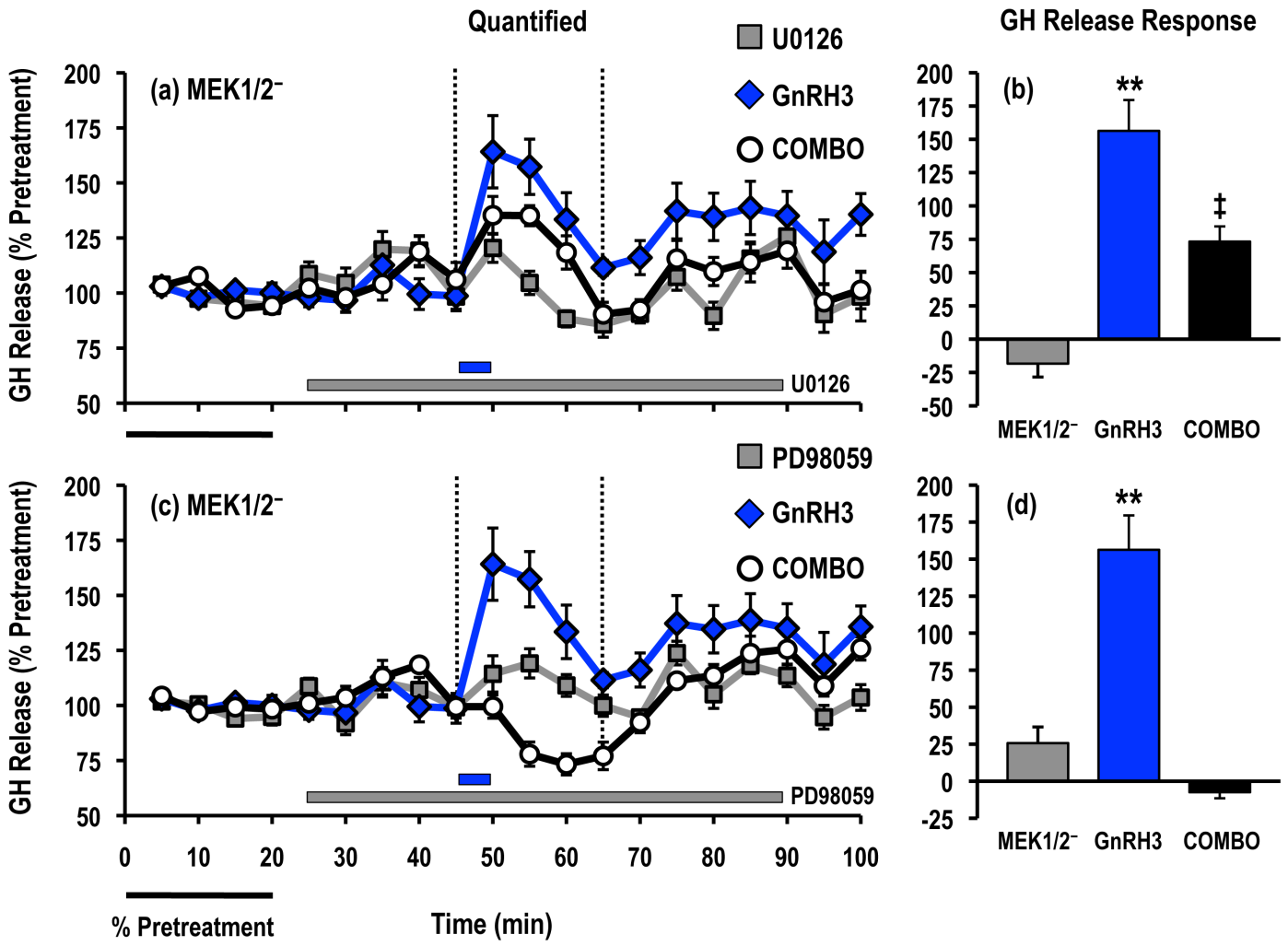

Quantified
GH Release Response
U0126
GnRH3
COMBO
PD98059
(a) MEK1/2⁻
(b)
(c) MEK1/2⁻
(d)
GH Release (% Pretreatment)
U0126
PD98059
MEK1/2⁻
GnRH3
COMBO
% Pretreatment
Time (min)

Figure 7.9 Effects of the selective MEK1/2 inhibitor U0126 (10 μM) on LH release responses stimulated by the novel and conventional PKC activators TPA (100 nM; a,b) and DiC8 (100 μM; c,d). LH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; orange solid diamond, PKC activator alone; open circle, PKC activator + inhibitor) and quantified net LH responses are shown on the right (b,d; mean ± SEM; MEK1/2^{-} denotes inhibitor alone treatment). Duration of LH release response quantification is indicated by the vertical dotted lines. LH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 3.20 ± 0.10 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with gonads at mid- to late recrudescence (February to March). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the orange horizontal bar represents the 5-min exposure to either PKC activator. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

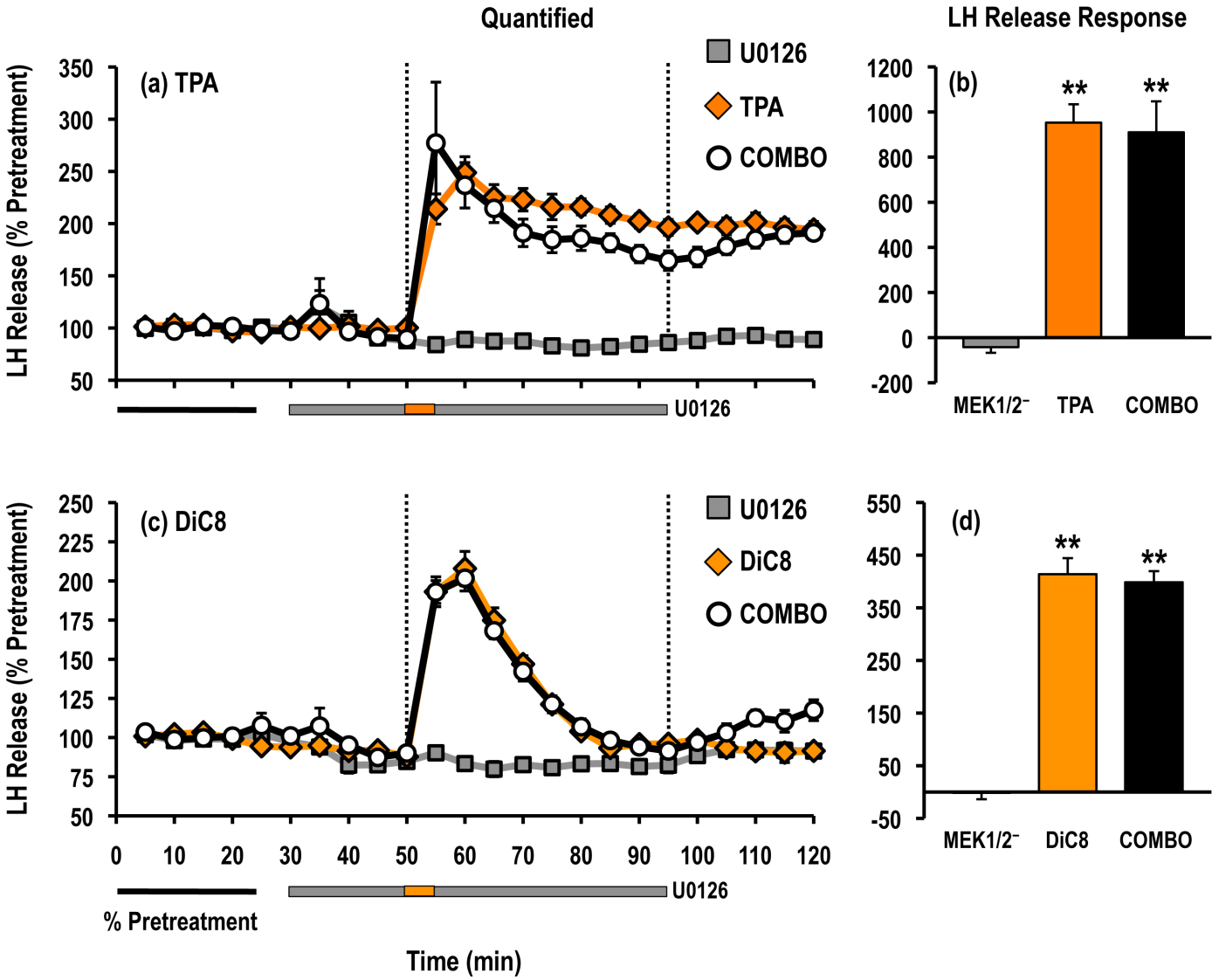

Quantified
LH Release Response
(a) TPA
U0126
TPA
COMBO
LH Release (% Pretreatment)
350
300
250
200
150
100
50
U0126
(b)
**
**
1200
1000
800
600
400
200
0
-200
MEK1/2⁻
TPA
COMBO
(c) DiC8
U0126
DiC8
COMBO
LH Release (% Pretreatment)
250
225
200
175
150
125
100
75
50
0
10
20
30
40
50
60
70
80
90
100
110
120
U0126
% Pretreatment
Time (min)
(d)
**
**
550
450
350
250
150
50
-50
MEK1/2⁻
DiC8
COMBO

Figure 7.10 Effects of the selective MEK1/2 inhibitor U0126 (10 μM) on GH release responses stimulated by the novel and conventional PKC activators TPA (100 nM; a,b) and DiC8 (100 μM; c,d). GH release profiles are shown on the left panel (a,c; gray solid square, inhibitor alone; orange solid diamond, PKC activator alone; open circle, PKC activator + inhibitor) and quantified net GH responses are shown on the right (b,d; mean ± SEM; MEK1/2^{-} denotes inhibitor alone treatment). Duration of GH release response quantification is indicated by the vertical dotted lines. GH release responses were expressed as a percentage of the pretreatment values (% pretreatment, average of the first five perifusion fractions collected; black horizontal bar; 7.50 ± 0.50 ng/ml). Pooled responses are shown from four experiments (n = 8) using individual cell preparations from goldfish with gonads at mid- to late recrudescence (February to March). The solid grey horizontal bar indicates the duration of the inhibitor treatment, whereas the orange horizontal bar represents the 5-min exposure to either PKC activator. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

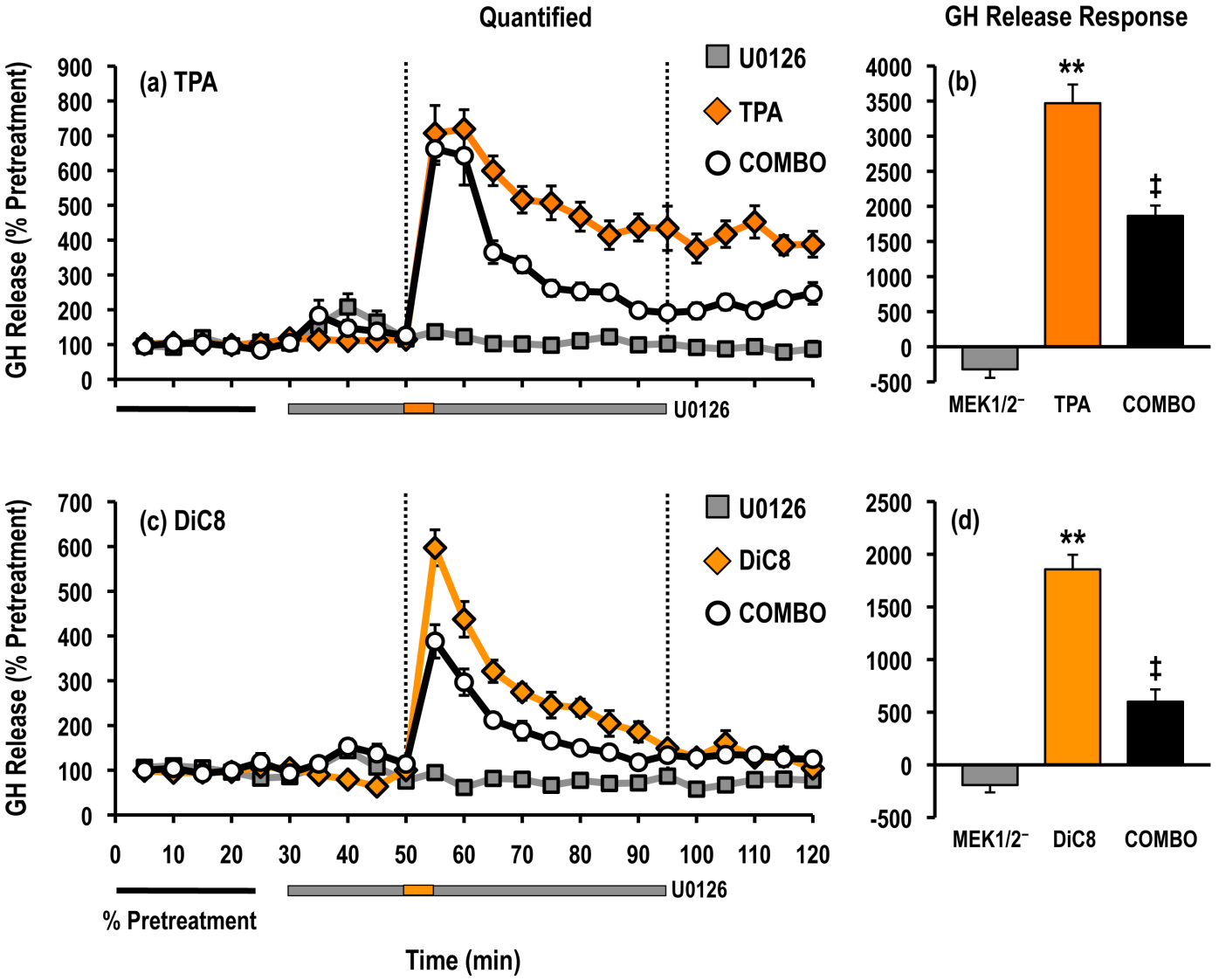

Quantified
GH Release Response
(a) TPA
(b)
(c) DiC8
(d)
U0126
TPA
DiC8
COMBO
GH Release (% Pretreatment)
% Pretreatment
Time (min)
MEK1/2⁻
**
‡

Figure 7.11 Effects of the selective MEK1/2 inhibitor U0126 (MEK1/2^{-}; 10 μM) on basal (a) LH and (b) GH release responses. Hormone release profiles are shown on the left panel and quantified responses are shown on the right (mean ± SEM). Basal release prior to inhibitor application (black bars) was quantified as the average percentage of the pretreatment values (average % pretreatment; black horizontal bar) of the first four perifusion fractions collected at the beginning of the experimental trial. The inhibitor alone basal release (gold bars) was quantified as the average % pretreatment value over the entire duration of U0126 treatment (30-95 min; the grey horizontal bar; $n = 16$, from eight independent cell preparations). Treatments marked with astericks (**) identify responses that are significantly different from the basal release prior to inhibitor application (paired Student's t-test; $p < 0.05$).

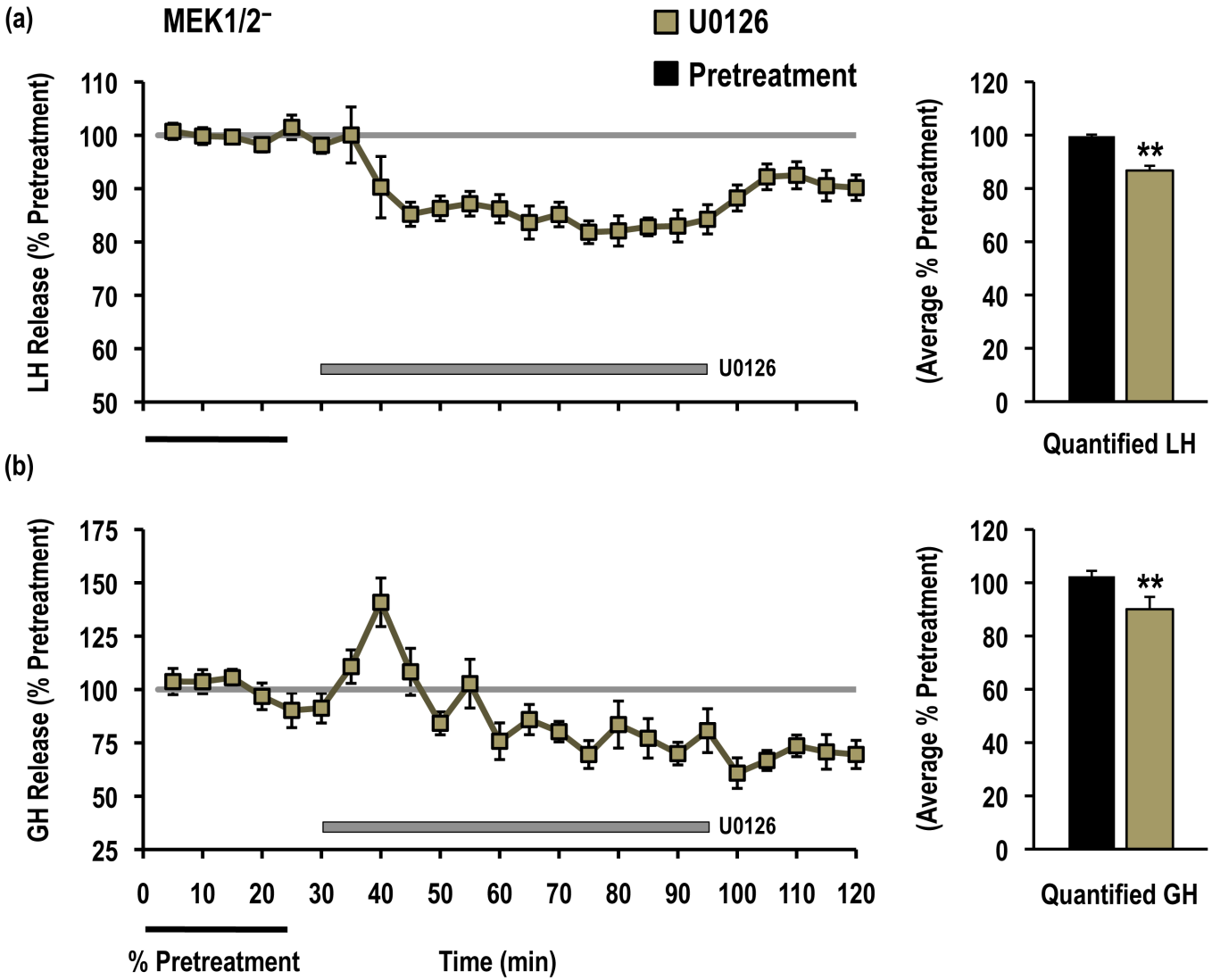

(a)
MEK1/2⁻
U0126
Pretreatment
LH Release (% Pretreatment)
110
100
90
80
70
60
50
U0126
(Average % Pretreatment)
120
100
80
60
40
20
0
**
Quantified LH
(b)
GH Release (% Pretreatment)
175
150
125
100
75
50
25
U0126
0
10
20
30
40
50
60
70
80
90
100
110
120
% Pretreatment
Time (min)
(Average % Pretreatment)
120
100
80
60
40
20
0
**
Quantified GH

Figure 7.12 Effects of the selective MEK1/2 inhibitor U0126 (10 μM) on basal, GnRH2-, and GnRH3-stimulated LH release and total availability over 2, 6, 12 and 24 hrs in static incubation. Treatment groups are identified by consecutive Arabic numerals, respectively: (1; black) untreated, (2; gray) U0126 (10 μM) alone, (3; blue) GnRH3 (100 nM) alone, (4; dark blue) GnRH3 + U0126, (5; red) GnRH2 (100 nM) alone, and (6; dark red) GnRH2 + U0126. Pooled results are shown with each treatment having been replicated three times using independently prepared dispersed pituitary cells (4×10^6 cells/ treatment) from goldfish at early gonadal recrudescence (October to December) and are expressed as the total μg LH present within the sample (mean ± SEM, n = 3). The amount of LH released into the media during treatment (LH release) and the cellular LH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay and expressed as the total μg LH present within the sample. The sum of the LH release and cellular LH content values in each matched treatment sample was calculated as an index of total LH available (total LH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Different letters of the alphabet identify treatment responses that are significantly different from one another (ANOVA followed by Fisher's LSD multiple comparisons where applicable; $P < 0.05$).

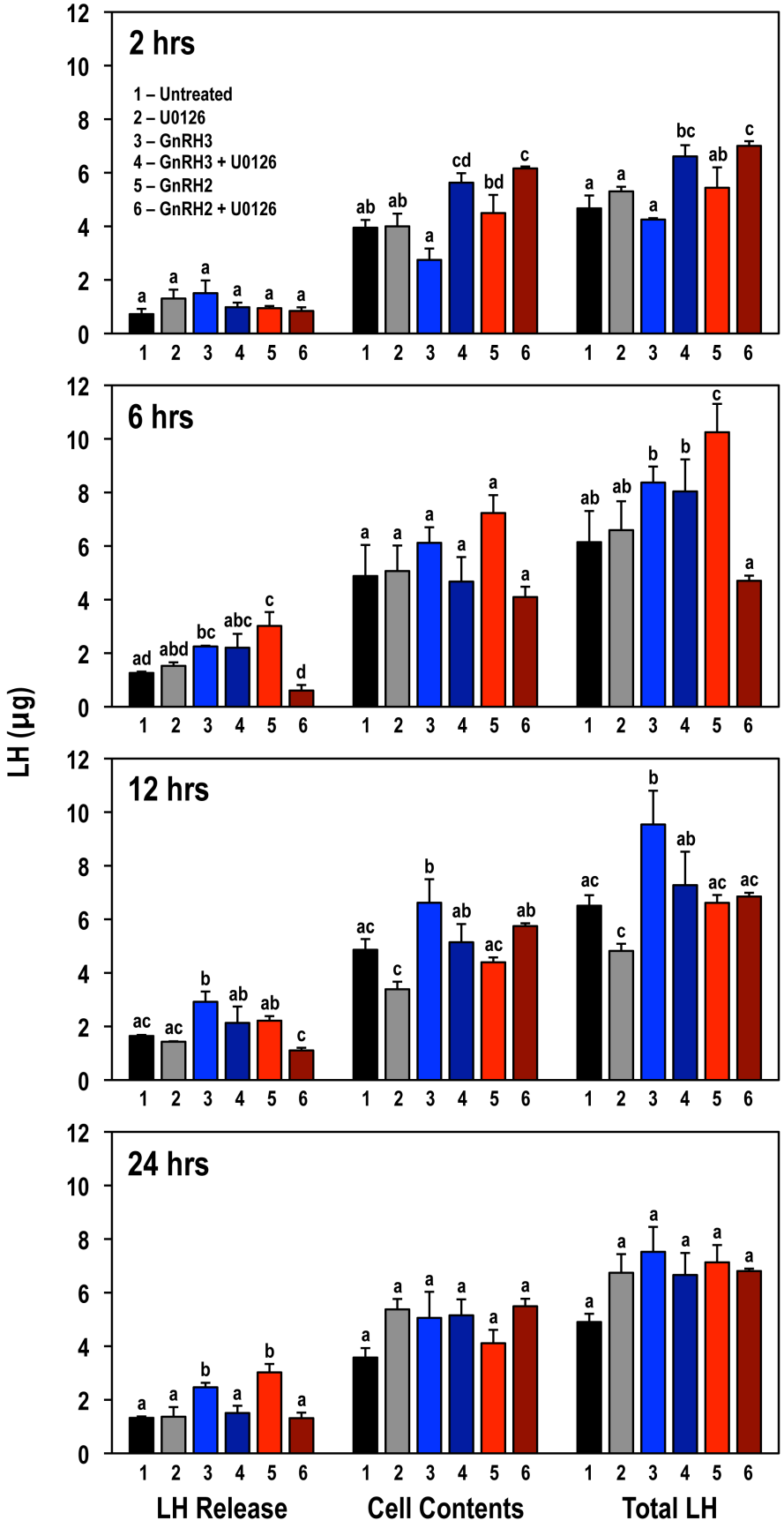

2 hrs
1 – Untreated
2 – U0126
3 – GnRH3
4 – GnRH3 + U0126
5 – GnRH2
6 – GnRH2 + U0126
6 hrs
12 hrs
24 hrs
LH (µg)
LH Release
Cell Contents
Total LH

Figure 7.13 Effects of the selective MEK1/2 inhibitor U0126 (10 μM) on basal, GnRH2-, and GnRH3-stimulated GH release and total availability over 2, 6, 12 and 24 hrs in static incubation. Treatment groups are identified by consecutive Arabic numerals, respectively: (1; black) untreated, (2; gray) U0126 (10 μM) alone, (3; blue) GnRH3 (100 nM) alone, (4; dark blue) GnRH3 + U0126, (5; red) GnRH2 (100 nM) alone, and (6; dark red) GnRH2 + U0126. Pooled results are shown with each treatment having been replicated three times using independently prepared dispersed pituitary cells (4×10^6 cells/ treatment) from goldfish at early gonadal recrudescence (October to December) and are expressed as the total μg GH present within the sample (mean ± SEM, n = 3). The amount of GH released into the media during treatment (GH release) and the cellular GH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay and expressed as the total μg GH present within the sample. The sum of the GH release and cellular GH content values in each matched treatment sample was calculated as an index of total GH available (total GH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Different letters of the alphabet identify treatment responses that are significantly different from one another (ANOVA followed by Fisher's LSD multiple comparisons where applicable; $P < 0.05$).

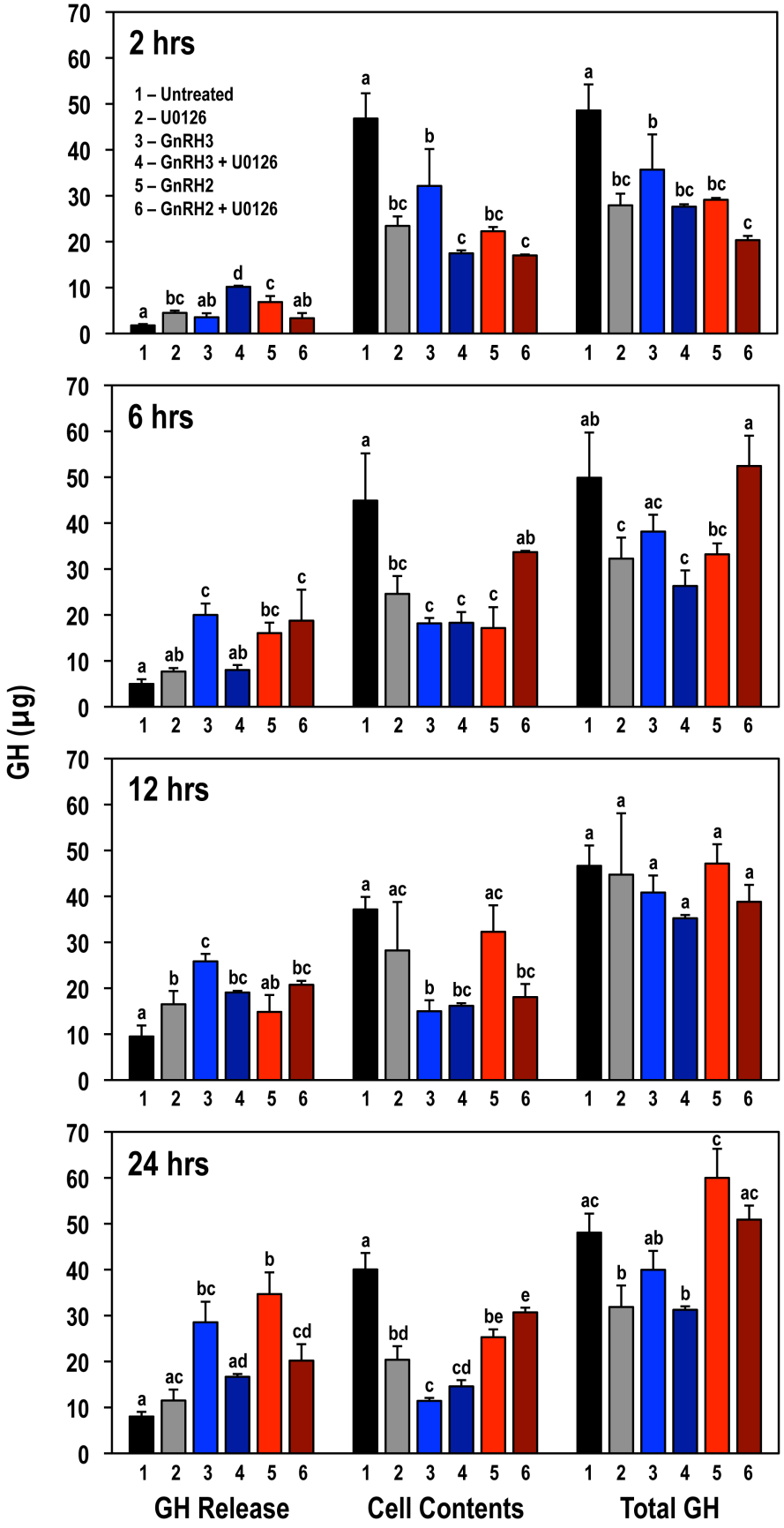

2 hrs
1 – Untreated
2 – U0126
3 – GnRH3
4 – GnRH3 + U0126
5 – GnRH2
6 – GnRH2 + U0126
6 hrs
12 hrs
24 hrs
GH (µg)
GH Release
Cell Contents
Total GH

Figure 7.14 Effects of the pan-PI3K inhibitor LY294002 (PI3K^{-}; 10 μM) as well as isoform-selective inhibitors of p110α (BYL719; p110α^{-}; 0.05 μM), p110β (TGX221; p110β^{-}; 0.05 μM), p110δ (IC87114; p110δ^{-}; 5 μM), and p110γ (CZC24832; p110γ^{-}; 0.5 μM) on the expression of phosphorylated ERK1/2 (Thr202/Tyr204) in dispersed goldfish pituitary cells following 2 hrs in static incubation. (a) Representative immunoblots of a goldfish pituitary lysate probed with a primary antibody that recognizes ERK1 and ERK2 phosphorylated at two conserved residues in the activation segment (Thr202 and Tyr204), and the same membrane probed with an ERK1/2 antibody (expected molecular mass of 42 and 44 kDa). (b) Quantified ratios of phosphorylated ERK1/2 (Thr202/Tyr204) to total immunoreactive ERK1/2 in treatments groups (black, untreated; gray, LY294002 alone; gold, isoform-selective p110 inhibitors) expressed as a percentage of unstimulated controls are presented (mean ± SEM, $n = 3$; with goldfish in April and May, a time of year when the gonads are at a sexually matured/pre-spawning stage). Treatments that are significantly different from one another are identified by different symbols (**; ANOVA followed by Fisher's LSD multiple comparisons where applicable; $p < 0.05$). For information about the specificity of the pan-PI3K and class I PI3K isoform-selective inhibitors, refer to Tables 3.1 and 4.1, respectively.

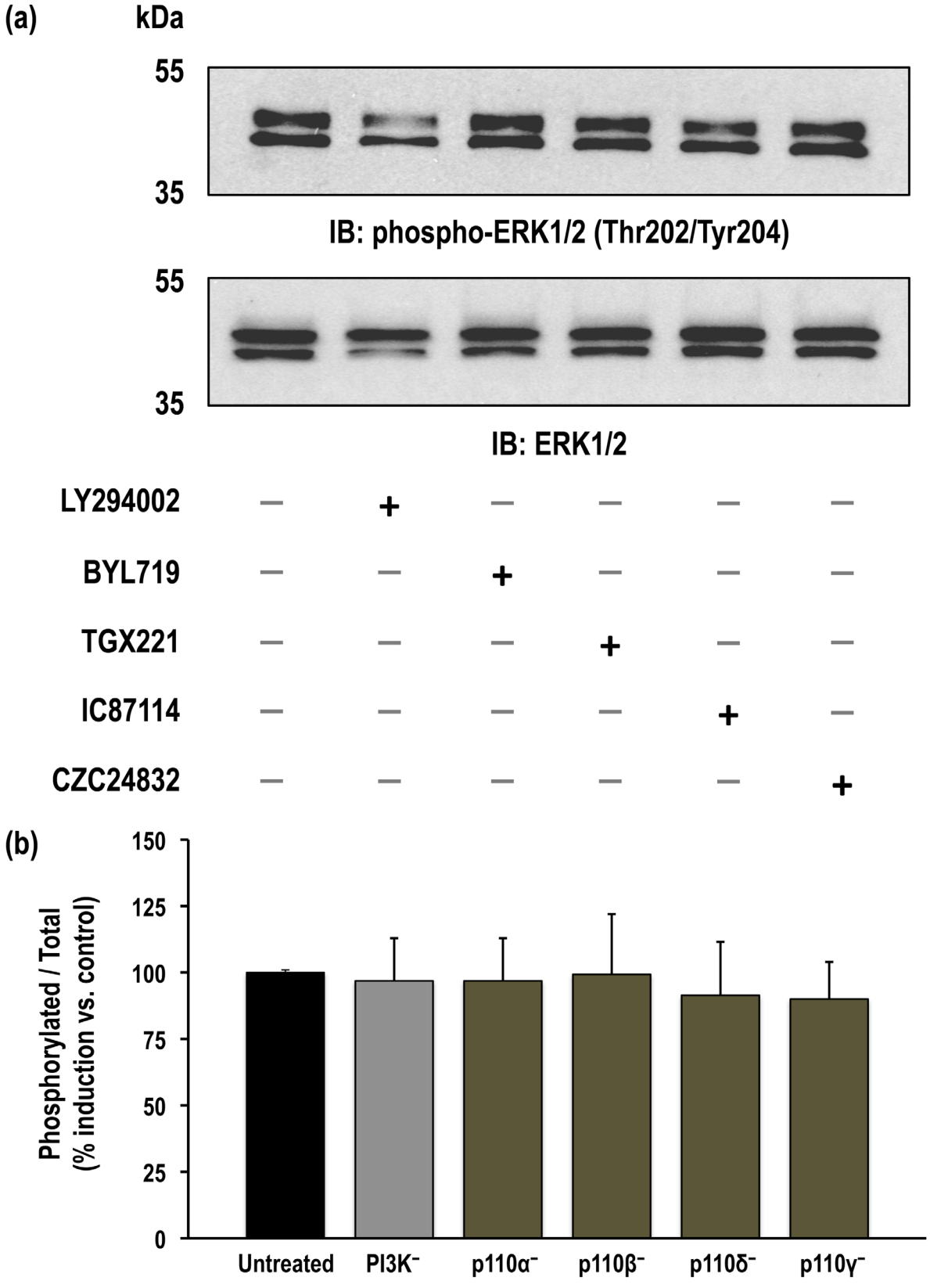

(a)
kDa
55
35
IB: phospho-ERK1/2 (Thr202/Tyr204)
55
35
IB: ERK1/2
LY294002 – + – – – –
BYL719 – – + – – –
TGX221 – – – + – –
IC87114 – – – – + –
CZC24832 – – – – – +
(b)
Phosphorylated / Total
(% induction vs. control)
150
125
100
75
50
25
0
Untreated
PI3K⁻
p110α⁻
p110β⁻
p110δ⁻
p110γ⁻

Figure 7.15 Summary model depicting the integration of Raf-MEK-ERK with PKC- and PI3K-dependent signalling during GnRH-stimulated LH release from goldfish gonadotropes. Results presented in this Chapter suggest that Raf-MEK-ERK signalling is involved in mediating acute GnRH2- and GnRH3-stimulated LH release responses. Activation of conventional or novel PKC isoforms is not upstream of Raf-MEK-ERK signalling in goldfish gonadotropes during LH release responses and Raf-MEK-ERK actions on LHβ and GTHα subunit expression (LH synthesis) are independent of PKC-dependent signalling. At this time, we cannot exclude the possibility that GnRHR-mediated activation of PI3Ks may also lead to intracellular events that integrate with Raf-MEK-ERK signalling. In fact, similar to the acute involvement of Raf-MEK-ERK, data from Chapter 3 strongly suggests that PI3K-dependent signalling is also either upstream or independent of PKC activation in goldfish gonadotropes during acute LH release.

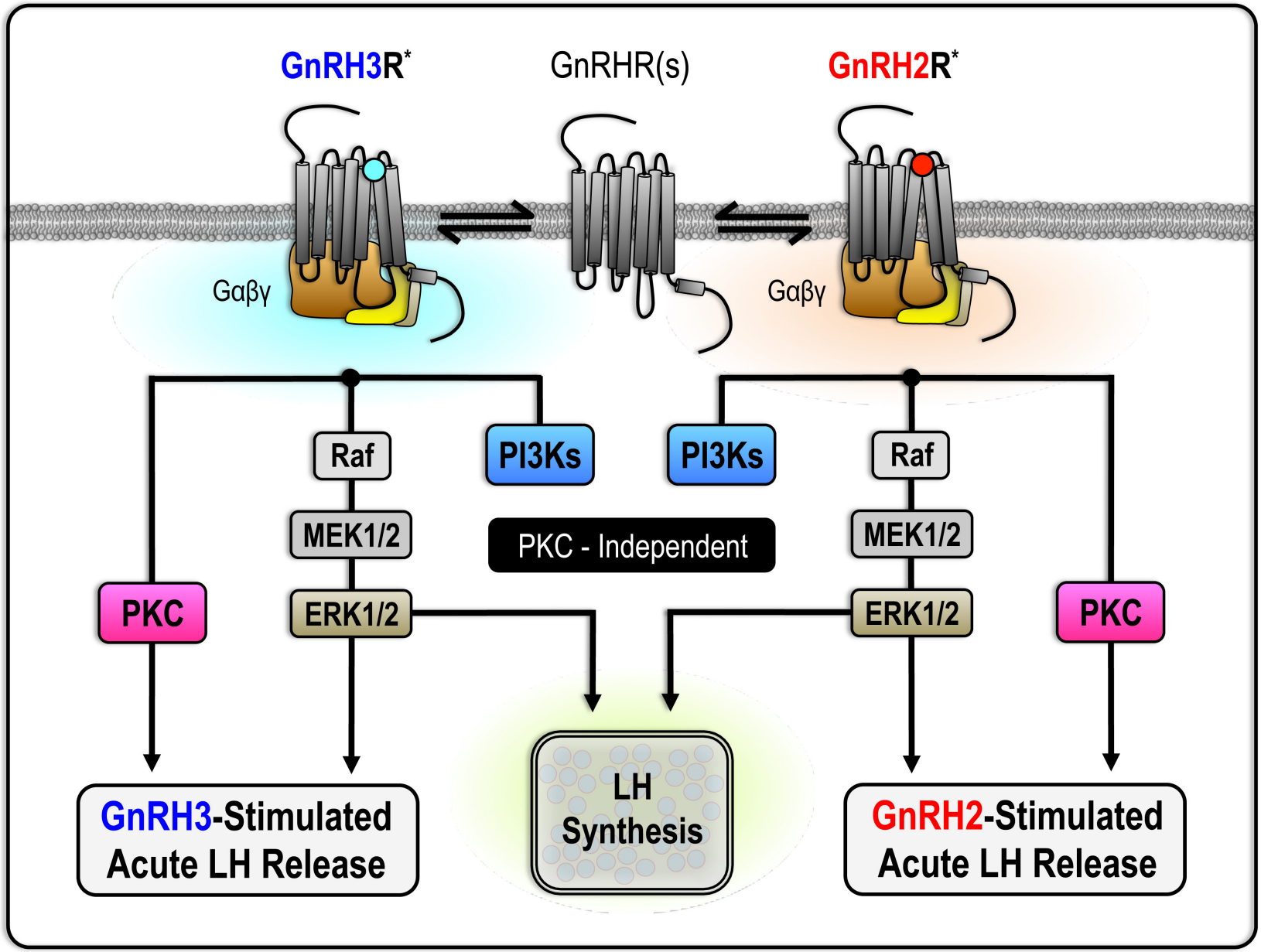
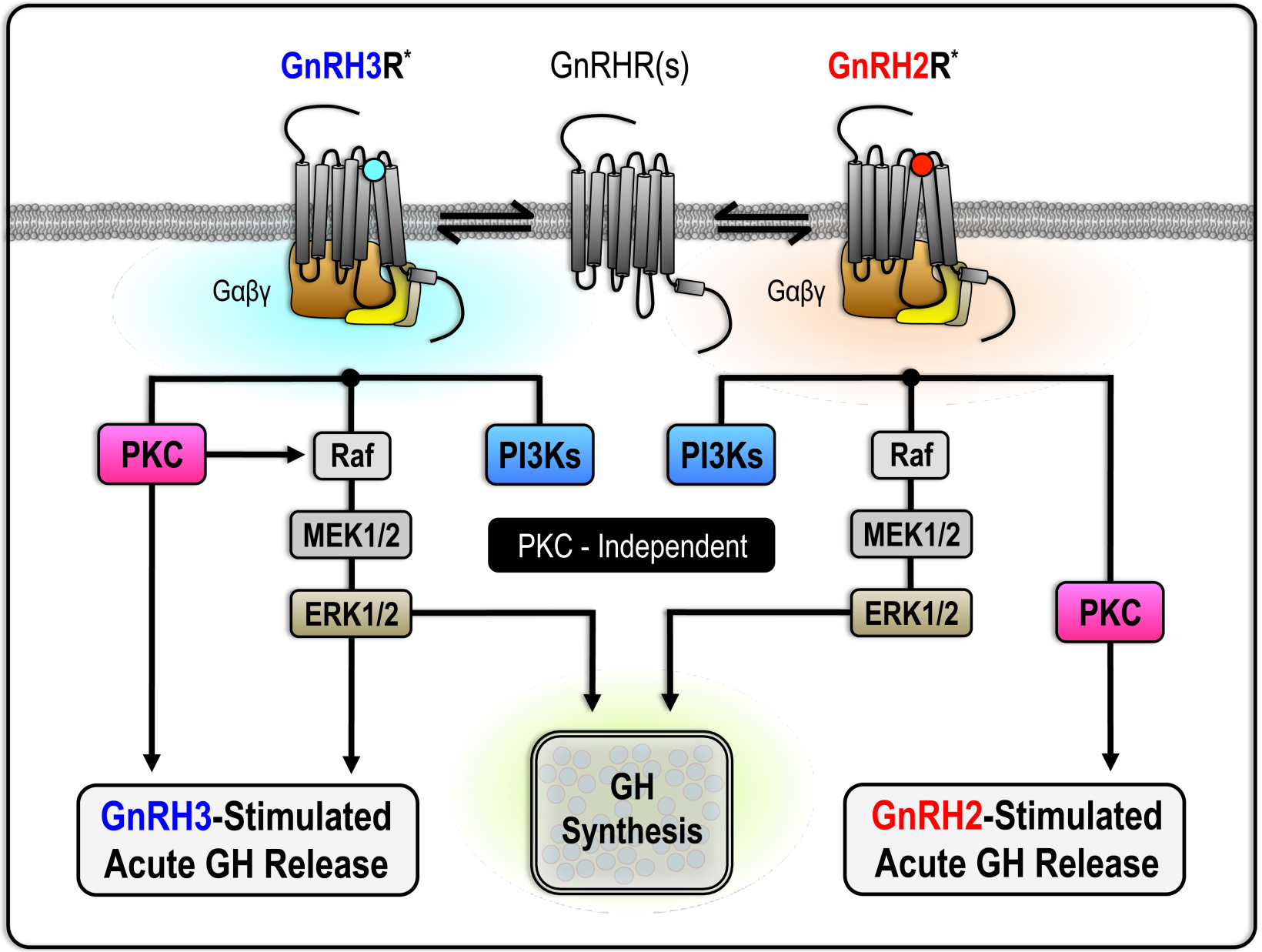
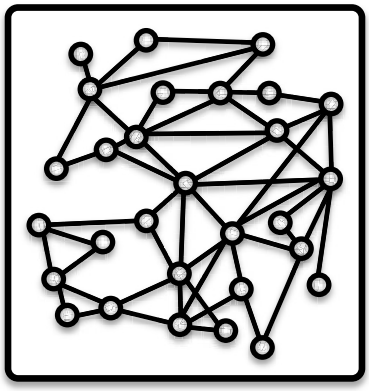

GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
Raf
PI3Ks
PI3Ks
Raf
MEK1/2
PKC - Independent
MEK1/2
PKC
ERK1/2
ERK1/2
PKC
LH
Synthesis
GnRH3-Stimulated
Acute LH Release
GnRH2-Stimulated
Acute LH Release

Figure 7.16 Summary model depicting the integration of Raf-MEK-ERK with PKC- and PI3K-dependent signalling during GnRH-stimulated GH release from goldfish somatotropes. Results presented in this Chapter suggest that Raf-MEK-ERK signalling is selectively involved in mediating acute GnRH3-stimulated GH release responses. Activation of Raf-MEK-ERK signalling also occurs downstream of conventional or novel PKC isoforms during GH release responses; however, GnRH2- and GnRH3-stimulated increases in GH subunit expression (GH synthesis) are mediated by Raf-MEK-ERK, but are also independent of PKC activation. These data strongly suggests that the coordinate control of hormone release and synthesis in somatotropes must involve both PKC-dependent and PKC-independent activation of Raf-MEK-ERK. Furthermore, at this time, we cannot exclude the possibility that GnRHR-mediated activation of PI3Ks may also lead to intracellular events that integrate with Raf-MEK-ERK signalling. That said, unlike the acute involvement of Raf-MEK-ERK, data from Chapter 3 strongly suggests that PI3K-dependent signalling is exclusively either upstream or independent of PKC activation in goldfish somatotropes during acute GH release.


GnRH3R*
GnRHR(s)
GnRH2R*
Gαβγ
Gαβγ
PKC
Raf
PI3Ks
PI3Ks
Raf
MEK1/2
PKC - Independent
MEK1/2
ERK1/2
ERK1/2
PKC
GH
Synthesis
GnRH3-Stimulated
Acute GH Release
GnRH2-Stimulated
Acute GH Release

Chapter Eight:

Involvement of the PI3K-PDK1-Akt-TORC signalling cascade during the long-term control of basal hormone secretion and cellular content in gonadotropes and somatotropes



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

8.1 Introduction

Results from Chapters 3, 4, and 5 indicate that PI3Ks, and class I PI3K isoforms in particular, as well as distinct downstream signalling elements, differentially mediate GnRH2 and GnRH3 stimulation of acute LH and GH release from goldfish pituitary cells in column perifusion. Adding to these results on acute LH and GH release, findings in Chapter 6 demonstrate that PI3K catalytic activity, in general, differentially regulates long-term basal as well as sustained GnRH2- and GnRH3-stimulated hormone release, as well as hormone availability, in a time-, cell type-, and GnRH-selective manner. However, whether the long-term regulation of goldfish gonadotrope and somatotrope functions specifically requires the activation of class I PI3Ks and how the canonical class I PI3K downstream effectors may participate in the chronic control of hormone release and production are not known.

As discussed in Chapters 1 and 5, common PtdIns(3,4,5)P_3-sensitive downstream effectors include PDK1 and Akt. Recruitment of both of these enzymes to the plasma membrane by PtdIns(3,4,5)P_3 allows PDK1 to activate Akt via phosphorylation of Thr308 (Alessi et al., 1997). Conversely, rapamycin-insensitive TORC2 also contributes to the activation of Akt by phosphorylating Ser473 (Sarbassov et al., 2005). Signalling downstream of Akt indirectly activates rapamycin-sensitive TORC1 by relieving the inhibitory influences of both the TSC complex and/or PRAS40 (reviewed in Chapter 1; Manning and Cantley, 2007). Interestingly, both TORC1 and TORC2 are well-characterized regulators of cellular protein synthesis (Shimobayashi and Hall, 2014). Thus, I also hypothesized that the PI3K-PDK1-Akt-TORC cascade also plays a role in

the regulation of hormone production and availability in goldfish gonadotropes and somatotropes.

In order to evaluate the signal transduction mechanisms that contribute to the long-term basal regulation of LH and GH release and production, I examined the effects of a pan-PI3K and a selective pan-class I PI3K inhibitor (LY294002 and GDC0941, respectively), as well as selective inhibitors of canonical PI3K-dependent signalling effectors (discussed in Chapter 4; Akt, Akt_i VIII; PDK1, GSK2334470; TORC1, rapamycin; TOR kinase activity within both TORC1 and TORC2, INK128) in 2 hr static incubation studies with goldfish pituitary cells. In addition, due to the uncoupling of hormone mRNA levels and cellular content observed following prolonged incubations with GnRHs (Chapters 6 and 7), I also evaluated the long-term effects of static incubation treatments with PDK1-, Akt-, TORC1- and TORC1/2-selective inhibitors at both 12 and 24 hrs. As described previously, hormone levels in the cell culture supernatants (released) and cellular protein extracts (cellular content) were measured. The sum of cellular hormone contents and released hormone for each individual treatment (total) were used as an index of hormonal protein availability and production.

8.2 Results

The efficacy and specificity of LY294002 (10 μM), GSK2334470 (3 μM), and Akt_i VIII (10 μM) have been described previously (Chapters 3 and 5). GDC0941 (250 nM) is highly-selective and ATP-competitive small molecule that specifically inhibits all class I PI3Ks when used in the nanomolar range (reported IC_{50}s for each of the p110 isoforms are between 2 to 75 nM; Folkes et al., 2008; Junttila et al., 2009). Rapamycin

(10 nM) is an extremely well-characterized allosteric inhibitor of the TORC1 signalling complex (Zheng et al., 1995; Oshiro et al., 2004; Laplante and Sabatini 2012); whereas, INK128 (50 nM) is an ATP-dependent inhibitor of TOR kinase catalytic activity that selectively inhibits TOR-dependent signalling when complexed in either TORC1 or TORC2 (Hsieh et al., 2012). Comparisons of the cellular actions of LY294002 and GDC0941 allows for the possible evaluation of class I PI3K-mediated effects relative to those regulated by the class II and/or III PI3Ks, while differences in the rapamycin- and INK128-dependent activities may be attributed to inhibition of TORC2 (Ballou and Lin, 2008; Feldman et al., 2009; Li et al., 2014). For clarity, a schematic of the signalling effectors targeted by the inhibitors used in this Chapter is provided in Figure 8.1. In general, the drug concentrations selected for use are based on published studies of the efficacy and selectivity of these reagents in mammalian cell-based assays.

8.2.1 Pharmacological mapping of PI3K-dependent signalling in 2 hr static incubation studies of basal LH release, cell content, and total available LH

Basal LH release was significantly increased by 2 hr static incubations with LY294002 (10 μM), GDC0941 (250 nM), GSK2334470 (3 μM), Akt_i VIII (10 μM), and rapamycin (10 nM), but not with INK128 (50 nM) treatment (Figure 8.2). Cellular LH content was also significantly increased following all drug treatments, except for the pan-class I PI3K inhibitor GDC0941 (Figure 8.2). Overall, total available LH was significantly increased by treatment with LY294002, GSK2334470, Akt_i VIII, and rapamycin (Figure 8.2). However, total LH availability was not altered by either GDC0941 or INK128 (Figure 8.2).

8.2.2 Pharmacological mapping of PI3K-dependent signalling in 2 hr static incubation studies of basal GH release, cell content, and total available GH

Basal GH release was significantly increased by 2 hr treatment with each of the inhibitors tested (Figure 8.3). On the other hand, with the exception of a stimulatory effect using Akt_i VIII and an inhibitory influence of GDC0941, the panel of inhibitors tested did not alter cellular GH content and total GH availability (Figure 8.3).

8.2.3 Effects of prolonged application of PDK1, Akt, and TORC inhibitors on basal LH release, cell content, and total available LH in 12 and 24 hrs static incubations

Treatment with GSK2334470 or Akt_i VIII over 12 hrs caused a significant increase, and rapamycin a significant decrease, in basal LH release relative to the untreated control (Figure 8.4). While both rapamycin and INK128 significantly reduced the cellular content and total availability of LH, Akt_i VIII reduced LH cell content and GSK2334470 elevated total LH availability (Figure 8.4).

Static incubation treatment with Akt_i VIII for 24 hrs significantly reduced LH release, cellular content, and total availability (Figure 8.5). Although GSK2334470 did not alter the total available LH, it significantly elevated basal LH release but decreased cellular LH content at 24 hrs (Figure 8.5). In addition, 24 hrs treatments with rapamycin, but not INK128, significantly reduced cellular LH content and total available LH (Figure 8.5).

8.2.4 Effects of prolonged application of PDK1, Akt, and TORC inhibitors on basal GH release, cell content, and total available GH in 12 and 24 hrs static incubations

Treatment with GSK2334470, Akt_i VIII, or INK128 for 12 hrs significantly increased basal GH release, while rapamycin significantly reduced unstimulated GH secretion (Figure 8.6). Except for the lack of effects on total GH availability with GSK2334470 treatment, inhibitors of PDK1-, Akt-, and TOR-dependent signalling also significantly reduced cellular GH content and total GH availability at 12 hrs.

At 24 hrs, GSK2334470, Akt_i VIII, rapamycin, or INK128 all significantly increased basal GH release. With the exception of INK128 treatment on total GH availability, these inhibitors also significantly reduced GH cellular content and total available GH (Figure 8.7).

8.3 Discussion

Across animal models, PI3K-dependent signalling is central to the cellular control of mRNA transcription and protein synthesis. To a large extent, PI3K-dependent regulation of cellular metabolism is due to Akt-mediated activation of the rapamycin-sensitive TORC1 complex (Engelman et al., 2006; Manning and Cantley, 2007; Laplante and Sabatini 2012). Results from this chapter identify PI3K-dependent signalling, and more specifically the Akt-TOR signalling node, as an important regulator of long-term basal pituitary hormone release and availability. In addition, observations from the pharmacological manipulations have also revealed novel and important details regarding the intracellular architecture of canonical PI3K-dependent signalling components in the

control of goldfish gonadotrope and somatotrope functions under unstimulated conditions.

8.3.1 Differential involvement of PI3K-dependent signalling in the control of basal hormone release and total hormone availability in 2 hr static incubations

Results reveal that the influences of PI3Ks and downstream PI3K-dependent effectors, as well as their relationships, in the control of basal hormone release and total hormone availability are function-specific and cell type-dependent.

8.3.1.1 Role of PI3Ks in general

Previously in this thesis, I have demonstrated a complex role for PI3Ks and their common downstream effectors in the control of acute hormone release responses when measured in column perifusion (Chapters 3, 4, and 5). Briefly, although pan-PI3K inhibitors transiently and significantly increase basal hormone release (Chapter 3), isoform-selective inhibitors of the class I PI3Ks caused a sustained decrease in basal LH and GH release (Chapter 4). These findings lead to the hypothesis that while endogenous class I PI3K enzymatic activities largely exerts a stimulatory influence on acute basal LH and GH secretion, class II and/or III PI3Ks may provide inhibitory inputs in this regard. In contrast, 2 hr treatment with either the pan-PI3K inhibitor LY294002 or the pan-class I PI3K inhibitor GDC0941 consistently elevated basal LH and GH release in the present thesis chapter (Tables 8.1 and 8.2), indicating that PI3Ks in general, and the class I PI3Ks in particular, exerts an inhibitory influence over long-term unstimulated LH and GH secretion. Whether these differences between acute and 2 hr studies are due to changes in

the function of all or select class I PI3K isoform(s) over time cannot be ascertained as GDC0941 is expected to inhibit all four class I PI3Ks at the concentration tested. Similarly, although it appears likely that class II and III PI3Ks also exert inhibitory influences on long-term basal LH and GH secretion as with class I PI3Ks, whether this is true is unknown.

Interestingly, divergent effects of treatments with LY294002 and GDC0941 on hormonal cell content and total availability were seen (Tables 8.1 and 8.2). Results with GDC0941 indicate that while class I PI3Ks, in general, have no effects on the regulation of cellular hormonal content and total availability in goldfish gonadotropes, they exert a positive influence on these parameters in somatotropes. On the other hand, when compared with the effects of GDC0941, results with LY294002 would suggest that class II and/or III PI3Ks exert inhibitory control over LH cellular content and total availability but are likely to provide a positive influence over GH cellular content and total availability to counteract the stimulatory effects of class I PI3K(s) on these functions in somatotropes. Overall, these data suggest that PI3K isoforms play unique roles in the coordinate control of pituitary hormone release and total availability, and in a cell type dependent manner. Future studies using isoform-specific class I PI3K inhibitors, as well as class II- and/or III-selective PI3K inhibitors will be necessary to further elucidate the time-dependent role of various PI3Ks in the control of basal pituitary hormone release, as well as cellular hormonal content and availability.

The effects of LY294002 static incubation treatment on basal GH release, cell content and total hormone observed in this thesis chapter are similar to those reported in Chapter 6. On the other hand, no stimulatory effects of LY294002 on basal LH release

(as demonstrated in the present chapter) were observed in Chapter 6; although a slight (insignificant) increases in unstimulated LH cell content and total availability are also seen following LY294002 incubation at 2 hrs in this earlier thesis chapter. It should be noted that experiments in Chapter 6 utilized a plating density of 4 x 10^6 cells in 10 ml of testing media while experiments in the present thesis chapter were performed at a plating density of 1.5 x 10^6 cells per 10 ml. In addition, due to the pooling of data across cell preparations, results in Chapter 6 were expressed in μg hormone values whereas the data in the present chapter were normalized as a percentage of untreated controls. Consequently, differences in the data normalization/expression, the dilution of samples needed to get the LH measurements within the functional range of the RIA (which would have an effect on the sensitivity for the detection of significant changes), and/or cell density effects might all be part of the reasons behind these discrepancies in the relative quantification of LH secretion observed across these experiments.

8.3.1.2 Involvement of canonical PI3K-dependent effectors

Although inhibition of BTK catalytic activity mirrors the actions of the selective class I PI3K inhibitors, inhibition of either PDK1 or Akt generally elevates basal hormone release (Chapters 3 and 5). In the case of PDK1, these actions are transient and selective for basal GH release, whereas inhibition of Akt results in a sustained increase in both basal LH and GH release over the perifusion treatment period (Chapter 5). Taken together, these observations suggest that the actions of PDK1- and Akt-dependent signalling can be uncoupled from the activity of class I PI3Ks during the acute regulation of basal hormone release and in a cell type dependent manner. In contrast, the pan-class I

PI3K inhibitor GDC0941, Akt_I VIII, and GSK2334470 all stimulated LH and GH release in 2 hr static incubation experiments in this chapter (Tables 8.1 and 8.2), suggesting that over a longer time period (2 hrs), class I PI3K inhibitory influences on hormone release from goldfish gonadotropes and somatotropes are mediated by PDK1 and Akt. Consistent with the idea that TORC1 can mediate Akt effects, static incubation treatment with rapamycin for 2 hrs likewise elevated LH and GH release. Interestingly, the TORC1/2 inhibitor INK128 enhanced basal GH, but not LH, secretion at 2 hrs. When viewed together, these results using rapamycin and INK128 treatments indicate that TORC2 may be part of the Akt activation pathway in the control of unstimulated GH release at 2 hrs. However, TORC2 is either not involved in, or exerts a stimulatory effect independent of Akt- or TORC1-dependent activation to counteract the canonical class I PI3K-PDK1-Akt-TORC1 inhibitory influence on, basal LH secretion. In addition to Akt, TORC2 has been recently shown to directly phosphorylate the turn motif of conventional PKC isozymes (Ikenoue et al., 2008; Facchinetti et al., 2008), and TORC2-mediated regulation of actin cytoskeleton dynamics is dependent upon activation of PKCα (Sarbassov et al., 2004). Although PKCs are not major regulators of acute basal hormone release in goldfish, perhaps in a manner similar to the class PI3Ks, PKCs exert differential cellular effects over the long-term that may account for TORC2-selective functions in somatotropes or gonadotropes.

In contrast to results on long-term basal hormone release at 2 hrs, treatment with Akt_i VIII, GSK2334470, and rapamycin effects on cellular LH content and the total LH available mimicked those of LY294002, but not GDC0941 (Table 8.1). These observations suggest that the general inhibitory influence of PI3Ks (and perhaps class II

and/or III PI3K-selective effects) on LH synthesis and replenishment within this time frame involves activation of PDK1-Akt-TORC1 signalling. Likewise, based on the observations with INK128 (Table 8.1), the TORC2 complex may also play a role in the activation of Akt in this regard. On the other hand, the dissimilarities between the results of Akt-, PDK1-, TORC1-, TORC1/2-, and class I PI3K-selective inhibition on cellular and total GH (Table 8.2) indicate that the stimulatory influence of class I PI3Ks on GH hormone synthesis and replenishment over this time frame (2 hrs) does not involve these canonical signalling elements.

Results presented in this chapter suggesting that Akt-dependent signalling can be uncoupled from class I PI3K catalytic activity and TOR activation in pituitary somatotropes are not at odds with previous results presented in Chapter 5. In addition, they implicate the presence of PtdIns(3,4,5)P_3-independent regulation of Akt activation in the control of basal somatotrope cellular functions. Interestingly, PI3K-independent activation of Akt is well-characterized in oncogenic signalling networks and this typically involves non-receptor tyrosine kinases, including c-Src (Mahajan and Mahajan, 2012). Furthermore, Akt can also be directly regulated by interacting with PtdIns(3,4)P_2 (Franke et al., 1997) and recent evidence suggest that class II PI3Ks might be able to produce PtdIns(3,4)P_2 through phosphorylation of PtdIns(4)P (Falasca and Maffucci, 2012). Signalling through PtdIns(3,4)P_2 could help to explain the differences in the effects of pan-PI3K and class I PI3K inhibitors, and also account for PtdIns(3,4,5)P_3-independent activation of Akt. However, results from *in vitro* studies strongly suggest that production of PtdIns(3)P from PtdIns appears to be the primary function of class II PI3Ks (Falasca and Maffucci, 2012). Nonetheless, it is clear from these data that understanding the

regulation of Akt-dependent signalling in goldfish gonadotropes and somatotropes will be an important area for future research. In particular, the involvement of tyrosine kinase-dependent signalling and the class II PI3Ks could represent novel intracellular regulators of basal hormone release and availability potentially through regulation of Akt catalytic activity.

8.3.2 Involvement of PDK1-, Akt-, and TORC-dependent signalling in the differential control of prolonged basal hormone release and availability over 12 or 24 hrs

Interestingly, results from the present chapter indicate that PDK1, Akt, and TORC1/2 interact in a canonical fashion, as well as independently of one another, to differentially modulate prolonged cellular functions in goldfish gonadotropes and somatotropes.

8.3.2.1 Prolonged regulation of basal LH and GH release

The data presented in this chapter strongly suggests that Akt, PDK1, and TORC1/2 play different roles in the prolonged regulation of LH and GH release over 12 and 24 hrs. With the exception of INK128 treatment at 12 hrs, application of either Akt_i VIII, GSK2334470, rapamycin, or INK128 largely exert a stimulatory influence on basal GH release (Table 8.2), suggesting that a classical PDK1-Akt-TORC1 (and perhaps TORC2) signalling cascade decreases prolonged basal GH secretion. On the other hand, the effects of PDK1-, Akt-, TORC1-, and TORC1/2-selective inhibitors are less consistent in terms of prolonged LH release. Inhibitors of TORC1 and TOR kinase

suppress basal LH release at 12 hrs but have no effects at 24 hr (Table 8.1), suggesting a stimulatory role for TOR complexes on unstimulated hormone release from goldfish gonadotropes only at 12 hrs. In contrast, results with Akt and PDK1 inhibitors indicates that PDK1- and Akt-dependent signalling exerts an inhibitory influence on basal LH at 12 hrs; while at 24 hrs, Akt and PDK1 play a stimulatory and inhibitory role, respectively (Table 8.1). Thus, in terms of prolonged basal LH secretion over 12 and 24 hrs, the PtdIns(3,4,5)P_3-sensitive effectors PDK1, Akt, and TORC1/2 are uncoupled from one another. Surprisingly, general inhibition of PI3Ks by LY294002 has no effect on prolonged LH and GH secretion in Chapter 6. Taking this earlier observation into account, it would be tempting to hypothesize that these canonical PtdIns(3,4,5)P_3-sensitive signalling elements may not only interact in a non-linear fashion in the differential modulation of basal LH and GH release, but they may also function independently of PI3K-mediated activation and subsequent PtdIns(3,4,5)P_3 generation. However, as discussed above, different classes of PI3K can activate Akt-dependent signalling in PtdIns(3,4,5)P_3-dependent and PtdIns(3,4,5)P_3-independent fashions; consequently, the idea that the influences of PDK1-Akt-TORC signalling on prolonged basal LH release are dissociated from the activation of PI3Ks remains to be confirmed.

8.3.2.2 Prolonged regulation of LH and GH cellular hormonal content and availability

Except for the inability of INK128 to alter cellular GH content and availability at 24 hrs, treatments with PDK1-, Akt-, TORC1- and TORC1/2-selective inhibitors all decreased the cellular content and availability of GH (Table 8.2). Interestingly,

experiments in Chapter 6 using LY294002, identify PI3K-dependent signalling as a positive regulator of cellular hormone content in pituitary somatotropes at both 12 and 24 hrs. When viewed together, these observations indicate that endogenous PI3K activity signals through a PDK1-Akt-TORC1 pathway to positively influence prolonged hormone production and replenishment of GH in goldfish somatotropes over 12 to 24 hrs. This conclusion is consistent with the well-characterized importance of the PI3K-PDK1-Akt-TOR cascade in the maintenance of cellular protein synthesis and availability in many vertebrate systems (Engelman et al., 2006; Manning and Cantley, 2007; Laplante and Sabatini 2012).

In terms of the prolonged regulation of basal cellular LH content and availability, results from Chapter 6 indicate that PI3K-dependent signalling positively influence these parameters at 12 hrs. In the present thesis chapter, treatment with rapamycin or INK128 both significantly reduced cellular LH content and total available LH at 12 hrs; while Akt inhibition similarly reduced cellular LH content at 12 hrs (Table 8.1). In contrast, GSK2334470 selectively increased the total available LH at 12 hrs. Taken together, these data suggest that PI3K-dependent signalling through Akt and TORC1, but independent of PDK1, is important for the positive regulation of basal LH content at 12 hrs. Conversely, TORC1/2 are also important for the maintenance of total LH availability, but by actions that are independent of Akt. However, it is important to note that inhibition with rapamycin suppresses basal LH content by roughly 75% relative to the untreated controls, whereas treatment with Akt_i VIII only reduces the cellular content by around 15%. These discrepancies in the magnitude of the responses could reflect differences in the

pharmacokinetics and potency of these inhibitors, but also highlight the importance of TORC1-mediated signalling to the control of cellular metabolism and protein synthesis.

The regulation of prolonged basal gonadotrope hormone availability at 24 hrs appears to be different from that at 12 hrs. Treatment with Akt_i VIII and rapamycin reduced LH cellular content and total availability at 24 hrs. Inhibition of PDK1 significantly decreased LH content but not the total available LH, while INK128 affected neither of these paramenters (Table 8.1). These results indicate that Akt- and TORC1-dependent signalling are important regulators of total LH availability and cellular LH content at 24 hrs and their effects on LH cellular content involves an upstream PDK1-sensitive component. In addition, these observations clearly demonstrate an uncoupling of TORC1- and TORC2-mediated cellular responses in goldfish gonadotropes at 24 hrs.

8.3.3 Uncoupling of PDK1- and Akt-dependent signalling in the control of long-term basal cellular functions of goldfish pituitary cells

Across the time points investigated, there were instances where treatments with Akt_i VIII and GSK2334470 resulted in distinct cellular responses. In particular, divergent responses were observed with regards to LH release at 24 hrs, cellular LH content at 12 hrs, and total LH availability at 12 and 24 hrs; as well as cellular GH content at 2 hrs and total GH availability at 2 and 12 hrs (Table 8.1 and 8.2). These results suggest that PDK1-dependent actions on basal cellular functions can be controlled by an Akt-independent mechanism.

Interestingly, previous characterization of GSK2334470 has revealed that Akt activation is much less sensitive to inhibition using this ATP-competitive PDK1 inhibitor

when compared with the activation of other PDK1-dependent effectors (Najafov et al., 2011; Knight, 2011). These properties of GSK2334470 are not due to problems with target specificity, since this compound has little or no effect on the activity of 95 protein kinases and 15 lipid kinases assayed in cell-free systems at concentrations 100-fold greater that the reported IC_{50} (Najafov et al., 2011; Chapter 5). Subsequent studies suggest that Akt resistance to PDK1 inhibition can be attributed to the Akt activation mechanism. Briefly, in addition to PDK1-mediated phosphorylation of Akt at Thr308, phosphorylation of Akt in the hydrophobic motif by TORC2 contributes to Akt activation through a unique mechanism that relies on the association of phosphorylated Ser473 with the PIF pocket of PDK1 (Najafov et al., 2012). The importance of the alternative PIF pocket-mediated activation mechanism for Akt-dependent signalling is revealed by observations that small molecule antagonists inhibiting the binding of substrates to the PDK1 PIF pocket increases the sensitivity of Akt to GSK2334470 (Rettenmaier et al., 2014). The current understanding is that interactions between PDK1 and Akt occur through two alternative mechanisms that can independently, as well as synergistically, facilitate efficient activation of Akt even in the presence of a PDK1 inhibitor (Najafov et al., 2012). Consequently, catalytically active Akt may remain following treatment with GSK2334470 and the cellular effects of PDK1 inhibition may also involve downstream effectors other than Akt. However, at the 3 μM dose used, GSK2334470 significantly reduces Akt catalytic activity within 5 min of treatment and sustained treatment inhibits cellular responses for up to 72 hrs in both primary mammalian cells and panels of human cancer cell lines (Najafov et al., 2011; Najafov et al., 2012). As a result, the use of Akt_i

VIII in parallel with GSK2334470 is critical in order to characterize the role of Akt-dependent signalling.

At this time, whether cellular responses that are sensitive to Akt_i VIII, but not GSK2334470, are due to PDK1-independent Akt signalling or are the result of Akt resistance to GSK2334470 treatments cannot be easily differentiated. Nonetheless, the fact that the differences in Akt_i VIII- and GSK2334470-sensitivity observed in this chapter are clearly cell- and function-specific strongly implies that the heterogeneity of the effects observed are due solely to global cellular resistance of Akt to GSK2334470-mediated inhibition.

8.3.4 Summary

Findings in this chapter add to the limited information available regarding the roles of PI3K-dependent or PtdIns(3,4,5)P_3-sensitive signalling elements in the regulation of basal pituitary hormone content and release. Overall, results highlight the intracellular complexity and central importance of the PI3K-PDK1-Akt-TOR signalling axis in the control of basal pituitary cell functions and, in particular, for prolonged GH release, cellular GH content, and total GH availability. As has been demonstrated in Chapter 6, the actions of these intracellular effectors are clearly time- and cell type-dependent. In addition to demonstrating, for the first time in any vertebrate study model, that PDK1- and Akt-dependent signalling elements exert a negative influence over long-term basal hormone release in both gondatotropes and somatotropes at various time points, TOR-dependent signalling is also identified as an important stimulatory signal for the long-term regulation of LH and GH cellular content. These data also support the idea that

long-term basal hormone release and hormone synthesis/replenishment can be regulated by dissimilar intracellular mechanisms. In addition, several novel examples of the uncoupling of intracellular effectors from the canonical PI3K-PDK1-Akt-TOR cascade are revealed. Though these results represents a first step in deciphering the complexity of intracellular signal transduction events modulating long-term functions in goldfish gonadotropes and somatotropes, the potential significance of these discrepancies to the understanding of integrated cellular control of hormone release and production cannot be ignored. Future studies will need to examine the involvement of these PtdIns(3,4,5)P_3-sensitive downstream effectors during agonist-stimulated hormone release and synthesis responses. In particular, due to the relatively novel role of TOR-dependent signalling in the control of basal hormone release observed in this study, it would be interesting to see if signalling through TORC1 and/or TORC2 is important for acute release responses mediated by GPCR-binding effectors like GnRHs.

Table 8.1 Summary of time-dependent effects of PI3K, PDK1, Akt, and TOR inhibition on LH release, cellular content, and total availability in primary cultures of goldfish pituitary cells.

Treatment	Duration (hrs)	LH release	LH content	Total LH availability
LY294002	**2**	↑	↑	↑
GDC0941	**2**	↑	↔	↔
Akt$_i$ VIII	**2**	↑	↑	↑
	12	↑	↓	↔
	24	↓	↓	↓
GSK2334470	**2**	↑	↑	↑
	12	↑	↔	↑
	24	↑	↓	↔
Rapamycin	**2**	↑	↑	↑
	12	↓	↓	↓
	24	↔	↓	↓
INK 128	**2**	↔	↑	↔
	12	↓	↓	↓
	24	↔	↔	↔

↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control.

Table 8.2 Summary of time-dependent effects of PI3K, PDK1, Akt, and TOR inhibition on GH release, cellular content, and total availability in primary cultures of goldfish pituitary cells.

Treatment	Duration (hrs)	GH release	GH content	Total GH availability
LY294002	**2**	↑	↔	↔
GDC0941	**2**	↑	↓	↓
Akt$_i$ VIII	**2**	↑	↑	↑
	12	↑	↓	↓
	24	↑	↓	↓
GSK2334470	**2**	↑	↔	↔
	12	↑	↓	↔
	24	↑	↓	↓
Rapamycin	**2**	↑	↔	↔
	12	↓	↓	↓
	24	↑	↓	↓
INK 128	**2**	↑	↔	↔
	12	↑	↓	↓
	24	↑	↓	↔

↔, not significantly different from untreated control; ↑, significantly increased compared to untreated control; ↓, significantly reduced compared to untreated control.

Figure 8.1 Schematic of the canonical PI3K-PDK1-Akt-TOR signalling cascade with specific focus on the targets of the selective inhibitors used in this thesis chapter. Pharmacological mapping of the PI3K-PDK1-Akt-TOR cascade during prolonged actions on basal hormone release and total availability utilized selective pan-PI3K (LY294002; 10 μM), pan-class I PI3K (GDC0941; 250 nM), Akt (Akt_i VIII; allosteric; 10 μM), PDK1 (GSK2334470; 3 μM), TORC1 inhibitor (rapamycin; allosteric; 10 nM), and TOR kinase (INK128; inhibits the catalytic activity of TOR in both TORC1 and TORC2; 50 nM) small molecule inhibitors. Note: although GSK2334470 is highly-selective, due to the direct association of PDK1 and Akt at the plasma membrane, inhibition of PDK1 may not acutely inhibit Akt (see discussion in Chapter 5 and Section 8.3.3).

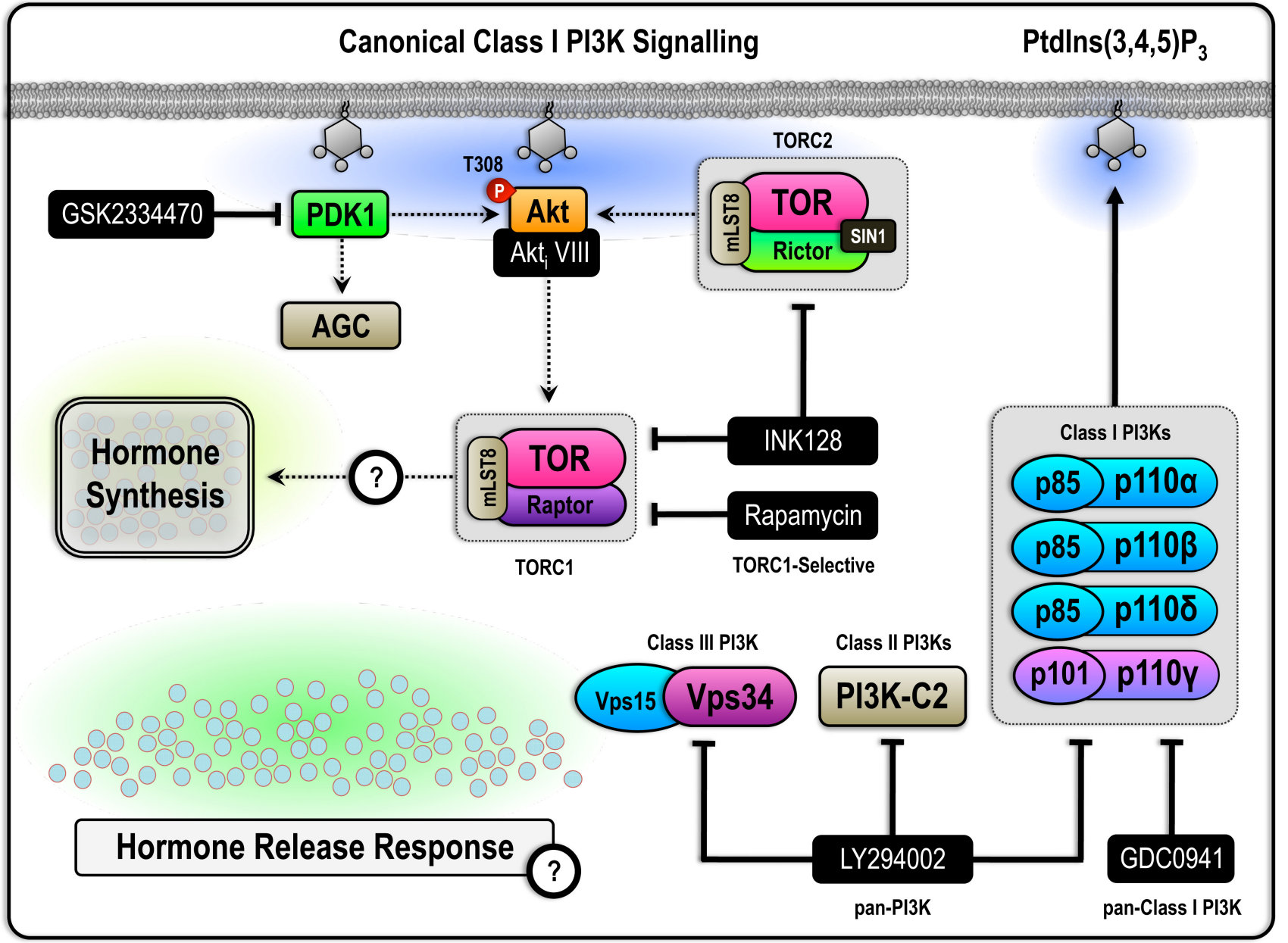

Canonical Class I PI3K Signalling
PtdIns(3,4,5)P3
TORC2
T308
GSK2334470
PDK1
Akt
Akti VIII
mLST8
TOR
Rictor
SIN1
AGC
Hormone Synthesis
?
mLST8
TOR
Raptor
TORC1
INK128
Rapamycin
TORC1-Selective
Class I PI3Ks
p85 p110α
p85 p110β
p85 p110δ
p101 p110γ
Class III PI3K
Vps15 Vps34
Class II PI3Ks
PI3K-C2
Hormone Release Response
?
LY294002
pan-PI3K
GDC0941
pan-Class I PI3K

Figure 8.2 Pharmacological mapping of the canonical PI3K-PDK1-Akt-TOR signalling cascade in the control of basal LH release and total availability following 2 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b,c; black), pan-PI3K inhibitor (a; LY294002; 10 μM; gold), pan-class I PI3K inhibitor (a; GDC0941; 250 nM; brown), Akt inhibitor (b; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (b; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (c; rapamycin; 10 nM; red), and TOR kinase inhibitor (c; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated five times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at early gonadal recrudescence (September to November) and are expressed as a percentage of untreated controls (mean ± SEM, n = 5). The amount of LH released into the media during treatment (LH release) and the cellular LH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the LH release and cellular LH content values in each matched treatment sample was calculated as an index of total LH available (total LH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

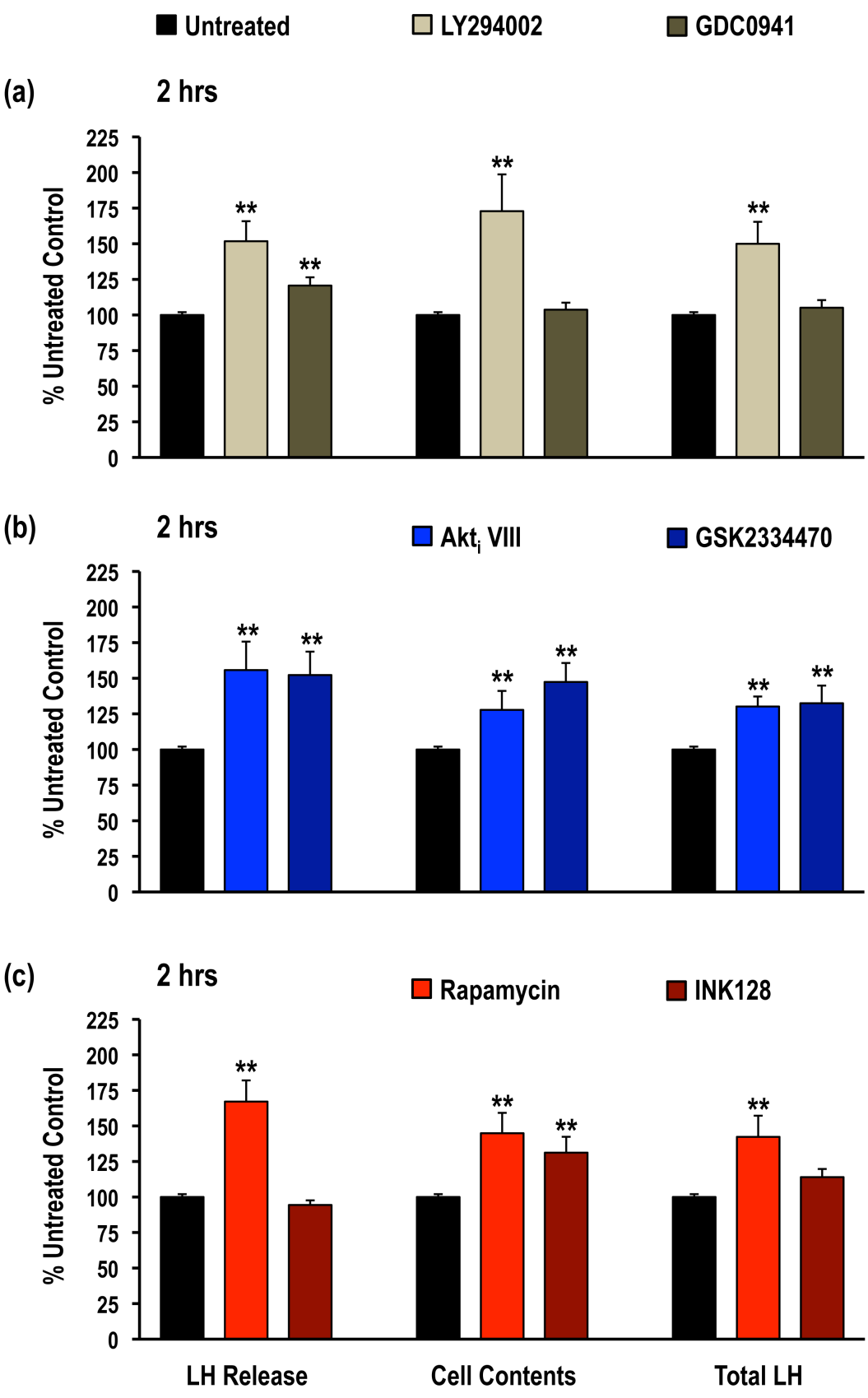

Untreated
LY294002
GDC0941
(a)
2 hrs
% Untreated Control
225
200
175
150
125
100
75
50
25
0
**
(b)
2 hrs
Akt$_i$ VIII
GSK2334470
(c)
2 hrs
Rapamycin
INK128
LH Release
Cell Contents
Total LH

Figure 8.3 Pharmacological mapping of the canonical PI3K-PDK1-Akt-TOR signalling cascade in the control of basal GH release and total availability following 2 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b,c; black), pan-PI3K inhibitor (a; LY294002; 10 μM; gold), pan-class I PI3K inhibitor (a; GDC0941; 250 nM; brown), Akt inhibitor (b; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (b; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (c; rapamycin; 10 nM; red), and TOR kinase inhibitor (c; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated five times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at early gonadal recrudescence (September to November) and are expressed as a percentage of untreated controls (mean ± SEM, n = 5). The amount of GH released into the media during treatment (GH release) and the cellular GH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the GH release and cellular GH content values in each matched treatment sample was calculated as an index of total GH available (total GH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

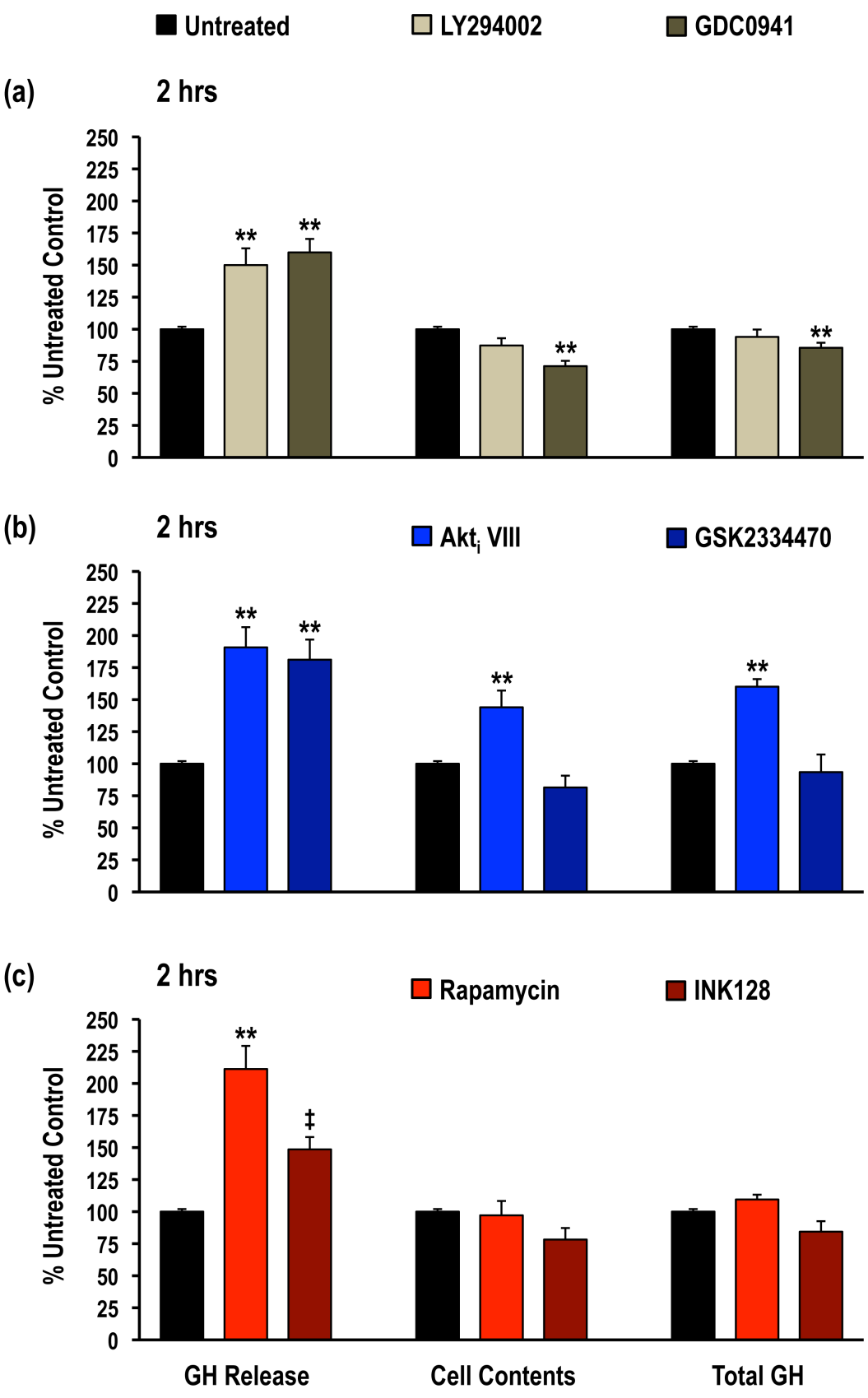

Untreated
LY294002
GDC0941
(a)
2 hrs
% Untreated Control
250
225
200
175
150
125
100
75
50
25
0
**
**
**
**
(b)
2 hrs
Akt$_i$ VIII
GSK2334470
% Untreated Control
**
**
**
**
(c)
2 hrs
Rapamycin
INK128
% Untreated Control
**
‡
GH Release
Cell Contents
Total GH

Figure 8.4 Pharmacological mapping of PDK1-, Akt-, and TOR-dependent signalling in the control of basal LH release and total availability following 12 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b; black), Akt inhibitor (a; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (a; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (b; rapamycin; 10 nM; red), and TOR kinase inhibitor (b; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated four times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at late gonadal recrudescence or with fully regressed gonads (June to September) and are expressed as a percentage of untreated controls (mean ± SEM, $n = 4$). The amount of LH released into the media during treatment (LH release) and the cellular LH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the LH release and cellular LH content values in each matched treatment sample was calculated as an index of total LH available (total LH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

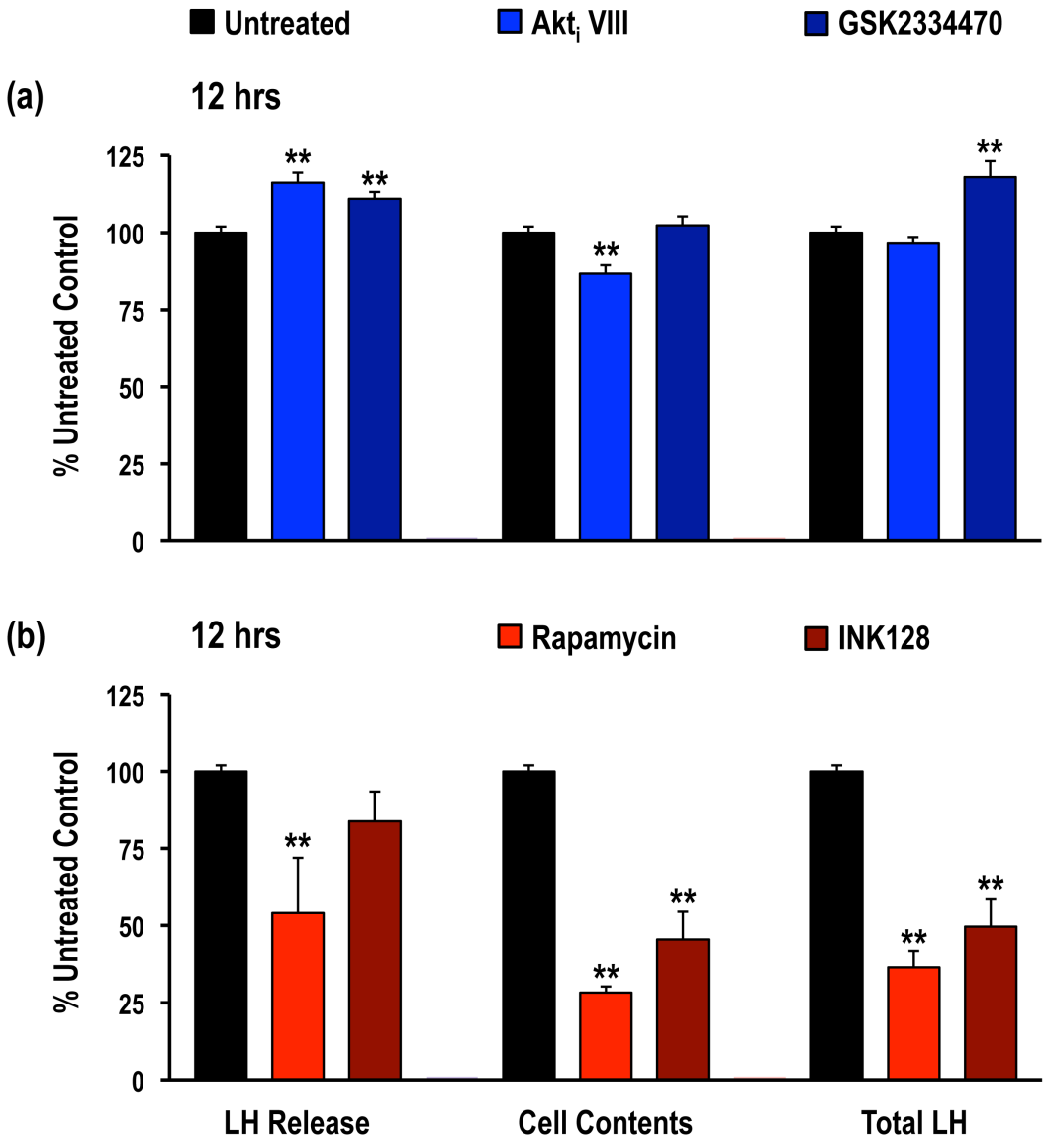

Untreated
Akt$_i$ VIII
GSK2334470
(a)
12 hrs
% Untreated Control
125
100
75
50
25
0
**
(b)
12 hrs
Rapamycin
INK128
LH Release
Cell Contents
Total LH

Figure 8.5 Pharmacological mapping of PDK1-, Akt-, and TOR-dependent signalling in the control of basal LH release and total availability following 24 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b; black), Akt inhibitor (a; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (a; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (b; rapamycin; 10 nM; red), and TOR kinase inhibitor (b; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated four times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at late gonadal recrudescence or with fully regressed gonads (June to September) and are expressed as a percentage of untreated controls (mean ± SEM, $n = 4$). The amount of LH released into the media during treatment (LH release) and the cellular LH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the LH release and cellular LH content values in each matched treatment sample was calculated as an index of total LH available (total LH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

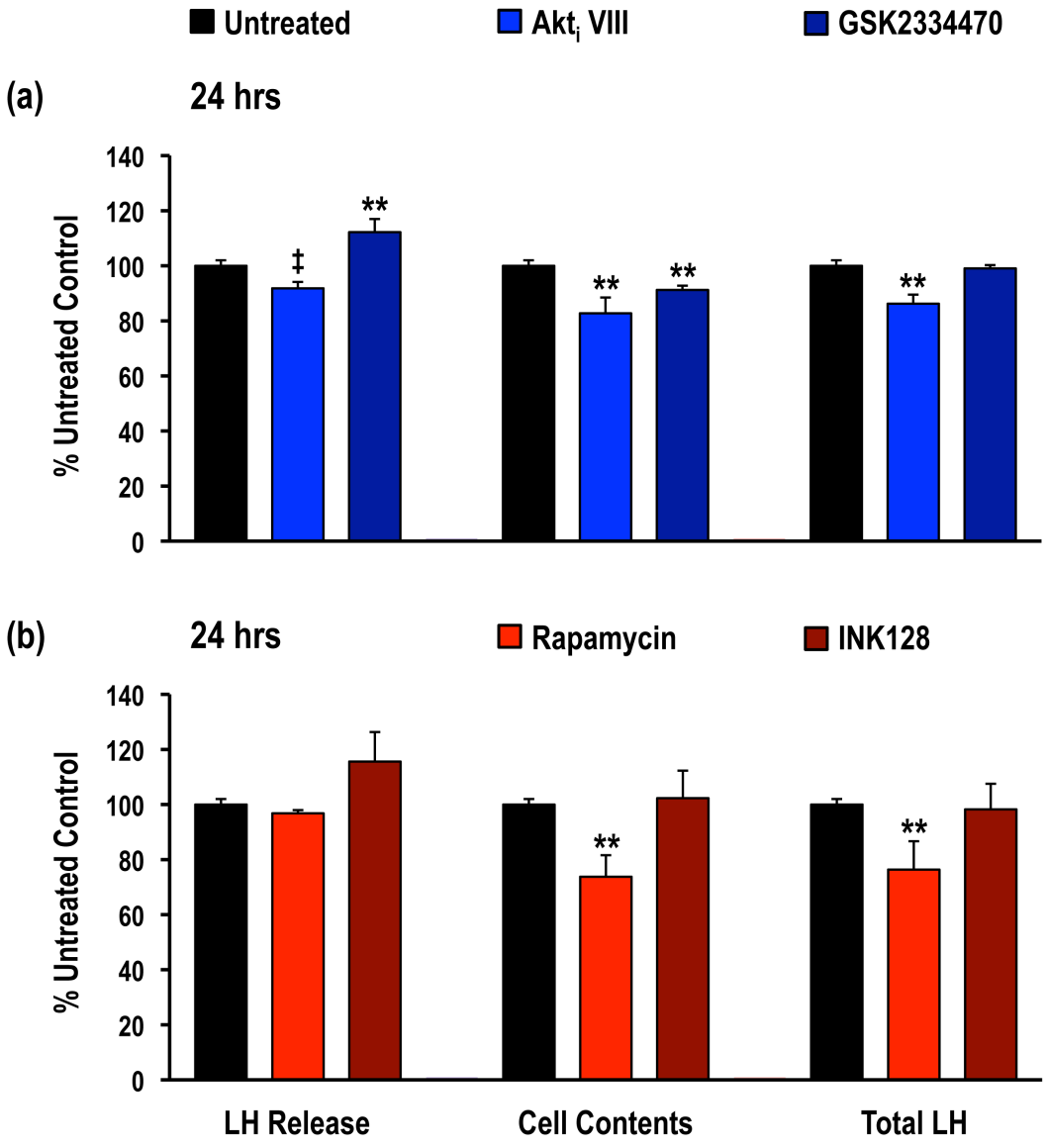

Untreated
Akt$_i$ VIII
GSK2334470
(a)
24 hrs
% Untreated Control
140
120
100
80
60
40
20
0
‡
**
**
**
**
(b)
24 hrs
Rapamycin
INK128
% Untreated Control
140
120
100
80
60
40
20
0
**
**
LH Release
Cell Contents
Total LH

Figure 8.6 Pharmacological mapping of PDK1-, Akt-, and TOR-dependent signalling in the control of basal GH release and total availability following 12 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b; black), Akt inhibitor (a; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (a; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (b; rapamycin; 10 nM; red), and TOR kinase inhibitor (b; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated four times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at late gonadal recrudescence or with fully regressed gonads (June to September) and are expressed as a percentage of untreated controls (mean ± SEM, $n = 4$). The amount of GH released into the media during treatment (GH release) and the cellular GH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the GH release and cellular GH content values in each matched treatment sample was calculated as an index of total GH available (total GH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).

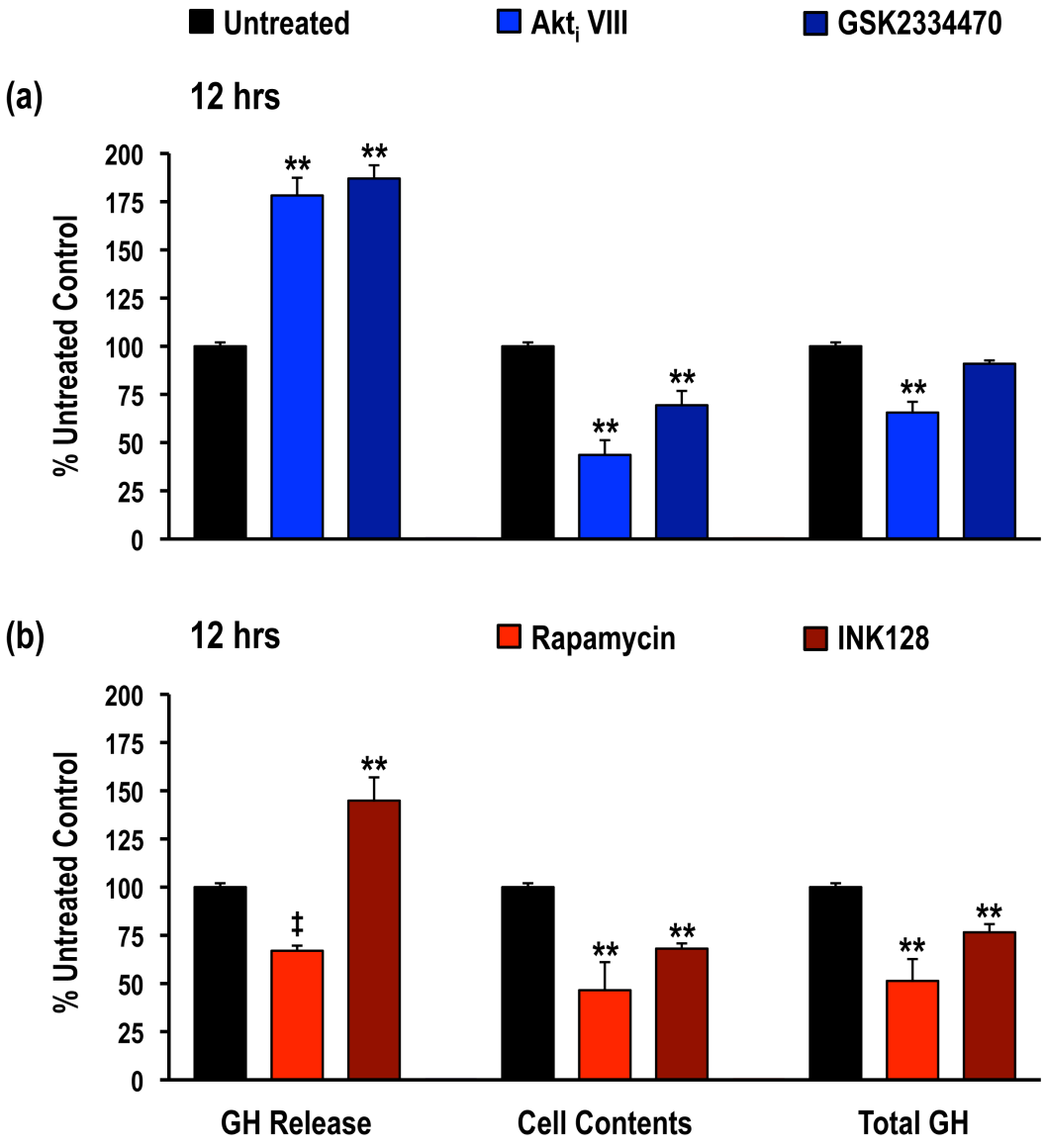
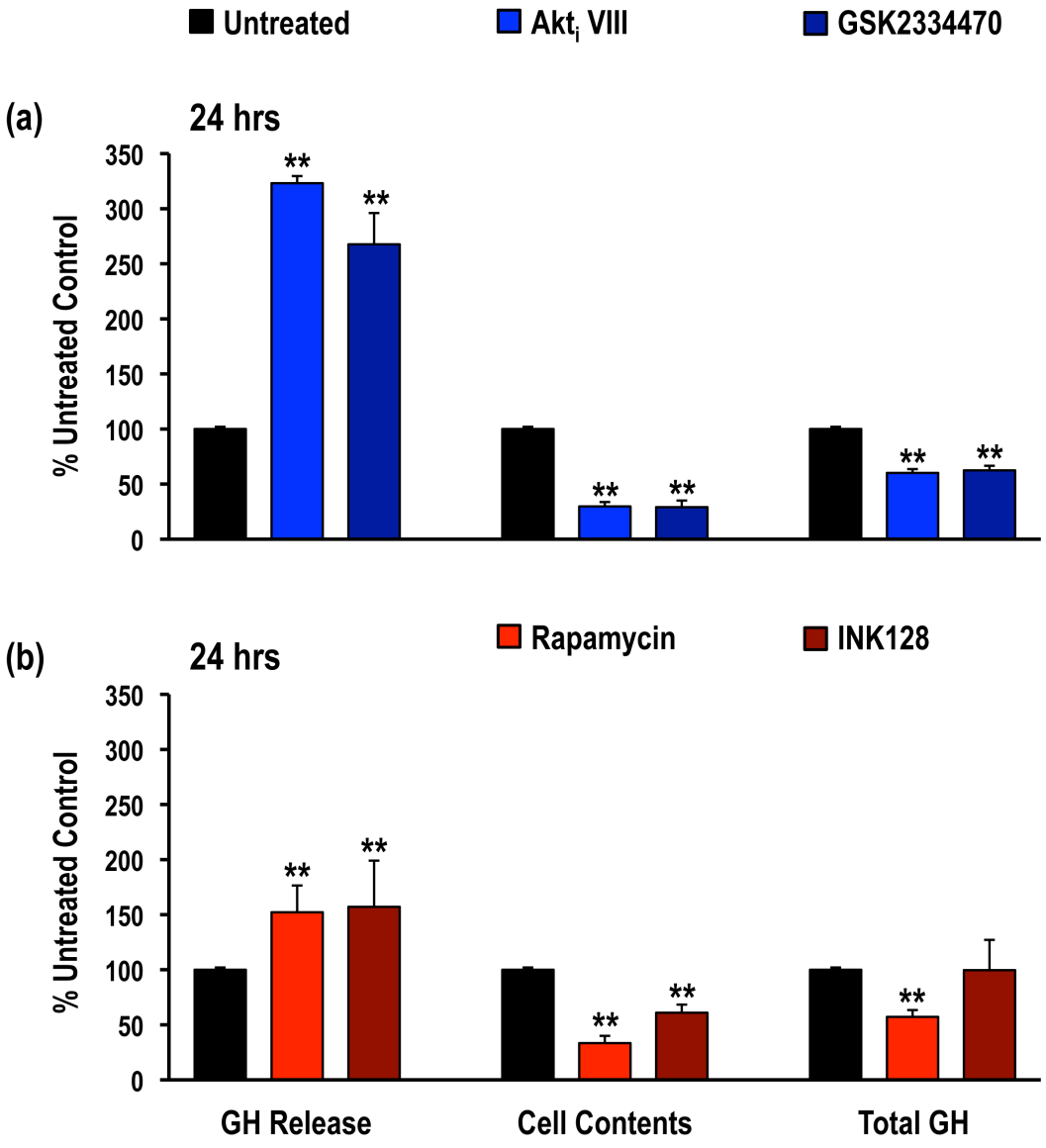
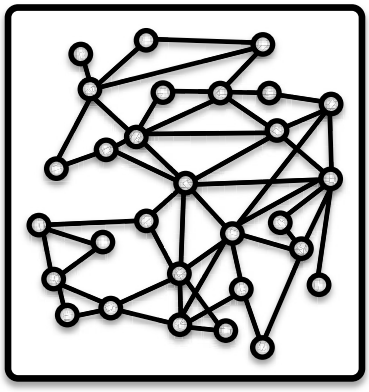

Untreated
Akt$_i$ VIII
GSK2334470
(a)
12 hrs
% Untreated Control
200
175
150
125
100
75
50
25
0
**
**
**
**
**
(b)
12 hrs
Rapamycin
INK128
% Untreated Control
‡
**
**
**
**
**
GH Release
Cell Contents
Total GH

Figure 8.7 Pharmacological mapping of PDK1-, Akt-, and TOR-dependent signalling in the control of basal GH release and total availability following 24 hrs in static incubation. Treatment groups are identified by different colors, respectively: untreated (a,b; black), Akt inhibitor (a; Akt_i VIII; 10 μM; blue), PDK1 inhibitor (a; GSK2334470; 3 μM; dark blue), allosteric TORC1 inhibitor (b; rapamycin; 10 nM; red), and TOR kinase inhibitor (b; INK128; 50 nM; dark red). Results are shown with each treatment having been replicated four times using independently prepared dispersed pituitary cells (1.5×10^6 cells/treatment) from goldfish at late gonadal recrudescence or with fully regressed gonads (June to September) and are expressed as a percentage of untreated controls (mean ± SEM, $n = 4$). The amount of GH released into the media during treatment (GH release) and the cellular GH content remaining at the end of the treatment period (cell content) were quantified by radioimmunoassay. The sum of the GH release and cellular GH content values in each matched treatment sample was calculated as an index of total GH available (total GH) during the treatment period in that sample. Measurements of release, cellular contents, and total hormone available were analyzed independently. Treatments that are significantly different from one another are identified by different symbols (** vs. ‡; ANOVA followed by Fisher's LSD multiple comparisons; $p < 0.05$).


Untreated
Akt$_i$ VIII
GSK2334470
(a)
24 hrs
% Untreated Control
350
300
250
200
150
100
50
0
**
Rapamycin
INK128
(b)
GH Release
Cell Contents
Total GH

Chapter Nine:

General Discussion



PI3K-Dependent Signalling and the Regulation of Pituitary Cell Functions

Results from experiments using hormone release and cellular content as physiologically-relevant endpoints in this thesis are consistent with the hypothesis proposed in Section 1.10 that PI3Ks, and class I PI3Ks in particular, participate in the regulatory effects of GnRH2 and GnRH3 on goldfish gonadotrope and somatotrope functions in a GnRH-isoform-, cell type-, function-, and time-dependent manner. In doing this research, I have also identified the PI3K superfamily of lipid kinases as an important intracellular platform for mediating biased cellular responses and elucidated their possible relationships with known signal transduction mechanisms mediating GnRH actions in goldfish pituitary cells, including Ca^{2+}-, PKC-, and Raf-MEK-ERK-dependent signal transduction. Overall, the data presented in this thesis not only establishes that biased signalling occurs in natural neuroendocrine systems, but also add to our understanding of how the complexity of biased intracellular responses induced by different endogenous hormone variants contributes to the endocrine control of reproduction and growth. In addition, insights into the influence of the constitutive activities of PI3Ks, PtdIns(3,4,5)P_3-sensitive effectors, and the Raf-MEK-ERK cascade on the differential control of agonist-stimulated and basal hormone secretion, as well as hormone availability, are provided.

9.1 Brief overview of findings and conclusions

This is the first comprehensive examination of PI3K-dependent signalling in any vertebrate pituitary cell type. Results from immunoblotting in Chapters 3 and 4 also provide the first evidence that class I PI3K isoforms are present and constitutively active in goldfish pituitary cells, as well as demonstrate, for the first time in any vertebrate study

model, that signalling downstream of these effectors contribute to basal and GnRH-dependent actions on acute LH and GH release. Observations also indicate that PI3K-dependent signalling is not activated downstream of novel or conventional isoforms of PKCs and reveal that PI3Ks can be involved upstream of GnRH-stimulated Ca^{2+} signals with the exception of GnRH2-elicited effects on somatotropes. This also represents the first time that cell type-specific and agonist-selective integration of PI3K-, PKC-, and Ca^{2+}-dependent signalling is elucidated in any endocrine secretion model. Furthermore, when taken together with the novel findings demonstrating the selective involvement of p110β, p110δ, and p110γ catalytic activity in GnRH2 and GnRH3 stimulation of LH and GH secretion, as well as results using a selective inhibitor of Gβγ-effector interactions, these observations provide the first demonstration of biased regulation of Gβγ-sensitive class I PI3Ks in any model, as well as suggest a novel physiological role for biased Gβγ-dependent signalling downstream of GnRHRs and, possibly, other GPCRs. Just as importantly, the involvement of p110α- and PDK1-dependent signalling in basal, but not agonist-stimulated, LH release provides novel evidence that class I PI3Ks are differentially involved in unstimulated and agonist-elicited hormone release.

Signalling downstream of agonist-stabilized GPCRs that regulate the activity of class I PI3Ks is generally thought to be mediated by PtdIns(3,4,5)P_3 production and recruitment of PH domain-containing canonical effectors such as PDK1, Akt, and BTK (Wymann and Marone, 2005; Hawkins et al., 2006; Burke and Williams, 2015; Chapter 1). However, results from Chapter 5 revealed that GnRH isoform-selective influences on GH release involved, at least in part, differential signalling by Akt and BTK during acute GH release, but not PDK1. Furthermore, GnRH-stimulated LH appears to require PH

domain-sensitive elements but PDK1-, Akt-, and BTK-dependent signalling are not involved. These observations suggest that novel non-canonical PtdIns(3,4,5)P_3-sensitive effectors regulate GnRH actions on acute LH release responses and also reveal the possibility that these non-canonical mechanisms may be involved in GnRH-selective stimulation of acute GH release, as well.

Because of the known importance of PI3Ks and MAPKs in the control of cellular metabolism and protein homeostasis (Engelman et al., 2006; Mendoza et al., 2011; Lavoie and Therrien, 2015), the role of PI3K- and Raf-MEK-ERK-dependent signalling in the long-term regulation of pituitary hormone secretion and availability were also examined in Chapters 6 and 7. Results demonstrate that differences in the catalytic activity of PI3Ks and MEK1/2 differentially contribute to the regulation of sustained GnRH-stimulated hormone release and cellular hormonal availability in a time-, cell type-, and GnRH-selective manner. These data add to the known differential involvement of PI3K-dependent signalling in acute LH and GH release responses demonstrated previously, as well as provide the first demonstration of biased regulation of MEK1/2-dependent signalling in a primary pituitary cell model. When compared to the reported time course for GnRH stimulation of hormonal mRNA expression, results from these two chapters indicate that agonist-induced hormonal gene expression and hormone synthesis in goldfish pituitary cells can be dissociated across time. Furthermore, although both GnRHs can increase total LH availability, they are generally not effective in elevating GH availability (and by implication, GH synthesis), with the possible exception of GnRH2 at 24 hrs. That the long-term GnRH2- and GnRH3-induced LH release at 6, 12, and/or 24 hrs, as well as GnRH3-stimulate GH release at 6 hrs (but not GnRH2-elicited

GH secretion), are similarly attenuated by pan-selective inhibitors of PI3Ks and MEK1/2 suggest that these pathways may exert parallel influence and/or work in an integrated fashion in mediating GnRH actions in a GnRH- and cell type-selective manner. Unexpectedly, time-dependent augmentations of the ability of GnRH2 and/or GnRH3 to increase hormone release, cellular content, and total hormonal availability were also seen with these general inhibitors of PI3K- and MEK1/2-dependent signalling, revealing that these pathways may also exert inhibitory regulatory influences on agonist actions.

When taken together, results from Chapters 3 to 7 clearly indicate the complexity of post-receptor signalling in the control of GnRH-stimulated actions on pituitary gonadotropes and somatotropes. More detailed characterizations of the relationship between the cascades discussed above in the integrated signalling networks within both gonadotropes and somatotropes will be required.

In addition to contributions to the understanding of PI3K-dependent signalling in the control of GnRH actions, pharmacological mapping revealed a complex role for PI3Ks during the regulation of basal hormone release, which is often dissociated from agonist-stimulated exocytosis. General inhibition of PI3Ks, PtdIns(3,4,5)P_3-sensitive PH domains, and Akt each elevates acute basal LH and GH release, at least transiently; in contrast, selective inhibition of individual class I PI3K isoforms is consistently inhibitory (Chapters 3 to 5). These observations suggest that while the catalytic activity of class I PI3Ks are stimulatory to acute basal hormone release, class II and/or III PI3Ks may exert inhibitory influences on unstimulated hormone secretion in goldfish gonadotropes and somatotropes over the short-term. Observations from Chapters 6 and 8 further reveal that while regulation of long-term (2 hrs) basal GH release generally involves the canonical

PI3K-PDK1-Akt-TORC1 cascade, there is an overall uncoupling of these elements in terms of long-term basal LH secretion. Likewise, regulation of long-term basal GH cellular content and availability, but not those for LH, largely involves the PI3K-PDK1-Akt-TORC1 transduction pathway (Chapter 8). These unique findings not only highlight the complexity of the control of basal hormone release and maintenance of hormone production by intracellular signalling processes, but also provide a cellular mechanism for the uncoupling of basal and agonist-stimulated hormone release in both gonadotropes and somatotropes. Whether the complexity of sustained PI3K-dependent actions involves modulation of *de novo* LH or GH synthesis will be an important area for future research.

Results from this thesis have contributed extensively to the knowledge of PI3K involvement during GnRH actions within natural pituitary cells, however, there are important aspects of PI3K-dependent signalling that remains unknown in this system. Although findings strongly implicate the receptor-mediated class I PI3Ks, they do not exclude the involvement of the class II or III PI3Ks in the control of basal or GnRH-stimulated actions. In fact, the recently established cellular functions of class II and III PI3Ks suggest these enzymes could potentially contribute to the integrated control of regulated exocytosis (Backer, 2008; Falasca and Maffucci, 2012; Chapter 1). As a result, data obtained using pan-PI3K inhibitors in Chapters 3, 6, and 8 are not at odds with the hypothesis that class II and/or III PI3Ks contribute to the control of basal and agonist-stimulated pituitary hormone release; however, this hypothesis needs to be verified using isoform-selective inhibitors of individual PI3Ks. In addition, results reveal the participation of PtdIns(3,4,5)P_3-sensitive signalling elements that are not part of the linear class I PI3K-PDK1-Akt cascade (Chapters 5 and 8). Future studies examining the roles of

non-canonical PtdIns(3,4,5)P_3-sensitive transduction components will also be important for understanding GnRH-selective signalling in goldfish gonadotropes and somatotropes. In particular, non-canonical PtdIns(3,4,5)P_3-dependent signalling could involve members of the cytohesin family of Arf-GEFs, including GRP1 and ARNO (Jackson et al., 2000; D'Souza-Schorey and Chavier, 2006). Additionally, comparisons of the cellular responses to inhibitors of PtdIns(3,4,5)P_3-sensitive PH domains, in general, with those elicited by ATP-dependent inhibitors of the class I PI3Ks or downstream kinases (Chapters 3 to 5), suggest additional non-catalytic roles for PtdIns(3,4,5)P_3-PH domain interactions; such as during the formation of scaffolds important for GnRHR-stimulated signalling or hormone-vesicle trafficking (Lemmon et al., 2008; Salamon and Backer, 2013; Burke and Williams, 2015). Whether PtdIns(3,4,5)P_3-sensitive effectors directly interact with GnRHRs is unknown, but there are established roles for class I PI3Ks in the recruitment of GRKs and arrestins to activated GPCRs (Naga Prasad et al., 2001, 2002, 2005; New et al., 2007; DeFea, 2008; Noor et al., 2011).

Overall, the studies presented within this thesis are deliberately broad in scope due to the relative lack of information available regarding the importance of PI3K-dependent signalling in the control of GnRHR-mediated signalling, or the biology of pituitary cells in general. As a result, in addition to the outstanding questions identified above, I would now like to closely examine a few of the significant avenues for future research while also highlighting the general importance of some of the novel data presented in this thesis. Where possible, a minimal model that accounts for the data presented in this thesis involving the selective control of basal and agonist-stimulated signalling will be provided.

9.2 Differential integration of PI3K-dependent signalling downstream of GnRHRs in goldfish gonadotropes and somatotropes

The results presented in this thesis provide strong evidence for the hypothesis that the intracellular mechanisms contributing to GnRH2 and GnRH3 actions on hormone release in goldfish gonadotropes and somatotropes are not identical. Furthermore, for the first time, we demonstrate GnRH-selective regulation of membrane-proximal signal transduction systems that are tightly coupled to receptor activation. Specifically, I propose that GnRH-induced dissociation of Gα subunits and Gβγ heterodimers, downstream of GnRHR activation, likely plays a central role in the differential signalling utilized by GnRH2 and GnRH3 to regulate acute LH and GH secretion. It is reasonable to hypothesize that $G\alpha_{q/11}$-mediated activation of PLCβ leads to PKC- and Ins(1,4,5)P_3-dependent events, while dissociated Gβγ heterodimers are part of the mechanism(s) leading to selective PI3K-dependent signal transduction by differential actions on p110β and p110γ (Figure 9.1). However, in addition to the bifurcation of $G\alpha_{q/11}$- and Gβγ-dependent signalling, whether activation of the atypical PKCζ isozyme occurs downstream of PI3K-dependent signalling and/or that PI3K catalytic activity also acts upstream of novel and conventional PKCs to control GnRH-stimulated hormone release are possibilities that require further study. Additionally, unlike the proposed Gβγ-dependent activation of p110β and p110γ, the mechanism coupling involvement of p110δ catalytic activity to GnRHR-mediated actions in both gonadotropes and somatotropes remains unresolved. Studies in mammals have also identified p110δ as a downstream component of GPCR-mediated signalling in immune cells, unfortunately the activation

mechanism controlling this relationship is unknown at this time, but it is thought to be independent of interactions with Gβγ heterodimers (Okkenhaug et al., 2013). In addition, although involvement of direct Gβγ-dependent activation of p110β and p110γ seems likely, we cannot exclude the possibility that transactivation of RTKs, likely via activation of MMPs, also contributes to the involvement of p110β- and/or p110δ-signalling downstream of GnRHRs. In fact, MMP-dependent transactivation has been proposed as a mechanism for GnRHR-mediated regulation of PI3K- and/or Akt-dependent signalling in GnRHR-transfected GT1-7 neurons (Shah et al., 2006), as well as ovarian (Ling Poon et al., 2011) and endometrial (Wu et al., 2013) cancer cells. GPCR-mediated transactivation of RTKs classically involves either PKC-, Ca^{2+}-, or SFK-dependent recruitment of MMPs (Liebmann, 2011; Figure 9.1). The inability of pan-PI3K inhibitors to alter hormone release responses triggered by either synthetic PKC activators or a Ca^{2+}-ionophore suggest that PKC- and Ca^{2+}-dependent signalling do not activate PI3Ks through GnRHR-mediated transactivation. However, future studies examining the involvement of SFKs, including c-Src, or the effects of directly inhibiting MMP catalytic activity will be important for addressing whether transactivation contributes to either GnRH2- or GnRH3-dependent recruitment of class IA PI3Ks.

Since inhibition of Akt and BTK catalytic activity increases GnRH3- and decreases GnRH2-induced GH release, respectively, PtdIns(3,4,5)P_3-sensitive effectors activated downstream of class I PI3Ks likely selectively modulate GnRH activities in somatotropes (Chapter 5). However, selective regulation of GnRH activity by transduction effectors downstream of class I PI3K signalling is not seen in gonadotropes. It is interesting to note that differential integration of PI3K- and Ca^{2+}-dependent

signalling is also only observed in somatotropes (Chapter 3). Although it is plausible that the GnRH-selective utilization of class I PI3K isoforms contributes to differences in how PI3K-dependent signalling integrates with downstream Ca^{2+}-dependent signalling, the relationships between specific class I PI3K isoforms and changes in $[Ca^{2+}]_i$ remain to be investigated. In addition to intracellular Ca^{2+}-mobilization, differences in the GnRH-selective usage of p110β, p110γ, and p110δ catalytic activity could also contribute to extracellular Ca^{2+} entry, likely through either L-type VSCCs or TRPCs (discussed in Chapter 3; Figure 9.2). However, most interestingly, the involvement of the class I PI3Ks strongly implicates members of the Ras, Rho, and Arf families of small GTPases through the regulation of their GEFs and GAPs (Hawkins et al., 2006; Vanhaesebroeck et al., 2010; Burke and Williams, 2015). In particular, observations using an inhibitor of PtdIns(3,4,5)P_3-PH domain interactions in Chapter 5 strongly implicates the involvement of the PH domain-containing Arf GEFs, GRP1 and ARNO, in the integrated control of GnRH actions in gonadotropes and, potentially, somatotropes. Interestingly, the activities of these signalling elements are all tightly associated with vesicle trafficking and regulation of the actin cytoskeleton (Lindmo and Stenmark, 2005; Vanhaesebroeck et al., 2010; Croisé et al., 2014; Figure 9.2). Studies of insulin secretion from pancreatic β-cells have demonstrated that agonists of GPCRs regulate hormone exocytosis through p110γ-dependent actin depolymerization leading to enhanced targeting of secretory granules to the plasma membrane (Pigeau et al., 2009; Kolic et al., 2014). Whether similar mechanisms are involved in GnRHR-mediated hormone secretion should be explored.

Outside of interactions with PKCs and changes in $[Ca^{2+}]_i$, results presented in this thesis, and summarized in Section 9.1, suggest that PI3K- and MEK1/2-dependent

signalling are activated in parallel to control long-term hormone release responses to GnRH in a time-dependent fashion. In addition, these two pathways may also act in concert in regulating basal GH release at 2 hrs (Chapters 6, 7, and 8). It is clear that additional studies are needed to address the communication between these pathways during agonist-stimulated actions. Although it has been shown that PI3K-dependent signalling can occur upstream of Raf-MEK-ERK signalling in many systems, this possibility can largely be ruled out (discussed in Chapter 7); although, at this time, we cannot exclude the alternative possibility that MEK1/2-dependent signalling may activate PI3Ks, perhaps through ERK1/2-dependent inhibition of the phosphoinositide 3′-phosphatase PTEN (Aksamitiene et al., 2012). Outside of acting upstream or downstream of one another, several lines of evidence suggest that cross-talk between PI3K-dependent signalling and the Raf-MEK-ERK pathway may contribute to GnRH selectivity, particularly in somatotropes, as well as provide a basis for the ability of Akt inhibition to augment GnRH3-elicited GH release (discussed in Chapter 7; Figure 9.3).

In addition to the potential cross-talk between PI3K- and MEK1/2-dependent signalling in the control of acute GnRH actions summarized above and in Chapter 7, it is likely that these pathways also converge during the long-term control of pituitary cell functions. Due to the narrow substrate specificity of many of the proximal signalling components, it is thought that the majority of the signalling interactions between these cascades occur at distal effectors, including Akt and ERK1/2, that phosphorylate a wide variety of effector proteins (Mendoza et al., 2011). In particular, Ras-dependent activation of the Raf-MEK-ERK cascade can lead to increased TORC1 activity through ERK1/2-mediated phosphorylation of TSC2 (Ma et al., 2005). Although the sites

phosphorylated by Akt and ERK1/2 are different, they both inhibit the GAP activity of the TSC complex and promote TORC1 activity (Ma et al., 2005; Zoncu et al., 2011). Furthermore, ERK1/2-dependent signalling can also stimulate TORC1 activity directly by phosphorylating RAPTOR (Carriere et al., 2011). Taken together, the Raf-MEK-ERK cascade can contribute to PI3K- and Akt-independent regulation of TORC1 activity (Mendoza et al., 2011; Figure 9.3). These studies are extremely interesting when considering the evidence for the uncoupling of TOR-dependent signalling from the canonical PI3K-PDK1-Akt machinery presented in Chapter 8. Thus, it is very likely that interactions between PI3K-dependent and Raf-MEK-ERK signalling converging on membrane-distal effectors such as TORC1 can explain the many of the parallel effects observed between the actions of pan-PI3K- and MEK1/2-selective inhibitors in the control of prolonged actions on basal and GnRH-stimulated cellular actions. Much like the activation status of Raf during acute actions (discussed in Chapter 7), future studies examining the prolonged functions of gonadotropes and somatotropes will need to focus on the convergence of signals on the TOR signalling machinery to truly understand the integrated control of hormone availability.

9.3 PtdIns(3,4,5)P_3 dynamics and the potential role of PtdIns phosphatases as modulators of GnRH action

Class I PI3Ks represent an important intracellular signalling locus for molecular crosstalk and are known to participate in intracellular feedback networks that dynamically modulate the magnitude or characteristics of signalling outputs (Carracedo and Pandolfi, 2008; Figure 9.4). Most importantly, activation of PTEN directly reduces

intracellular concentrations of PtdIns(3,4,5)P_3 and attenuates class I PI3K-dependent signalling (Song et al., 2012). The dynamic control of class I PI3K and PTEN activity creates a unique spatial and temporal code of PtdIns(3,4,5)P_3 production that compartmentalizes PtdIns(3,4,5)P_3-responsive effectors in membrane microdomains (Funamoto et al., 2002; Gao et al., 2011; Karunarathne et al., 2013a,b; Naguib et al., 2015). Furthermore, the cellular concentrations of PtdIns(3,4,5)P_3 are also directly regulated by the sequential activity of 5′- and 4′-phosphatases that produce PtdIns(3,4)P_2 and PtdIns(3)P, respectively (Leslie et al., 2008). As outlined in Chapter 1, PtdIns(3,4)P_2 and PtdIns(3)P can both act as distinct intracellular signals; furthermore, results on the cellular consequences of PtdIns(3,4,5)P_3 removal by PTEN or PtdIns 5′-phosphatases suggest that these routes are functionally distinct (Leslie et al., 2008; Xie et al., 2013; Figure 9.4). However, whether the spatiotemporal dynamics of PtdIns(3,4,5)P_3 production and turnover are unique to different class I PI3K isoforms remains unresolved. On the other hand, the possibility that spatiotemporal segregation of PtdIns(3,4,5)P_3 production contributes to biased class I PI3K signalling is supported by results presented in Chapter 3 demonstrating that the dynamics of intracellular Ca^{2+} signals generated by GnRH2 and GnRH3 are differentially altered by broad-spectrum inhibitors of PI3Ks in isolated pituitary cells. When considered together with the demonstration of the differential involvement of unique class I PI3K catalytic subunits (Chapter 4), as well as the recruitment of distinct PtdIns(3,4,5)P_3-sensitive effectors (Chapter 5), it is likely that the membrane dynamics of the GnRH-stimulated PtdIns(3,4,5)P_3 signal contribute to the selective control of hormone release responses in goldfish gonadotropes and somatotropes. Future studies using selective inhibitors of

specific lipid and protein phosphatases in GnRH-dependent signalling will be important for understanding the potential role for PtdIns(3,4,5)P_3 dynamics in agonist-selective cellular functions, as well as for the integrated control of basal hormone availability. Regardless of what future investigations on the role of cellular phosphatases in modulating PI3K-dependent signalling may reveal, the complex roles for class I PI3K signalling identified in this thesis already make goldfish pituitary cells an attractive platform for examining agonist-selective activation of PtdIns(3,4,5)P_3-sensitive signal transduction, as well as identify PI3Ks as ideal targets for signal integration during the multifactorial control of endocrine cell functions (see Section 9.9 below).

9.4 Uncoupling of basal- and agonist-stimulated hormone release

Heterogeneity in the hormone vesicle release pool is thought to be an important feature of neurons and neuroendocrine cells (Rizzoli and Betz, 2005; Crawford and Kavalali, 2015), and agonist-selective coupling to unique intracellular Ca^{2+} stores has been extensively characterized in goldfish gonadotropes and somatotropes (reviewed in Chapter 1). Furthermore, at least two agonist-selective release pools that are either PKA- or PKC-dependent exist in both gonadotropes and somatotropes of goldfish and these pools both participate in the mutifactorial neuroendocrine regulation of LH and GH secretion by multiple stimulatory and inhibitory release factors (Chang et al., 2000). However, there is no available evidence for heterogeneity within either of the PKC- or PKA-sensitive release pools, and results using GnRH2 and GnRH3 show that the release responses triggered by known PKC-dependent effectors are not additive (Chang et al., 1993). However, in general, basal LH and GH release in goldfish are controlled primarily

by PKA- and not PKC-dependent signalling (Wong et al., 1994; Jobin et al., 1996; Chang et al., 2000), suggesting that basal and agonist-sensitive hormone release pools can be different. Results presented throughout this thesis are in agreement with this hypothesis and identify PI3K-dependent signalling as an important regulatory element allowing for the selective control of basal and agonist-stimulated hormone release. In particular, data presented in Chapter 5 demonstrates that PtdIns(3,4,5)P_3- and Akt-dependent signalling are major regulators of basal LH and GH release. With this in mind, it is interesting to speculate on the selective control of the cytohesin Arf-GEFs during basal and agonist-induced hormone release responses. When the results presented in this thesis are considered together with previous studies in goldfish, it seems likely that differences in PKA- or Akt-dependent signalling are involved in the integrated control of basal secretion, while stimulation of PKCs by $G\alpha_{q/11}$-coupled GPCRs would mediate GnRH-stimulated hormone release responses. Interestingly, phosphorylation of the cytohesins GRP1 and ARNO by PKCs contributes to their plasma membrane localization and activity (DiNitto et al., 2007; Frank et al., 1998). Perhaps, the relative strength of the signalling input from PKC following GnRHR activation results in local increases in Arf-GEF activity resulting in GnRH-selective increases in hormone release responses. Likewise, GnRH-stimulated translocation of GRP1 and ARNO would likely be facilitated by transient increases in receptor-mediated activation of class I PI3Ks, whereas high constitutive recruitment and activation of Akt would control basal secretion. Whether the dynamic control of this system would also be subject to cross-talk between Akt- and PKA-dependent signalling (discussed in Chapter 5) should also be investigated. Similarly, whether uncoupling of the PI3K-PDK1-Akt-TOR cascade, as observed for

long-term studies of hormone release, also contributes to the acute control of basal hormone release needs to be addressed.

In addition to a clear role in the control of GnRH-stimulated hormone release responses, Gβγ also appears to participate in the control of basal hormone secretion; treatment with an allosteric inhibitor of Gβγ-effector interactions decreases basal LH and GH release (Chapter 4). Interestingly, the magnitude of NSC8668 effects on basal LH release mirrored those observed using selective inhibitors of p110β, p110γ, BTK, and MEK1/2 catalytic activity. However for somatotropes, although NSC8668 again mirrored the responses observed for the class I PI3K and BTK inhibitors, basal GH release was only sensitive to inhibition of p110γ, but not p110β, and the kinetics of MEK1/2-dependent basal GH release were dissimilar to those see with inhibition of Gβγ-effector interactions (Chapters 4, 5, and 7). Taken together, these observations suggest that p110β and p110γ may be redundant Gβγ-sensitive signals during the constitutive control of basal LH release and that their downstream signalling may include the activation of BTK and/or MEK1/2. Conversely, Gβγ heterodimers may selectively target p110γ to control basal GH release. The pattern of involvement observed for Gβγ-dependent effectors during basal hormone release is quite distinct from GnRH-stimulated responses; whereas the two GnRHs selectively activate p110β or p110γ to control LH release, both GnRHs are sensitive to inhibition of both p110β and p110γ in terms of GH secretion. As a result, we hypothesize that the repertoire of heterodimeric Gβγ subunits involved in the control of basal and GnRH-stimulated actions might be different (see also Section 9.6).

9.5 PtdIns(3,4,5)P_3 production and the control of exocytosis

To date, studies of the roles of PI3Ks in the control of exocytosis have relied on the use of wortmannin and LY294002. As outline in Chapter 3, although these are useful tools for screening cellular responses for the involvement of PI3K-dependent signalling, results from experiments using these compounds can be difficult to interpret. Major concerns when using these compounds in isolation can include the lack of inhibitory effects against class II isoform PI3K-C2α and changes in acute PtdIns(4,5)P_2 availability or inhibition of PI4Ks (Osbourne et al., 2006; Wen et al., 2011). Furthermore, these compounds do not allow for the selective targeting of individual PI3K isoforms and, as a result, the precise roles for class I PI3K catalytic activity and PtdIns(3,4,5)P_3 production in the control of exocytosis remain largely uncharacterized. Results presented in this thesis demonstrate that catalytic activities of the class I PI3Ks are positive regulators of basal and GnRH-stimulated hormone secretion in pituitary cells. Work done using *Drosophila melanogaster*, as well as in mammalian systems, implicate PtdIns(3,4,5)P_3 as an essential component of neurotransmitter release. In particular, PtdIns(3,4,5)P_3 production is critical to the control of secretory vesicle exocytosis by inducing the clustering of syntaxin1A, a protein essential for synaptic vesicle fusion (Khuong et al., 2013). Similarly, direct binding of the p85-type regulatory subunits of the class IA PI3Ks to synapsin-I controls PI3K-dependent catalytic activity and promotes the delivery of synaptic vesicles from the reserve pool to the readily-releasable pool for exocytosis (Cousin et al., 2003). Interestingly, synapsins are synaptic vesicle-associated phosphoproteins known to integrate stimulatory signals from both PKA and CaMKI that promote exocytosis (Hosaka et al., 1999; Menegon et al., 2006). Perhaps, the PKA-dependent regulation of basal LH and GH release in goldfish is dependent upon the

associated activity of class I PI3Ks; whereas signals from CaMKs mediate the selective actions of PI3Ks during agonist-stimulated release. In addition to synapsin, synaptic vesicles also contain the Ca^{2+}-sensor synaptotagmin, which is essential for regulated control of Ca^{2+}-dependent exocytosis. Interestingly, increases in the $[Ca^{2+}]_i$ observed during stimulation of neurotransmitter release switch the specificity of the C2B phosphoinositide-binding domain of synaptotagmin from PtdIns(3,4,5)P_3 (resting $[Ca^{2+}]_i$) to PtdIns(4,5)P_2 ($[Ca^{2+}]_i$ required for transmitter release; Schiavo et al., 1996). The dynamics of the switch from PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 binding have been proposed to play a role in the coordinate control of vesicle docking and fusion (Schiavo et al., 1996). Taken together, these studies suggest a novel role for PtdIns(3,4,5)P_3 production, independent of kinase recruitment and activation, during the control of regulated exocytosis.

Outside of the general roles in vesicle docking, it is also possible that PtdIns(3,4,5)P_3 production acts to acutely reduce PtdIns(4,5)P_2 availability. Plasma membrane depletion of PtdIns(4,5)P_2 has been shown to be mandatory for exocytosis and is regulated by the activation of PLCs (Hammond et al., 2006). Interestingly, independent of its ability to generate Ins(1,4,5)P_3 and stimulate the release of Ca^{2+} from intracellular stores, PLC actions on exocytosis have also been shown to be, at least in part, DAG-mediated (Hammond et al., 2006). These data are in agreement with studies using goldfish pituitary cells demonstrating a role for PLC- and DAG-dependent, but Ins(1,4,5)P_3-insensitive, signalling in the control of GnRH2-stimulated hormone release (Chang et al., 2009, 2012). Consequently, it is interesting to hypothesize that PtdIns(3,4,5)P_3 production by receptor-activated class I PI3Ks also contributes to the

transient depletion of plasma membrane PtdIns(4,5)P_2 in goldfish pituitary cells. This mechanism would be particularly relevant for GnRH2 although it may also be applicable for GnRH3 actions for goldfish considering that both GnRHs utilize $G\alpha_{q/11}$-dependent activation of PLCs, as well as Gβγ-sensitive isoforms of class I PI3Ks (Chapter 4). Regardless of whether one or more of the proposed linkages to exocytosis occurs during GnRH activation of GnRHRs in goldfish pituitary cells, results from the available literature suggest an emerging role for PtdIns(3,4,5)P_3 production during exocytosis, primarily through the recruitment and docking of vesicles at the plasma membrane. Whether the activity of class I PI3Ks is associated with the maintenance of hormone release pools, or is required for the control of vesicle docking and trafficking in pituitary cells will be important areas for future research.

9.6 Gβγ-mediated signalling bias: a novel GPCR signalling paradigm

In addition to its role in the assembly and trafficking of GPCR signalling complexes, Gβγ subunits can modulate the activity of numerous cellular effectors via direct interactions (Khan et al., 2013). As discussed earlier, results in Chapter 4 suggest that GnRHR activation leads to activation of the Gβγ-sensitive p110β and p110γ isoforms of class I PI3K in a GnRH- and cell type-specific manner. Interestingly, MEK1/2-dependent signalling also participate in the control of GnRH-stimulated hormone release (Chapter 7; Klausen et al., 2008; Chang et al., 2009) while BTK modulates GnRH actions on GH secretion (Chapter 5). Regulation of BTK (Tsukada et al., 1994) and the Raf-MEK-ERK (Crespo et al., 1994; Pumiglia et al., 1995) transduction cascade can also be Gβγ-dependent. Taken together, these data suggest that GnRHR-mediated signalling

involves GnRH-selective interactions with Gβγ-dependent effectors and provide the first evidence for the direct coupling of Gβγ-mediated signalling to GnRHR activation in any system. These findings are also extremely novel in the sense that they provide evidence for the possible involvement of Gβγ subunits in the manifestation of GnRHR-mediated signalling bias, as well as represent the first demonstration of biased Gβγ-dependent signal transduction responses for any natural peptide-GPCR system.

Conceptually, in the proposed biased Gβγ-dependent signalling paradigm, structurally-related ligands would shift the conformational equilibium to specific receptor state(s) that selectively activate some Gβγ-sensitive effectors but not others. Undoubtedly, the conformational dynamics of this process would be tightly associated with the Gα subunit. As a result, unlike previous studies suggesting that promiscuous coupling of a single GPCR to multiple families of Gα subunits, Gβγ-mediated signalling bias would allow for heterogeneity in the signalling downstream of GPCRs that signal through a single family of Gα. Interestingly, the heterotrimeric Gαβγ complex is assembled from a pool of 16 Gα subunits, 5 Gβ subunits, and 12 Gγ subunits (Khan et al., 2013). However, recent evidence suggests that a single GPCR can discriminate between different Gαβγ complexes and different Gα subunits display preferences for the identity of the Gγ subunits within the heterotrimer (Hillenbrand et al., 2015). Furthermore, variation among the basic and hydrophobic residues present in different Gγ subunits contributes to differences in Gβγ-membrane dissociation rates and translocation kinetics in response to GPCR activation (O'Neill et al., 2012). Therefore it is possible that heterogeneity in the molecular identity of the Gαβγ complex, and in particular the Gβγ heterodimer, could contribute to signal-specific downstream responses that are dependent

upon the agonist-stabilized conformation of the GPCR. This would also allow for the biased regulation of signalling outside of, or in concert with, Gαβγ-independent mechanisms such as the recruitment of arrestins (Reiter et al., 2012).

In support of biased signalling mediated by Gβγ subunits, a recent report has identified a novel allosteric modulator that selectively inhibits Gβγ-dependent signalling without influencing transduction downstream of Gα subunits upon ligand-dependent activation of the Gαβγ heterotrimer (Blättermann et al., 2012). Although this finding is difficult to reconcile with the current structural models for Gαβγ nucleotide exchange and dissociation, these data suggest that GPCR-Gβγ interactions may contribute to the activation of Gβγ-dependent signalling (Smrcka, 2013). Thus, the presence of a clear structure-activtiy relationship between GnRH variants and GnRHRs in goldfish (reviewed in Chapter 1), coupled with the GnRH-selective regulation of Gβγ-sensitive effectors demonstrated in this thesis, strongly implies that agonist binding could result in the stabilization of receptor conformations that promote biased signalling outcomes dependent upon Gβγ, and not Gα, subunits. Understanding the structural biology and cellular significance of Gβγ-mediated signalling bias will be an exciting avenue for future studies of functional selectivity downstream of activated GPCRs.

9.7 Biased GnRHR signalling and goldfish: future directions

Results presented in this thesis provide further evidence demonstrating that the native GnRH2 and GnRH3 isoforms utilize ligand-biased intracellular signalling mechanisms to stimulate hormone release responses from goldfish somatotropes and gonadotropes (Figure 9.5, GnRH2-stimulated LH release; Figure 9.6, GnRH3-stimulated

LH release; Figure 9.7, GnRH2-stimulated GH release; Figure 9.8, GnRH3-stimulated GH release). In addition, they strongly suggest that goldfish GnRHRs adopt ligand-selective conformations to differentially control pituitary cell functions. As outlined in Chapter 1, goldfish express two unique isoforms of GnRHRs. thus, it is possible that the differences in GnRH2 and GnRH3 selectivity observed both between and within gonadotropes and somatotropes are due, at least in part, to the differential activity and/or localization of the GfA and GfB GnRHRs. On the other hand, the demonstration that the LH- and GH-releasing activities of both GnRHs require the same population of GnRHs on either gonadotropes or somatotropes, respectively, suggests that distinct GnRH2- or GnRH3-selective receptors are not present on either cell type. Interestingly, preliminary immunoneutralization studies done previously using antibodies reported to be selective for the 3rd extracellular loop of GfA and GfB GnRHRs, respectively (provided by Robert P. Millar, Department of Molecular and Cell Biology, University of Cape Town, South Africa; Parhar et al., 2002; Peter et al., 2003), indicate that the polyclonal antibody raised against GfA GnRHR abrogates both GnRH2 and GnRH3 stimulation of GH, but not LH, release from goldfish pituitary cells in static incubation; conversely, the antibody raised against GfB GnRHR reduced GnRH2 and GnRH3 effects on both LH and GH secretion; the GfA GnRHR antibody also did not stain morphologically identified goldfish gonadotropes (J.P. Chang and R.E. Peter, unpublished results). These results would suggest that only one type of GnRHR is present on goldfish gonadotropes but both isoforms are present on somatotropes. However, these results should be taken with caution since a detailed analysis of the antigen sequences used for immunization with the predicted amino acid identities from the reported cDNA sequence of these receptors

revealed the presence of one mismatched amino acid in each of the GfA and GfB GnRHR antigenic peptides (J.G. Pemberton, personal observation). Future studies characterizing the GnRHR complement on goldfish gonadotropes and somatotropes will be needed for understanding the endogenous contributions of biased signalling by the GfA and GfB GnRHRs. In particular, the production of new selective GfA and GfB GnRHR antibodies, and especially those designed to target the extracellular domains of these receptors, would facilitate studies on GnRHR localization, trafficking, and association with other membrane proteins or intracellular effectors; as well as for functional studies using immunoneutralization approaches as described above.

Interestingly, early studies demonstrated that GnRH2 causes a greater degree of down-regulation of receptor number when compared to GnRH3 (Habibi, 1991a). GnRH2 is also more effective in causing desensitization of hormone release when given as either continuous or extended pulses, or as a pulse every 20 min; conversely, GnRH3 is more effective when administered as pulses every 60 min (Habibi, 1991b). Furthermore, alternate pulsatile treatments with GnRH2 and GnRH3 in 30 min intervals cause lower desensitization compared to repeated pulses of GnRH2 or GnRH3, or repeated pulses of the two combined (Khakoo et al., 1994). These data can be interpreted as that GnRH2 and GnRH3 stimulate distinct patterns of GnRHR trafficking in pituitary cells. The regulation of GPCR-dependent signalling networks by endocytotic membrane trafficking is well characterized (Sorkin and von Zastrow, 2009; Calebiro et al., 2010). Whether GnRH2- and GnRH3-stimulated signalling differentially regulates the internalization and/or recycling of GnRHRs in gonadotropes and somatotropes will be important areas for future research. Similarly, due to their intricate roles in receptor internalization,

studies of GnRHRs should also look at the relative importance of GRKs and arrestins during agonist-induced desensitization as well as intracellular signalling.

9.8 Implications for PI3K-dependent signalling during multifactorial neuroendocrine regulation of goldfish gonadotrope and somatotrope functions

The finding that PI3K-dependent signalling is an important transduction target for the differential actions of GnRHs on LH and GH release significantly contributes to the understanding of GnRHR-mediated intracellular mechanisms important for the physiological control of reproduction and growth. In addition these studies also provide the intracellular basis for understanding the neuroendocrine control of gonadotrope and somatotrope functions by multiple regulators, as well as the possible functional significance of the two endogenous variants of GnRH.

For example, in terms of GnRH functions, both GnRH2 and GnRH3 reliably elevate LH and GH secretion, but GnRH2 is generally more effective as an LH secretogogue (reviewed in Chapter 1). Interestingly, the *in vitro* kinetics of the GnRH2-induced LH release is consistently biphasic in nature whereas the LH responses to GnRH3 are largely monophasic during times of sexual regression or early gonadal recrudescence, but becomes biphasic at times of late gonadal recrudescence (Lo and Chang, 1998b). These changes in LH response kinetics can be attributed to increased positive feedback from sex steroids (including testosterone and estradiol) directly on pituitary cells during gonadal maturation that serves to enhance PKC-dependent cellular responses (Lo and Chang, 1998a; Chang et al., 2009). It is possible that differences in the use of PI3K isoforms and the related signalling mechanisms revealed in this thesis also

contributes to these kinds of kinetic differences; and accordingly, whether steroid feedback affects GnRHR-mediated activation of these transduction pathways will be an important area in future studies. Interestingly, brain GnRH mRNA expression studies also indicate that GnRH3 is more important than GnRH2 in inducing the ovulatory LH surge in goldfish (Canosa et al., 2008). On the other hand, of the two GnRHs, GnRH2 may be more effective than GnRH3 in increasing both GH mRNA expression and hormone production (Chapters 6 and 7). Considering that GH release potentiates LH-stimulated gonadal steroidogenesis (Van der Kraak et al., 1990), these findings would suggest that GnRH2 is more important than GnRH3 for the regulation of gonadal functions during early stages of gonadal recrudescence. Furthermore, since brain GnRH2 also regulates feeding behaviour in addition to stimulating GH secretion (Hoskins et al., 2008; Matsuda et al., 2008), it may be part of the mechanisms integrating energy intake and metabolism. Therefore, the differential effects of PI3K- and Raf-MEK-ERK signalling on long-term hormone availability are also likely to be important signalling targets coordinating GnRH2 and GnRH3 actions on the integration of gonadotrope and somatotrope functions throughout the yearly reproductive and growth cycle.

The complexity of the physiological control of reproduction is further compounded by the fact that numerous GPCR-interacting neuropeptides and neurotransmitters contribute to the multifactorial control of goldfish gonadotrope and somatotrope functions. In addition to GnRHs, ghrelin and pituitary adenylate cyclase-activating peptide (PACAP) have also been shown to stimulate both LH and GH release in goldfish. Briefly, ghrelin utilizes PKC-, Ca^{2+}-, and NO-dependent signalling to increase LH and GH release, and also selectively recruits PKA-dependent signalling

during stimulation of LH release (Chang et al., 2009, 2012; Grey and Chang, 2012, 2013), while PACAP relies on PKA- and Ca^{2+}-dependent signalling to elevate LH and GH secretion (Wong et al., 1994). However, in other model systems, both ghrelin (Delhanty et al., 2006; Waseem et al., 2014) and PACAP (Castorina et al., 2014; May et al., 2010) also signal through PI3K-dependent activation of Akt to control cellular responses. On the other hand, dopamine (DA) is known to increase GH release via D1-type receptors (DRD1), and to suppress LH release via D2-type receptors (DRD2; Chang et al., 1990b). In mammalian models, DA actions through the DRD2 are associated with mental illness as a result of negative regulation of the canonical PI3K-Akt-TOR signalling cascade (Beaulieu et al., 2007; Kitagishi et al., 2012). Similarly, somatostatin (SST) is an important inhibitory signal for GH release, and activation of the type 2 SST receptor negatively regulates class IA PI3K signalling through direct interactions with p85-type regulatory subunits (Bousquet et al., 2006). Accordingly, components of the PI3K-sensitive signal transduction cascade likely represent important targets by which multiple stimulatory and inhibitory neuroendocrine factors affect and coordinate the secretion of LH and GH. By identifying the importance of PI3Ks in GnRH2 and GnRH3 actions on goldfish gonadotropes and somatotropes, including the differential involvement of specific PI3K isoforms and downstream signalling elements, the results of this thesis also provide the basis for future studies of how PI3Ks and other signalling cascades participate in the multifactorial control of goldfish pituitary cell functions.

9.9 Summary

The results presented in this thesis represent the first steps in understanding the

involvement of PI3K-dependent signal transduction in the control of GnRH actions on pituitary hormone release, storage, and synthesis. Findings also add to the general understanding of functional selectivity in GPCR actions by demonstrating, for the first time, the complexity of ligand-biased signaling downstream of class I PI3K catalytic activity. Research of this nature has not been attempted at this level of detail in any pituitary cell model system and serves as the basis for future studies examining the integrated regulation of PI3K isoforms, as well as downstream PI3K-dependent effectors, by multiple stimulatory and inhibitory pituitary cell regulators. Furthermore, by using natural GnRH variants in a primary pituitary cell culture system, results from this thesis demonstrate how the complexity of biased signalling participates in the neuroendocrine control of reproduction and growth by modulating gonadotrope and somatotrope functions regulated by endogenous hypothalamic neuropeptide isoforms. Studies using basal vertebrate models will continue to provide important evolutionary insights into the molecular mechanisms that couple GPCR activation to biased intracellular signal transduction responses and reveal how this phenomenon can ultimately impact whole-organism physiology. Overall, this thesis makes a significant contribution to our understanding of the information transfer across cellular membranes by illustrating novel aspects of the heterogeneity inherent within the mechanisms that couple GPCR activation and phosphoinositide-dependent intracellular signalling.

Figure 9.1 Classical model of ligand-dependent transactivation of RTKs downstream of GPCRs. G protein-coupled receptor (GPCR) activation can lead to the stimulation of different receptor tyrosine kinases (RTKs) and the subsequent recruitment of canonical RTK-associated transduction mechanisms; including class IA phosphoinositide 3-kinases (PI3Ks). The process is commonly referred to as GPCR-RTK transactivation and classically involves GPCR-mediated activation of either protein kinase Cs (conventional (c), novel (n), or atypical (a)PKCs), Src family tyrosine kinases (SFKs; predominantly, either c-Src, Fyn, or Lck), or Ca^{2+}-dependent signalling, depending on the cell type. Briefly, activation of these intracellular pathways stimulates the catalytic activity of a matrix metalloproteinase (MMP) which cleaves a surface-bound growth factor (pro-ligand) that is no able to bind and activate the appropriate RTK. Signalling through GPCR-RTK transactivation is not universal for GPCRs, but allows for the recruitment of non-canonical signalling effectors. In goldfish gonadotropes and somatotropes, gonadotropin-releasing hormone receptors (GnRHRs) likely activate class I PI3Ks through direct binding of Gβγ heterodimers to the p110β and p110γ catalytic subunits. This effectively splits GnRH-dependent signals into Gα- and Gβγ-dependent transduction mechanisms. Additionally, it is possible that GnRHR-mediated transactivation could recruit the class IA PI3K isoforms p110β and p110δ, likely through the activation of SFKs and not PKC- or Ca^{2+}-dependent signalling. Adapted from Wetzker and Böhmer, *Nat. Rev. Mol. Cell Biol.*, 2003.

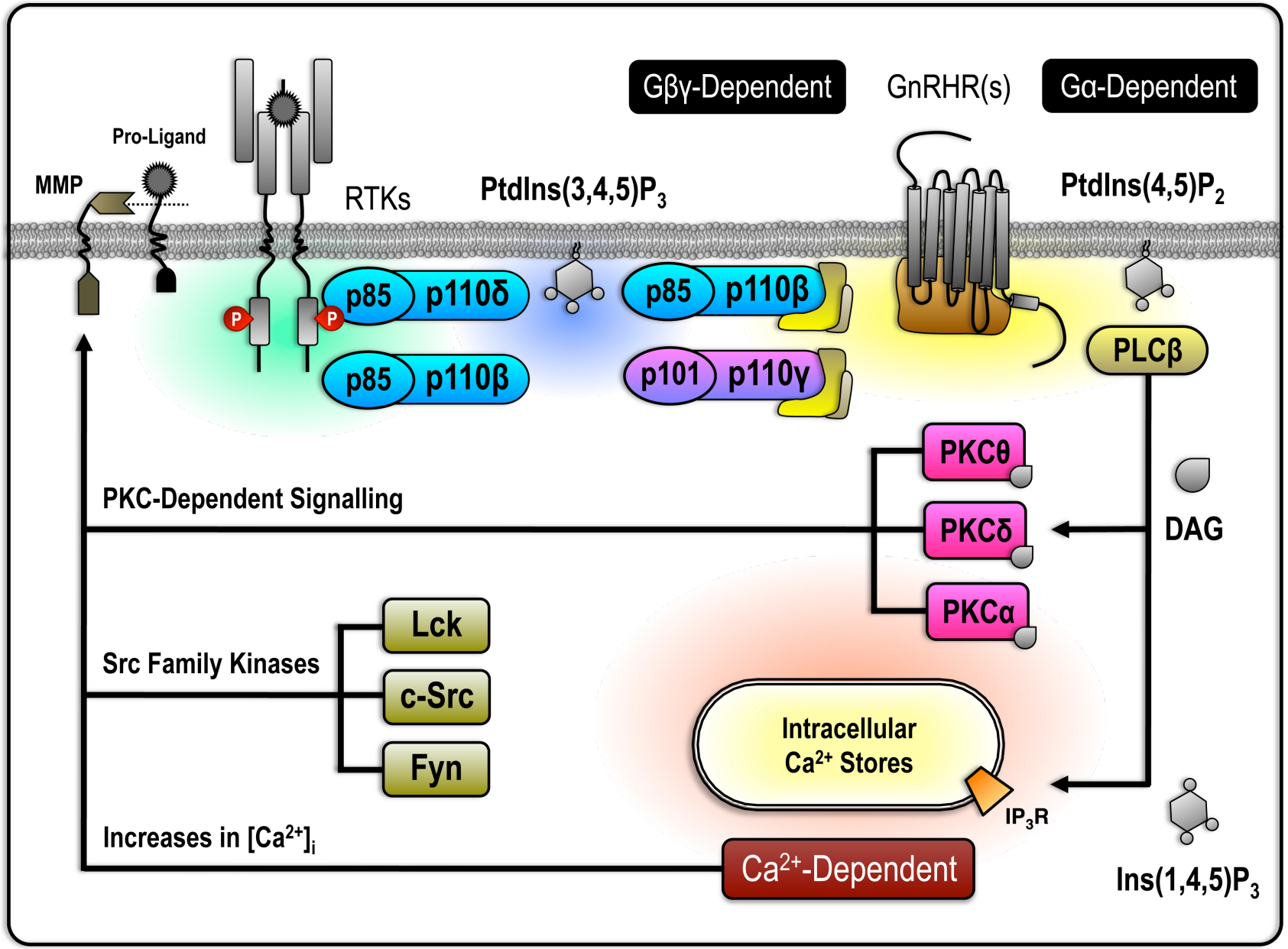

Gβγ-Dependent
GnRHR(s)
Gα-Dependent
Pro-Ligand
MMP
RTKs
PtdIns(3,4,5)P3
PtdIns(4,5)P2
p85
p110δ
p85
p110β
p85
p110β
p101
p110γ
PLCβ
PKCθ
PKCδ
PKCα
DAG
PKC-Dependent Signalling
Lck
c-Src
Fyn
Src Family Kinases
Intracellular
Ca2+ Stores
IP3R
Increases in [Ca2+]i
Ca2+-Dependent
Ins(1,4,5)P3

Figure 9.2 Diversity of non-canonical class I PI3K signal transduction. The class I phosphoinositide 3-kinases (PI3Ks) activate downstream signalling through the production of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). In addition to classical targets such as PDK1, Akt, and BTK, PtdIns(3,4,5)P_3-sensitive pleckstrin homology (PH) lipid-binding domains are present in a wide variety of intracellular signalling proteins. Examples of important non-canonical class I PI3K signalling effectors are shown. Results from this thesis strongly implicate the involvement of the ADP-ribosylation factor (Arf) guanine nucleotide exchange factors (GEFs) general receptor for phosphoinositides 1 (GRP1) and Arf nucleotide-binding-site opener (ARNO) in the control of basal and GnRH-stimulated hormone release. Alternatively, PtdIns(3,4,5)P_3 is also known to recruit the Rac-GEF PtdIns(3,4,5)P_3-dependent Rac exchanger protein 1 (P-Rex1) to control actin remodeling (in concert with Arfs) and activate the Raf-MEK-ERK mitogen-activated protein kinase subfamily. Acute stimulation of PtdIns(3,4,5)P_3 production has also been implicated in the rapid trafficking of L-type voltage-sensitive Ca^{2+} channels and the direct gating of some isoforms of transient receptor potential (TRP) channels to enhance extracellular Ca^{2+} entry. Lastly, isoforms of phospholipase C γ (PLCγ) directly bind and are activated by PtdIns(3,4,5)P_3 at the plasma membrane; although catalytic activity is also tightly regulated by Src family tyrosine kinases (such as c-Src). PLCγ activation results in the activation of diacylglycerol (DAG)-sensitive isoforms of protein kinase C (conventional (C) and novel (n)PKCs) as well as stimulates intracellular Ca^{2+} release from inositol 1,4,5-trisphosphate (Ins (1,4,5)P_3)-gated (IP_3R-containing) Ca^{2+} stores. Overall, these non-canonical mechanisms for the control of PtdIns(3,4,5)P_3-dependent signalling could result in the coordinate control of actin cytoskeleton dynamics and alter intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) to facilitate basal and agonist-stimulated hormone release responses.

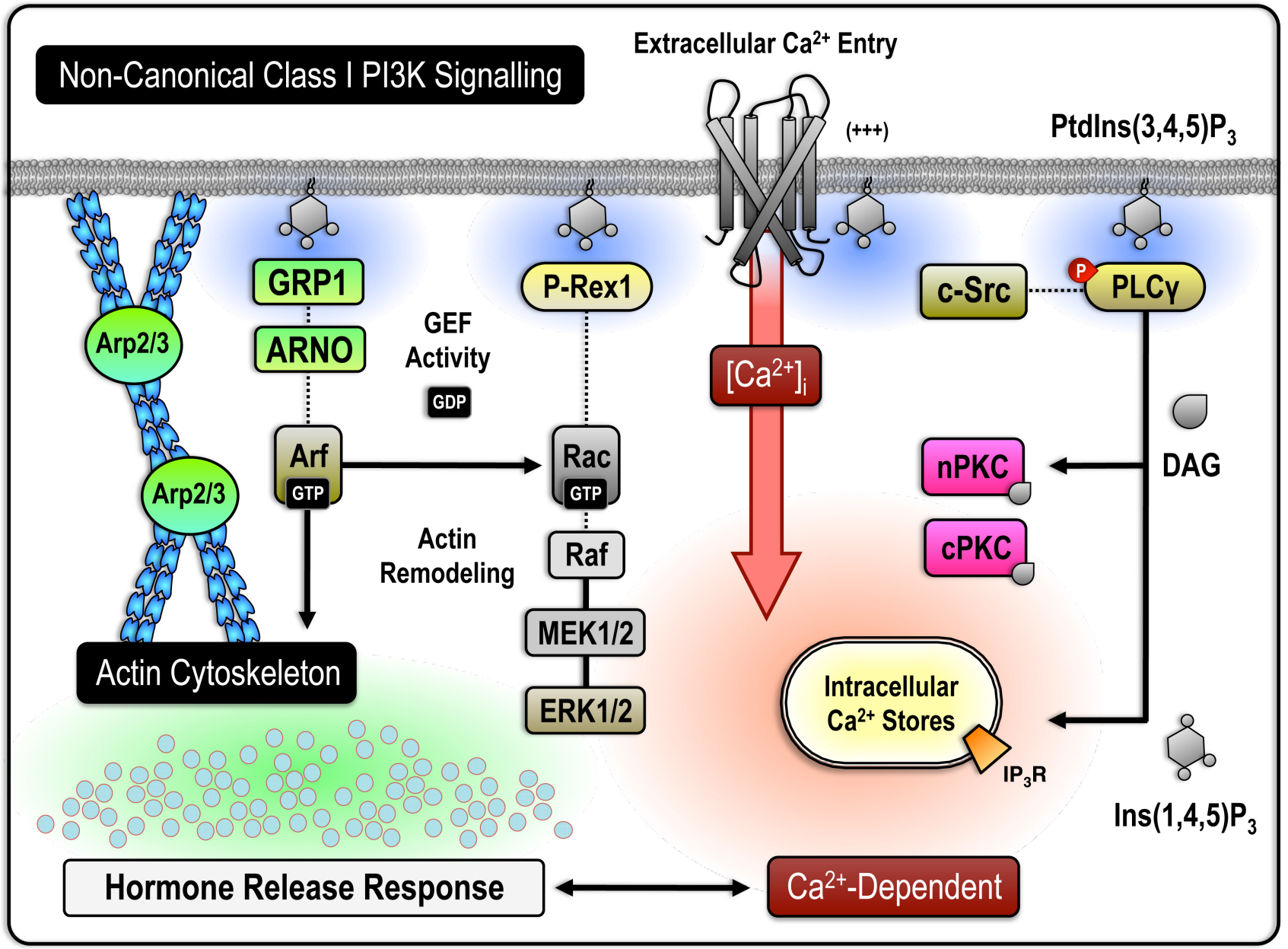

Non-Canonical Class I PI3K Signalling
Extracellular Ca2+ Entry
(+++)
PtdIns(3,4,5)P3
GRP1
ARNO
GEF
Activity
GDP
P-Rex1
c-Src
P
PLCγ
Arp2/3
Arp2/3
Arf
GTP
Rac
GTP
[Ca2+]i
DAG
nPKC
cPKC
Actin
Remodeling
Raf
MEK1/2
ERK1/2
Actin Cytoskeleton
Intracellular
Ca2+ Stores
IP3R
Ins(1,4,5)P3
Hormone Release Response
Ca2+-Dependent

Figure 9.3 Integration of PI3K-dependent and Raf-MEK-ERK signalling. Phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (Akt) are canonical PI3K-dependent intracellular effectors that are recruited to the plasma membrane through phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5)P_3)-selective pleckstrin homology lipid-binding domains. PDK1 functions as an important intermediate during PtdIns(3,4,5)P_3-dependent signalling by phosphorylating the T-loop activation segment of numerous AGC family serine/threonine kinases, including isozymes of: Akt, protein kinase C (conventional (cPKC), novel (nPKC), and atypical (aPKC) isozymes), protein kinase A (PKA), serum- and glucocorticoid-induced protein kinase (SGK), S6 kinase (S6), ribosomal S6 kinase (RSK), and G protein-coupled receptor kinase (GRK). In addition to phosphorylation of the activation loop by PDK1, activation of Akt, as well as conventional isozymes of PKC (cPKC), also requires phosphorylation within the hydrophobic motif by the target of rapamycin (TOR) complex 2 (which consists of TOR kinase bound to the core components rapamycin-insensitive companion of TOR (RICTOR), SAPK-interacting protein 1 (SIN1), and mammalian lethal with SEC thirteen 8 (mLST8); TORC2). Phosphorylation of Akt results in activation of downstream effectors, including the rapamycin-sensitive TOR complex 1 (consisting of the core components TOR kinase, regulatory-associated protein of TOR (RAPTOR), and mLST8; TORC1). Akt activation of TORC1 is indirect and requires the phosphorylation and inactivation of two major negative regulators: tuberous sclerosis complex 2 (TSC2) and phosphorylating proline-rich Akt substrate of 40 kDa (PRAS40). Alternatively, PKC is known to activate the Raf-MEK-ERK cascade through either direct phosphorylation of Raf, or indirectly through the activation of the Ras superfamily guanine nucleotide exchange factor Ras guanyl nucleotide-releasing protein (RasGRP). Activation of Raf-MEK-ERK signalling can also activate TORC1 independently of Akt, through both direct phosphorylation and activation of RAPTOR, as well as phosphorylation and inactivation of TSC2 (ERK-mediated phosphorylation occurs at residues distinct from those targeted by Akt). Interestingly, in addition to convergence on intracellular effectors, activation of Akt directly inhibits the activation of Raf-MEK-ERK signalling by phosphorylating a negative regulatory site on Raf. The balance between PKC- and Akt-dependent phosphorylation of Raf may balance intracellular activation of the Raf-MEK-ERK cassette and thereby modulate Akt-independent inputs into TORC1. In addition, PDK1 and TORC2 are likely to be involved in Akt-independent signalling through the phosphorylation and regulation of AGC kinases; consequently, additional Akt-independent cross-talk with Raf-MEK-ERK may also occur. Overall, it is obvious that PI3K-dependent and Raf-MEK-ERK signalling is extremely complex, and results from this thesis strongly suggest that intracellular signalling through these pathways is likely to alter the basal hormone release and synthesis in gonadotropes and somatotropes.

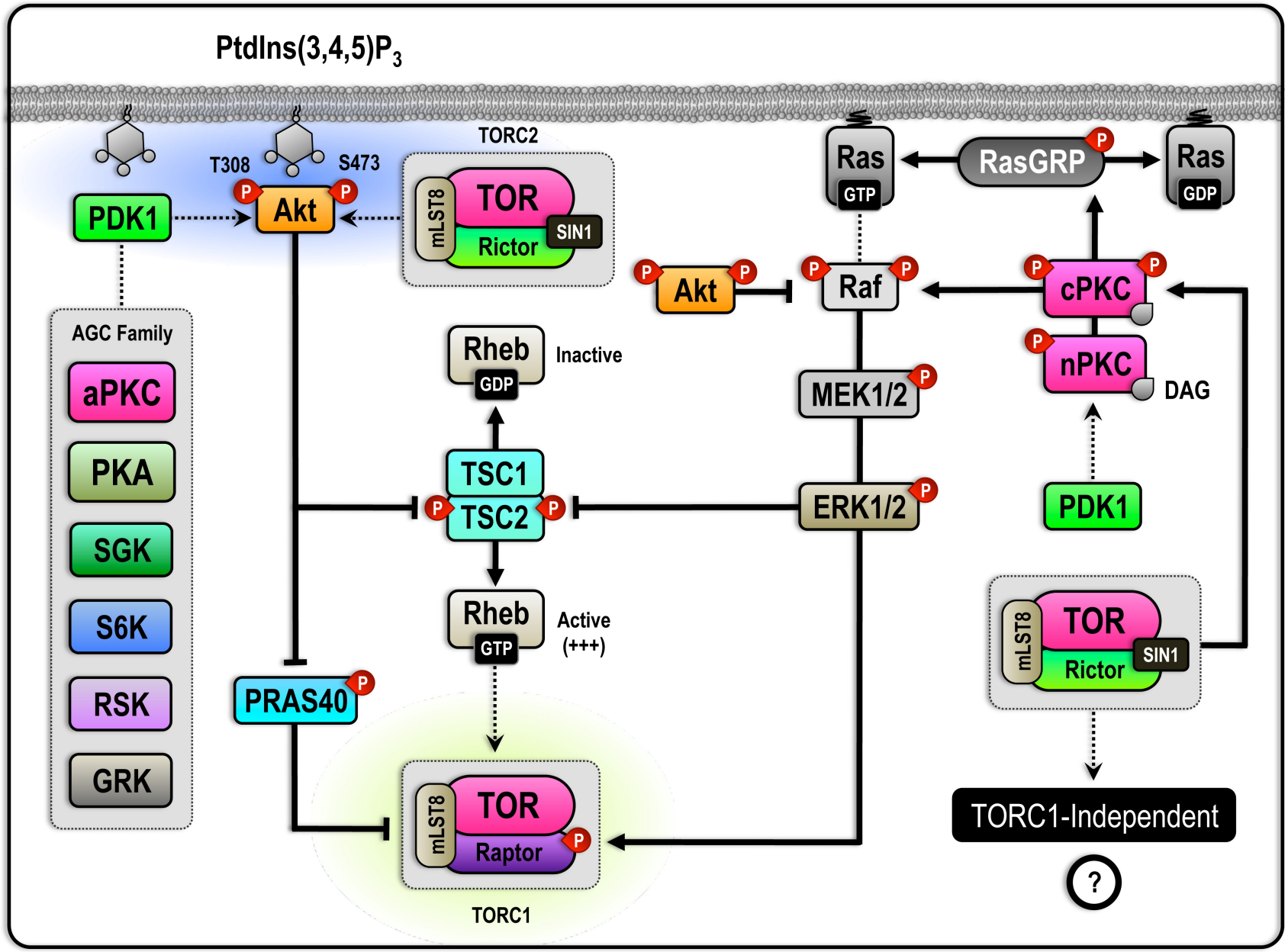

PtdIns(3,4,5)P3
TORC2
T308
S473
PDK1
Akt
mLST8
TOR
Rictor
SIN1
AGC Family
aPKC
PKA
SGK
S6K
RSK
GRK
Ras
GTP
RasGRP
Ras
GDP
Akt
Raf
cPKC
nPKC
DAG
Rheb
GDP
Inactive
MEK1/2
TSC1
TSC2
ERK1/2
PDK1
Rheb
GTP
Active
(+++)
PRAS40
mLST8
TOR
Rictor
SIN1
mLST8
TOR
Raptor
TORC1
TORC1-Independent
?

Figure 9.4 PtdIns(3,4,5)P_3 metabolism and intracellular signalling domains. Class I PI3Ks (p110α, p110β, p110δ, and p110γ) phosphorylate the 3′-hydroxyl group of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to produce the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). Activation of the 3′-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten) stimulates hydrolysis of PtdIns (3,4,5)P_3 back into PtdIns(4,5)P_2 and attenuates PtdIns(3,4,5)P_3-dependent signal transduction. Alternatively, activation of the 5′-phosphatases SH2-containing inositol polyphosphate-5-phosphatase 1 and/or 2 (SHIP1/2), convert PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2. Interestingly, PtdIns(3,4,5)P_3 and PtdIns (3,4)P_2 share several downstream targets, including PDK1 and Akt, but they also bind and activate distinct intracellular targets. Consequently, SHIP1/2-mediated metabolism of PtdIns(3,4,5)P_3 can facilitate continued signalling through PDK1 and Akt, while also preventing the activation of other PtdIns (3,4,5)P_3-sensitive effectors. Recent evidence suggests that the class II PI3K catalytic subunits (PI3K-C2α, PI3K-C2β, and PI3K-C2γ) might be able to produce PtdIns(3,4)P_2 from PtdIns(4)P; providing an additional pathway leading to PtdIns(3,4)P_2-dependent signalling. Taken together, the cellular localization and coordinate activity of class I PI3Ks, PTEN, and SHIP1/2 create dynamic PtdIns(3,4,5)P_3 signalling domains at the plasma membrane which have been shown to contribute to the spatiotemporal control of downstream signal transduction responses. Adapted from Leslie et al., *Oncogene*, 2008.

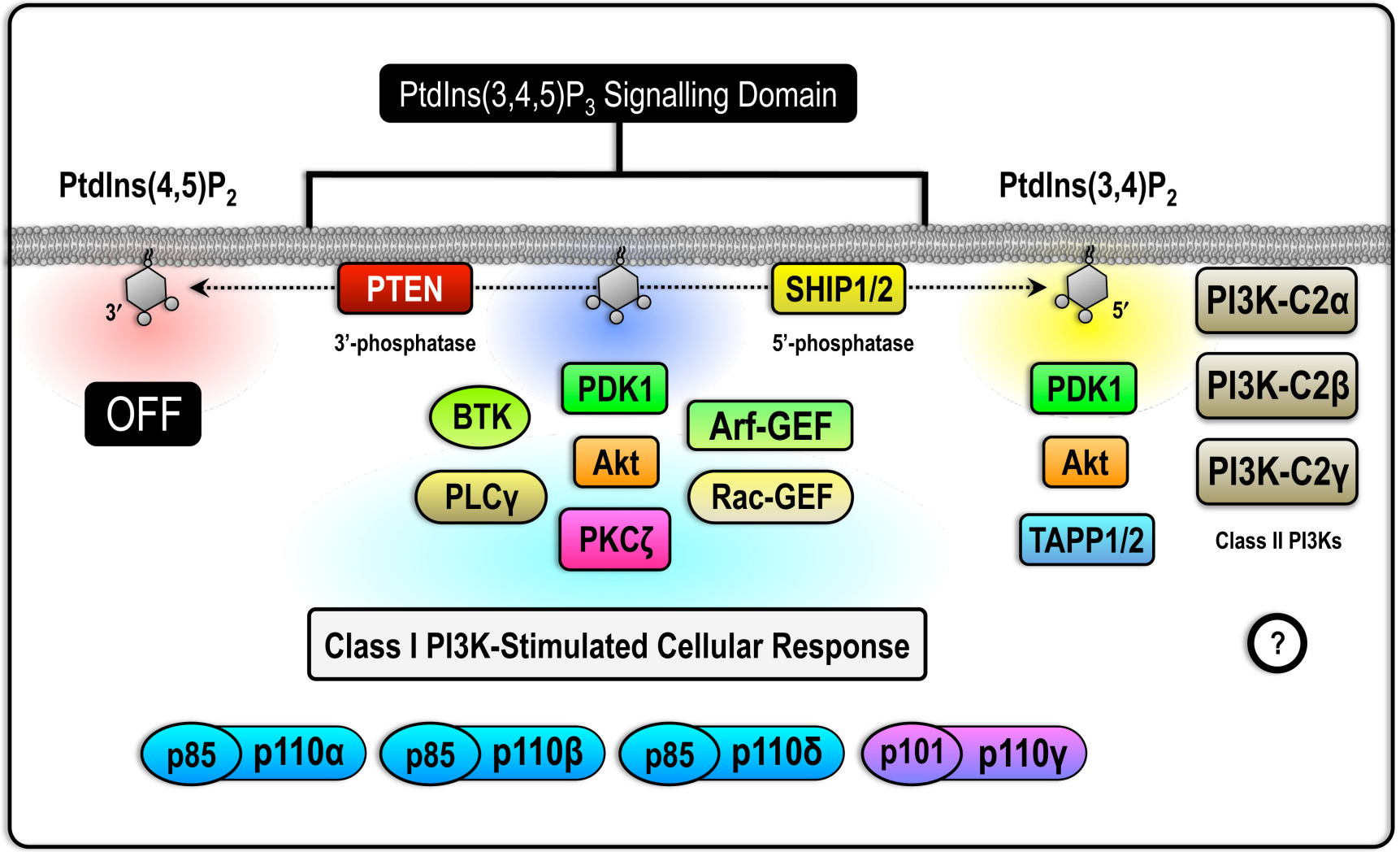

PtdIns(3,4,5)P3 Signalling Domain
PtdIns(4,5)P2
PtdIns(3,4)P2
3′
5′
PTEN
3'-phosphatase
SHIP1/2
5'-phosphatase
OFF
BTK
PDK1
Arf-GEF
Akt
PLCγ
Rac-GEF
PKCζ
PDK1
Akt
TAPP1/2
PI3K-C2α
PI3K-C2β
PI3K-C2γ
Class II PI3Ks
?
Class I PI3K-Stimulated Cellular Response
p85 p110α
p85 p110β
p85 p110δ
p101 p110γ

Figure 9.5 Summary schematic of acute GnRH2-stimulated LH release. In goldfish (*Carassius auratus*), the canonical $G\alpha_{q/11}$-coupled effectors phospholipase Cβ (PLCβ), protein kinase C (PKC), and Ca^{2+}-dependent signalling, as well as the Raf-MEK-ERK subfamily of mitogen-activated protein kinases (MAPKs), are consistently involved in mediating gonadotropin-releasing hormone (GnRH)-stimulated LH release responses. Results from this thesis demonstrate that GnRH2 actions on gonadotropes also involves the selective activation of the class IA PI3K catalytic subunit p110β and shared regulation of p110δ activity with GnRH3-stimulated responses. PI3K-dependent signalling was also upstream or independent of PKCs as well as increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and GnRH2-stimulated LH release did not involve the catalytic activity of the canonical phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) transduction targets PDK1, Akt, and BTK. As a result, we propose that non-canonical PtdIns(3,4,5)P_3-sensitive effectors likely mediate GnRH actions on LH release (see main text and Figure 9.2). Lastly, PKC-independent activation of Raf-MEK-ERK occurs in gonadotropes, which may or may not be controlled by acute signalling responses downstream of PtdIns(3,4,5)P_3 production.

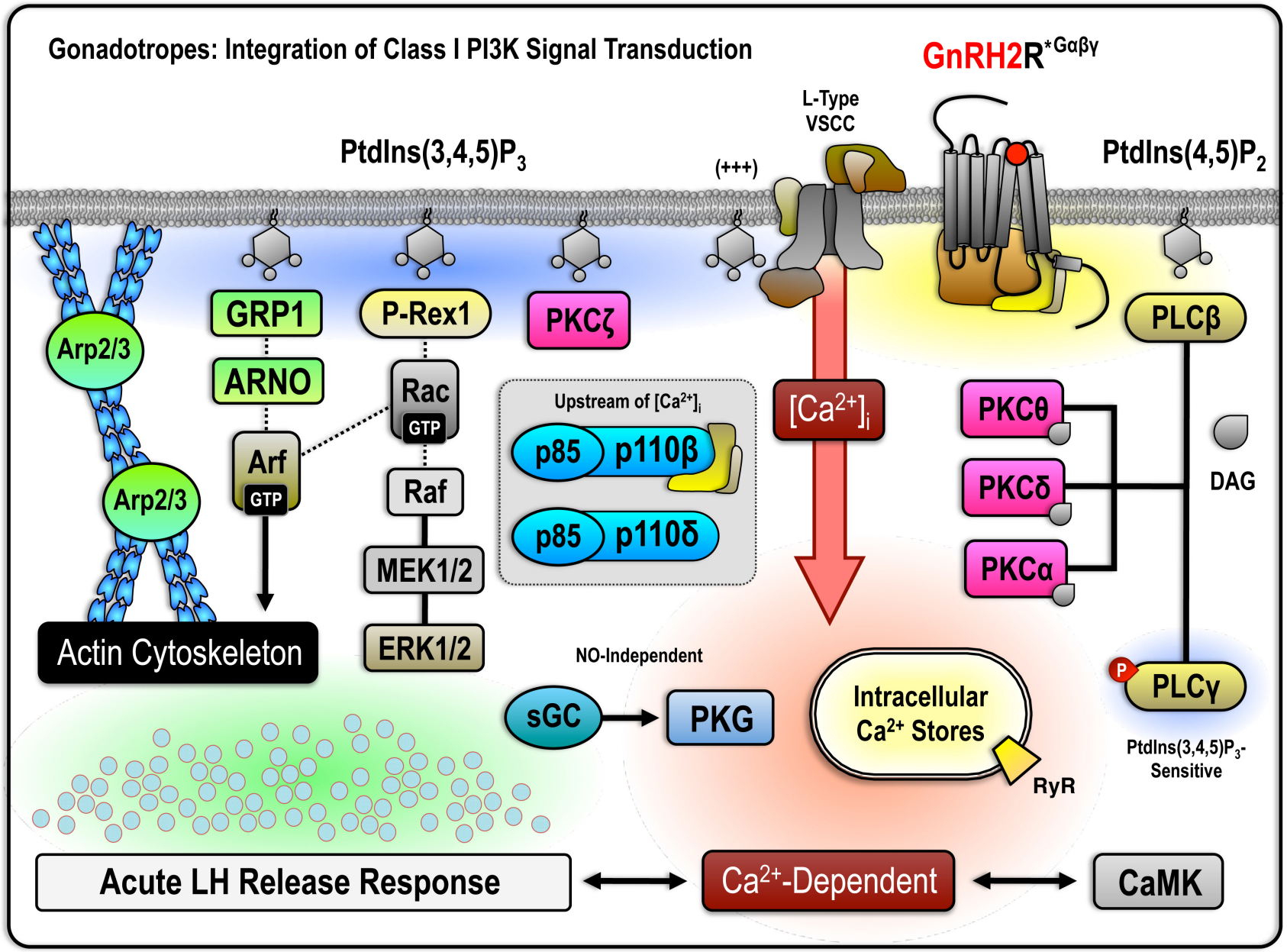

Gonadotropes: Integration of Class I PI3K Signal Transduction
GnRH2R*Gαβγ
L-Type VSCC
(+++)
PtdIns(3,4,5)P3
PtdIns(4,5)P2
GRP1
P-Rex1
PKCζ
PLCβ
Arp2/3
ARNO
Rac
GTP
Arf
GTP
Arp2/3
Upstream of [Ca2+]i
p85
p110β
p85
p110δ
[Ca2+]i
PKCθ
PKCδ
PKCα
DAG
Raf
MEK1/2
ERK1/2
Actin Cytoskeleton
NO-Independent
sGC
PKG
Intracellular Ca2+ Stores
RyR
P
PLCγ
PtdIns(3,4,5)P3-Sensitive
Acute LH Release Response
Ca2+-Dependent
CaMK

Figure 9.6 Summary schematic of acute GnRH3-stimulated LH release. In goldfish (*Carassius auratus*), the canonical $G\alpha_{q/11}$-coupled effectors phospholipase Cβ (PLCβ), protein kinase C (PKC), and Ca^{2+}-dependent signalling, as well as the Raf-MEK-ERK subfamily of mitogen-activated protein kinases (MAPKs), are consistently involved in mediating gonadotropin-releasing hormone (GnRH)-stimulated LH release responses. Results from this thesis demonstrate that GnRH3 actions on gonadotropes also involves the selective activation of the class IB PI3K catalytic subunit p110γ and the shared regulation of p110δ activity with GnRH2-stimulated responses. PI3K-dependent signalling was also upstream or independent of PKCs as well as increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and GnRH2-stimulated LH release did not involve the catalytic activity of the canonical phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) transduction targets PDK1, Akt, and BTK. As a result, we propose that non-canonical PtdIns(3,4,5)P_3-sensitive effectors likely mediate GnRH actions on LH release (see main text and Figure 9.2). Lastly, PKC-independent activation of Raf-MEK-ERK occurs in gonadotropes, which may or may not be controlled by acute signalling responses downstream of PtdIns(3,4,5)P_3 production.

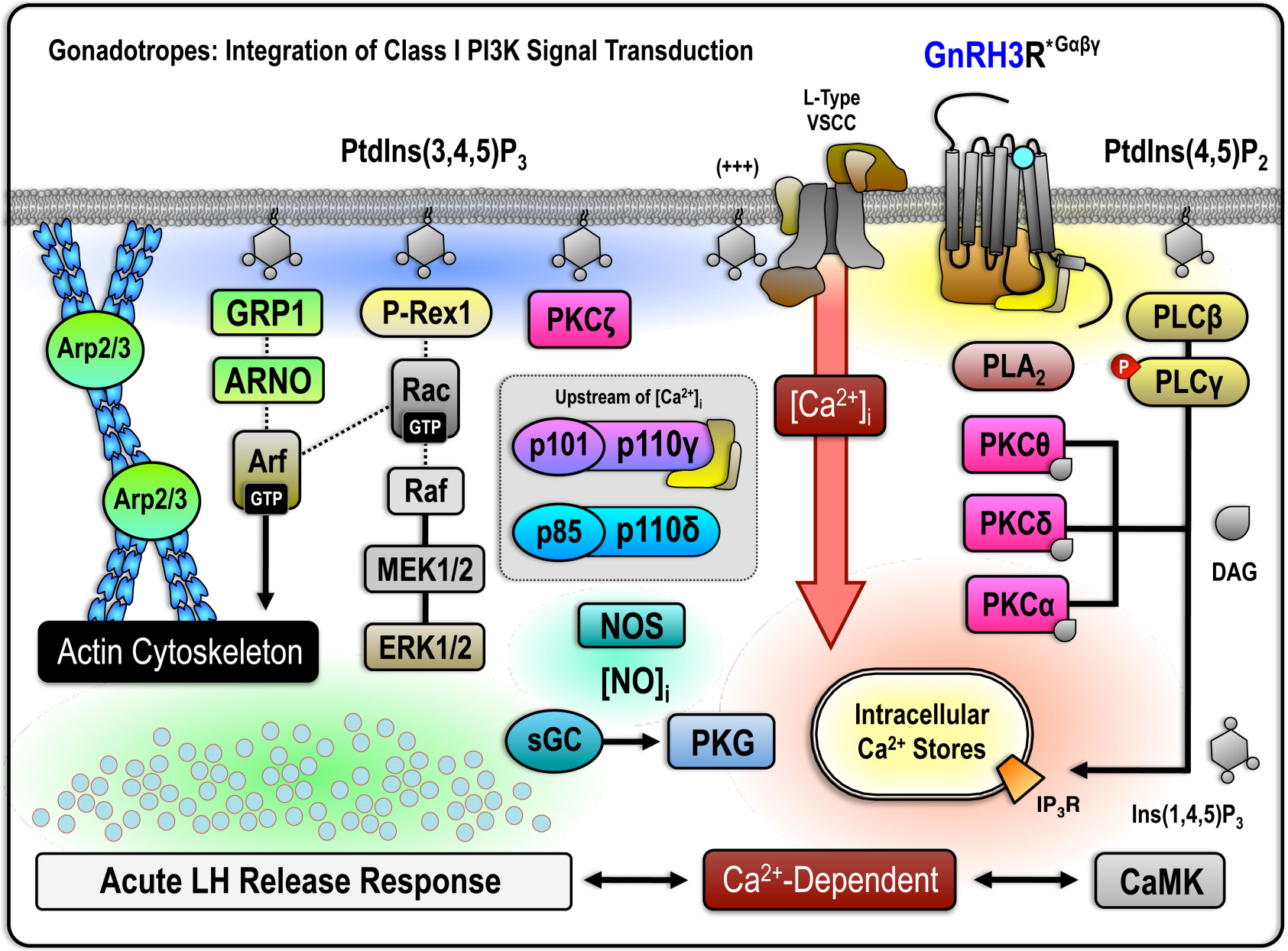

Gonadotropes: Integration of Class I PI3K Signal Transduction
GnRH3R*Gαβγ
L-Type VSCC
(+++)
PtdIns(3,4,5)P3
PtdIns(4,5)P2
GRP1
P-Rex1
PKCζ
Arp2/3
ARNO
Rac
GTP
Arf
GTP
Arp2/3
Raf
MEK1/2
ERK1/2
Actin Cytoskeleton
Upstream of [Ca2+]i
p101
p110γ
p85
p110δ
[Ca2+]i
PLCβ
PLA2
P
PLCγ
PKCθ
PKCδ
PKCα
DAG
NOS
[NO]i
sGC
PKG
Intracellular Ca2+ Stores
IP3R
Ins(1,4,5)P3
Acute LH Release Response
Ca2+-Dependent
CaMK

Figure 9.7 Summary schematic of acute GnRH2-stimulated GH release. In goldfish (*Carassius auratus*), the canonical $G\alpha_{q/11}$-coupled effectors phospholipase Cβ (PLCβ), protein kinase C (PKC), and Ca^{2+}-dependent signalling, as well as nitric oxide (NO) production by NO synthases (NOS), are consistently involved in mediating gonadotropin-releasing hormone (GnRH)-stimulated GH release responses. Results from this thesis demonstrate that GnRH2 actions on somatotropes also involves the selective activation of Bruton's tyrosine kinase (BTK), likely downstream of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) production catalyzed by the class I PI3K catalytic subunits p110β and p110γ. In general, PI3K-dependent signalling was also upstream or independent of PKCs as well as increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). BTK signalling is likely involved in the phosphorylation and activation of phospholipase Cγ (PLCγ; which can also directly bind to PtdIns(3,4,5)P_3). PLCγ activity produces diacylglycerol (DAG), which binds to and activates several families of effector proteins including conventional PKC (nPKC) and novel PKC (nPKC) isozymes. Alternatively, GnRH2-stimulated LH release did not involve the Raf-MEK-ERK subfamily of mitogen-activated protein kinases (MAPKs) or the catalytic activity of the canonical PtdIns(3,4,5)P_3-sensitive transduction targets PDK1 or Akt. As a result, in addition to BTK, we propose that non-canonical PtdIns (3,4,5)P_3-sensitive effectors mediate GnRH actions on GH release (see main text and Figure 9.2).

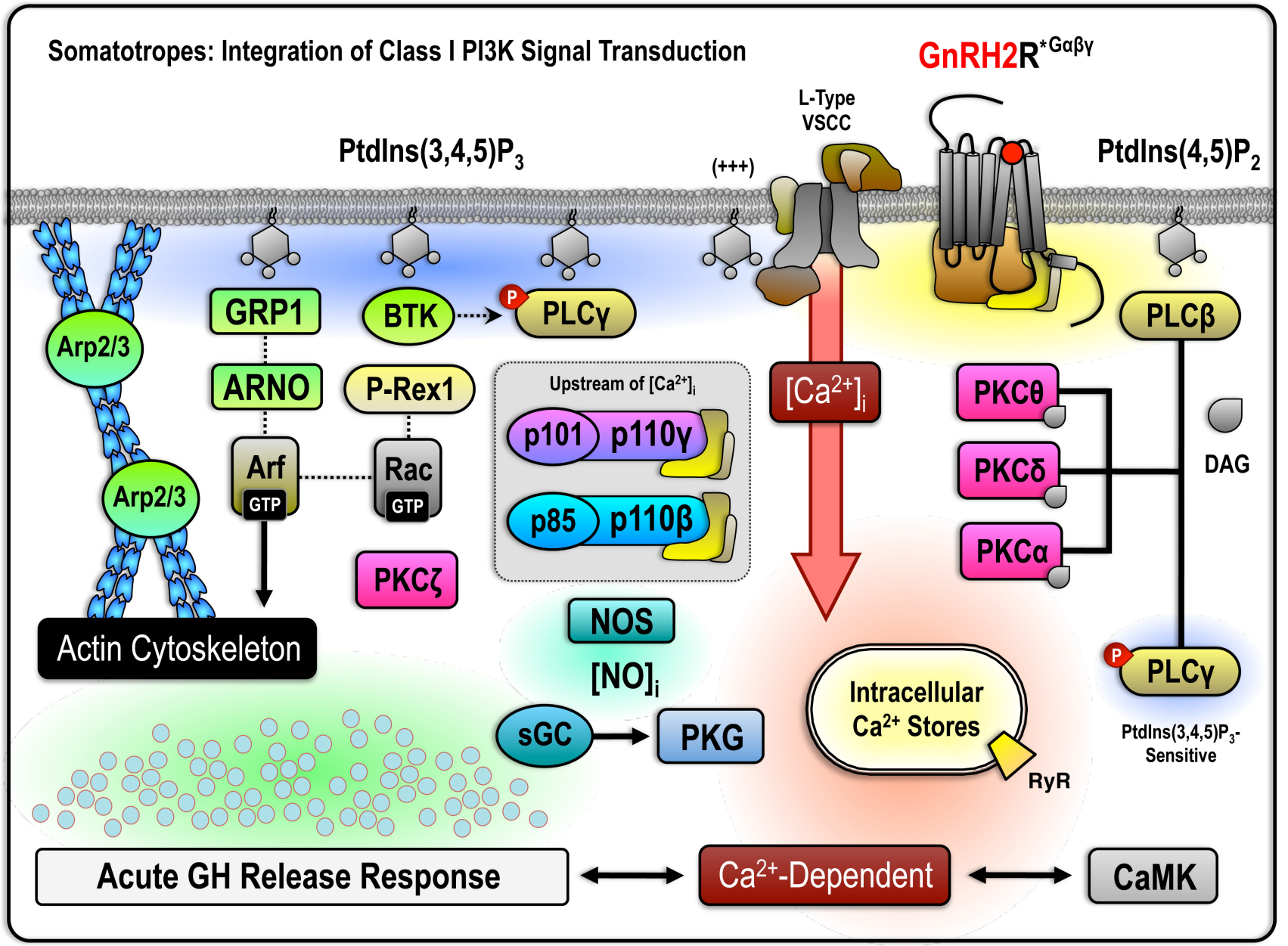
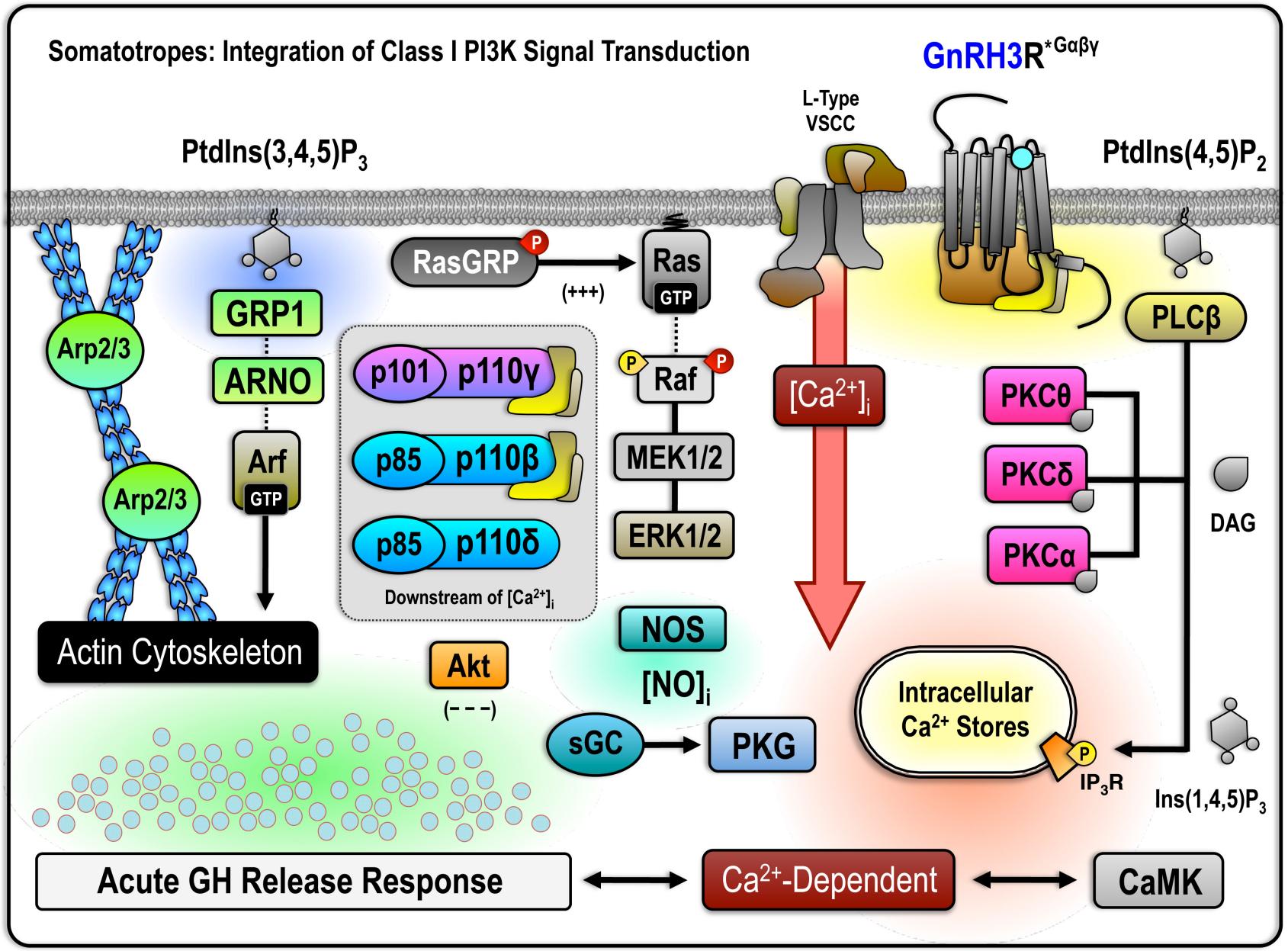

Somatotropes: Integration of Class I PI3K Signal Transduction
GnRH2R*Gαβγ
L-Type VSCC
(+++)
PtdIns(3,4,5)P3
PtdIns(4,5)P2
GRP1
BTK
PLCγ
PLCβ
Arp2/3
ARNO
P-Rex1
Upstream of [Ca2+]i
[Ca2+]i
PKCθ
PKCδ
PKCα
DAG
Arf
GTP
Rac
GTP
p101
p110γ
p85
p110β
PKCζ
Arp2/3
Actin Cytoskeleton
NOS
[NO]i
sGC
PKG
Intracellular Ca2+ Stores
RyR
PLCγ
PtdIns(3,4,5)P3-Sensitive
Acute GH Release Response
Ca2+-Dependent
CaMK

Figure 9.8 Summary schematic of acute GnRH3-stimulated GH release. In goldfish (*Carassius auratus*), the canonical $G\alpha_{q/11}$-coupled effectors phospholipase Cβ (PLCβ), protein kinase C (PKC), and Ca^{2+}-dependent signalling, as well as nitric oxide (NO) production by NO synthases (NOS), are consistently involved in mediating gonadotropin-releasing hormone (GnRH)-stimulated GH release responses. Results from this thesis demonstrate that GnRH3 actions on somatotropes also involves the selective activation of the class IA PI3K catalytic subunit p110δ in addition to the shared regulation of the p110β and p110γ subunits with GnRH2-stimulated responses. In general, PI3K-dependent signalling was also upstream or independent of PKCs but is likely downstream of increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Alternatively, GnRH3-stimulated GH release also involved the Raf-MEK-ERK subfamily of mitogen-activated protein kinases (MAPKs), but not the catalytic activity of the canonical phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3)-sensitive transduction targets PDK1 or BTK. As a result we propose that non-canonical PtdIns(3,4,5)P_3-sensitive effectors might also mediate GnRH actions on GH release (see main text and Figure 9.2). However, the canonical PtdIns(3,4,5)P3-binding target Akt was shown to be a negative regulator of GnRH3-stimulated GH release. Inactivation of Akt could potentially facilitate GnRH-selective signalling by removing inhibitory inputs at regulatory phosphorylation sites on Raf and receptors for inositol 1,4,5-trisphosphate (IP_3Rs; yellow "P" symbols on targets). Lastly, PKC-dependent activation of Raf-MEK-ERK occurs in somatotropes, likely through either direct phosphorylation of Raf, or indirectly through the activation of the Ras superfamily guanine nucleotide exchange factor Ras guanyl nucleotide-releasing protein (RasGRP; red "P" symbols on targets).


Somatotropes: Integration of Class I PI3K Signal Transduction
GnRH3R*Gαβγ
L-Type VSCC
PtdIns(3,4,5)P3
PtdIns(4,5)P2
RasGRP
P
(+++)
Ras
GTP
GRP1
ARNO
Arf
GTP
Arp2/3
Arp2/3
Actin Cytoskeleton
p101
p110γ
p85
p110β
p85
p110δ
Downstream of [Ca2+]i
Raf
P
P
MEK1/2
ERK1/2
[Ca2+]i
PLCβ
PKCθ
PKCδ
PKCα
DAG
NOS
[NO]i
Akt
(- - -)
sGC
PKG
Intracellular Ca2+ Stores
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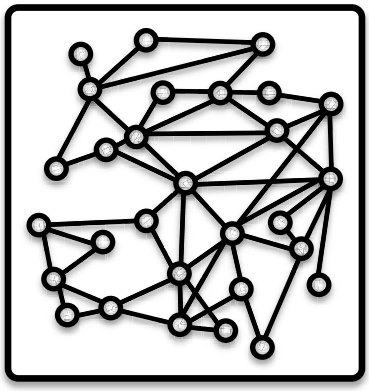
IP3R
Ins(1,4,5)P3
Acute GH Release Response
Ca2+-Dependent
CaMK

Chapter Ten:

Literature Cited



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