12-1-2014 12:00 AM

Pathway-Specific Signaling and its Impact on Fertility: A Focus on the Kisspeptin Receptor, Kiss1r

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

PATHWAY-SPECIFIC SIGNALING AND ITS IMPACT ON FERTILITY: A FOCUS ON THE KISSPEPTIN RECEPTOR, Kiss1r

(Thesis format: Monograph)

by

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Graduate Program in Physiology

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

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Abstract

Hypothalamic gonadotropin-releasing hormone (GnRH) is the master regulator of the neuroendocrine reproductive (HPG) axis and its secretion is regulated by various afferent inputs to the GnRH neuron. Activation of some $G\alpha_{q/11}$-coupled receptors leads to GnRH neuronal membrane depolarization and GnRH secretion. The most potent trigger of GnRH secretion is kisspeptin (KISS1), the ligand for the $G\alpha_{q/11}$-coupled receptor, KISS1R. The critical role for the KISS/KISS1R system in reproduction is revealed in patients bearing KISS1R mutations causing reproductive axis dysfunction. Furthermore mice carrying targeted KISS1R deletion phenocopy human patients in being infertile and hypogonadotropic. While studies have shown that Kiss1r signaling is essential for attaining and maintaining fertility the underlying mechanisms have not been fully elucidated. Our overall goal of this study was to reveal novel signaling mechanisms downstream of Kiss1r that regulate GnRH secretion and fertility. We recently demonstrated that in addition to $G\alpha_{q/11}$, Kiss1r also signals to ERK1/2 via the β-arrestin pathway. Here we investigated the role of this β-arrestin signaling in Kiss1r-dependent Gnrh secretion. Using mice lacking β-arrestin, we determined that Kiss1r triggers significant Gnrh secretion via the β-arrestin pathway. Thus, mice use both the Kiss1r-coupled $G\alpha_{q/11}$ and β-arrestin cascades to regulate Gnrh secretion and thereby, fertility. In addition to β-arrestins, Kiss1r potentially uses other $G\alpha_{q/11}$-independent signaling pathways to regulate fertility. To test whether these pathways are sufficient to attain and maintain fertility we created Gnrh neuronal-specific $G\alpha_{q/11}$ knockout mice and characterized their reproductive development and capacity to trigger Gnrh secretion following KISS1 administration. While these mice were hypogonadotropic and had delayed pubertal onset they retained partial fertility and ability to trigger Gnrh secretion in response to KISS1 stimulation confirming that Kiss1r-$G\alpha_{q/11}$-independent signaling is sufficient for attaining and maintaining fertility. Overall, we show that Kiss1r signals via both $G\alpha_{q/11}$-dependent and -independent mechanisms to regulate Gnrh secretion and fertility. We also demonstrated that one of these $G\alpha_{q/11}$-independent pathways is mediated by β-arrestins in mice. Based on these novel findings, the potential now exists to develop new molecular strategies to treat infertility in humans.

Keywords: KISS1R, IHH, L148S, β-arrestin, $G\alpha_{q/11}$, GnRH, LH, FSH, HPG axis

Co-Authorship Statement

This dissertation incorporates material that is the result of collaborative research efforts and all studies were carried out under the supervision of Dr. Andy Babwah. The following additional collaborators have made a significant contribution to the completion of particular studies and deserve recognition:

1. Dr. Stuart Tobet and Connor Nash evaluated GnRH neuronal populations in perfused brains of embryonic and adult mice (Figs. 2.1, 2.2 and 2.16).
2. Dr. Ursula Kaiser's lab verified preliminary data from the Babwah laboratory that L148S triggers ERK1/2 phosphorylation in HEK 293 cells and also demonstrated the inability of L148S to trigger kisspeptin stimulated $InsP_3$ formation compared to WT KISS1R (Figs. 2.5D, 2.6 and 2.9).
3. Dr. Macarena Pampillo performed the L148S-dependent ERK1/2 phosphorylation assay in mouse embryonic fibroblasts (MEFs) lacking β-arrestin (Fig. 2.8).
4. Dr. Macarena Pampillo crossed parental strains to obtain the experimental *Gnaq*$^{fl/fl}$*;Gna11*$^{-/-}$*;Gnrh-Cre* mice and control littermates as well as assisted with blood collection and surgeries.
5. Serum was analyzed for luteinizing hormone, follicle stimulating hormone and testosterone by the Endocrine Technology and Support Lab, Oregon National Primate Research Center, Beaverton, OR, USA. (Figs. 2.3, 2.4, 2.22 and 2.23).

Animals were housed at the London Regional Cancer Program Animal Facility where vivarium staff provided general animal maintenance. I was responsible for the crossing, weaning, genotyping and maintenance of each mouse colony used in this dissertation. In addition to this I actively participated in all animal procedures and treatments including brain perfusions, dissections, surgeries tissue cryosectioning and blood collection and processing for hormone assays. Finally, unless otherwise stated above, I directly performed all other cell-related studies using a variety of cell lines (HEK 293, GT1-7, MEFs, and COS-7).

Dedication

To the graduate student who happens across this dissertation one day. Pressure makes diamonds so do not give up and remember that we have all been exactly where you are right now.

Acknowledgments

I am incredibly overwhelmed with the amount of love and support that I have been blessed to receive over the years. My deepest gratitude goes out to the teachers, professors, friends, family and colleagues who have shared knowledge or just inspired me to keep working. This accomplishment is as much yours as it is mine – thank you.

Words alone are not enough to express the unfailing support I received from my parents. Although they were miles away and I missed them dearly, their prayers and words of encouragement went a long way in carrying me through the rough times. At times when I was at my weakest they reminded me that if "God brings you to it He will bring you through it" and I've come to realize this truth regardless of religious persuasion.

I also want to thank the members of my advisory committee for all the helpful suggestions with special thanks to Dr. Bonnie Deroo for words of encouragement and Dr. Daniel Hardy who patiently read and offered corrections for my thesis.

Dr. Macarena Pampillo was invaluable in the laboratory for sharing skills, expertise and to troubleshooting new techniques. The lab could not function without her and I cannot thank her enough for all the assistance and guidance over the years.

Last but not least, thank you Dr. Babwah for opening your lab to give an amazing opportunity to an international student. Your generosity, guidance and advice over the years will never be forgotten and working with you has truly been a rewarding experience.

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List of Abbreviations

7TMR	7 transmembrane receptor (referred to as GPCR in this publication)
α_2AR	alpha-2-adrenergic receptor
An	antide
AP-2	adaptin protein-2
ARC	arcuate nucleus
Arrb	gene encoding for β-arrestin protein
$AT_{1A}R$	angiotensin type 1A receptor
AVPV	anteroventral periventrivular nucleus
β_2AR	beta-2-adrenergic receptor
β-arr	beta-arrestin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCR	chemokine receptor (C-C motif)
CCP	clathrin coated pit
CHO cells	chinese hamster ovary cell line
COS-7	african green monkey kidney fibroblast cell line
CPP	central precocious puberty
Cre	Cre recombinase enzyme
C-tail	carboxy-terminal tail
CXCR	chemokine receptor (CXC motif)
D2R	dopamine receptor type 2
DAG	diacylglycerol
DKO	double knockout
DRY	conserved sequence (aspartic acid-arginine- tyrosine) on the IL2 of GPCRs
E_2	estrogen
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK1/2	extra-cellular signal regulated kinase 1/2

FSH	follicle stimulating hormone
$G\alpha_{q/11}$	G-protein alpha subunit for the G_q and G_{11} protein families
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
Gnaq	gene encoding for $G\alpha_q$
Gna11	gene encoding for $G\alpha_{11}$
GnRH	gonadotrophin releasing hormone
GnRH-RI	gonadotropin releasing hormone receptor type I
G-protein	guanine nucleotide binding protein
GTP	guanosine triphosphate
GRK	G-protein receptor kinase
GT1-7	immortalized GnRH secreting mouse hypothalamic cell line
HBSS	Hanks' balanced salt solution
HEK 293	human embryonic fibroblast 293 cell line
HPG axis	hypothalamic-pituitary-gonadal axis
Icv	intra-cerebroventricular (for drug administration)
i.p.	intra-peritoneal (for drug administration)
IHH	idiopathic hypogonadotropic hypogonadism
IL2	intracellular loop 2 of GPCRs
$InsP_3$	inositol (1,4,5)-triphosphate
KISS1	kisspeptin (also Kp)
KISS1R	Kisspeptin receptor formally called GPR54: G-protein coupled receptor 54
KO	knockout
KP-10	kisspeptin-10
KP-54	kisspeptin-54
LH	luteinizing hormone,
MAPK	mitogen activating protein kinase,
mAChR	muscarinic acetylcholine receptors
MDA-MB-231	breast carcinoma cell line
ME	median eminence
MEF	mouse embryonic fibroblast cell line
mGluR	metabotropic glutamate receptor

mRNA	messenger ribonucleic acid
NK_3B	neurokinin B (substance P) receptor
OVLT	organum vasculosum of the lamina terminalis
PAR1 or 2	protease activating receptor 1 or 2
PBS	phosphate buffered saline
pERK	phosphorylated ERK1/2
PIP_2	phospatidylinositol (4,5)-bisphosphate
PKA or PKC	protein kinase A or C
PTH1R	parathyroid hormone type 1 receptor
PTX	pertussis toxin
SST_{2A}	somatostatin type 2A receptor
T	testosterone
V2R	vasopressin receptor 2
WT	wild-type

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Chapter 1: Literature Review

1.1 The reproductive axis

The three tiered reproductive axis consists of coordinated hormonal signals relayed between the hypothalamus, pituitary and gonads (HPG axis) (Fig. 1.1). Hypothalamic gonadotropin-releasing hormone (GnRH) is established as the master control of the HPG axis and in the mammalian brain there are fewer than 1500 GnRH neurons which are not organized in distinct nuclei but scattered throughout the medial septum, preoptic area and basal hypothalamus (Wray 2002, Silverman et al. 1994, Wu et al. 1995). Regardless of location most GnRH neurons project their axons into the median eminence. The median eminence is one of seven areas in the brain devoid of a blood-brain-barrier (BBB). When stimulated, GnRH is released in a pulsatile fashion into a fenestrated capillary bed called the primary plexus. This primary plexus which is nearer the hypothalamus is connected to a secondary plexus nearer the anterior pituitary; by a hypophysial portal vein. Once in the secondary plexus, GnRH diffuses out of the blood and activates GnRH receptors on gonadotropes in the anterior pituitary resulting in the synthesis and release of gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH) into systemic circulation (Fig. 1.1) (Wray 2002).

1.2 Gonadotropin Hormone Releasing (GnRH) Neurons

In the early 1970's hypothalamic GnRH was established as the prime regulator of the reproductive hormonal cascade in vertebrates, including mammals (McCann et al. 2002, Schally et al. 1971, Baba et al. 1971). Mammals have two forms of this 10 amino acid neuropeptide GnRH-1 and GnRH-2. The GnRH receptor (GnRHR) is expressed on the plasma membrane of gonadotropin releasing cells in the anterior pituitary. It is a seven-transmembrane spanning receptor (7TMR) but unlike most other receptors in this category, it lacks a carboxy tail which prevents rapid desensitization and internalization. While the GnRH-2 agonist modulates sexual behaviour, GnRH-1, (Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly) activates GnRH-R1 on anterior pituitary gonadotropes and triggers gonadotropin secretion and is thus central regulator of the reproductive axis (Millar 2005). GnRHR couples to and signals via several G proteins in a cell-type

dependent manner (Armstrong et al. 2009). In the gonadotrope it primarily signals via $G\alpha_{q/11}$ and activation by GnRH leads to the PIP_2 hydrolysis, $InsP_3$ formation, protein kinase C (PKC) activation and ERK1/2 phosphorylation (Kaiser et al. 1995). GnRHR expression on pituitary gonadotropes is dependent on pulsatile GnRH stimulation. However, continuous GnRH exposure leads to non-β-arrestin mediated desensitization of the receptor, which is utilized in some clinical treatments (Kaiser et al. 1995, Nathwani et al. 2000, An et al. 2005). Pulsatile GnRH stimulates gonadotropes in an amplitude and frequency-dependent manner. The primary targets for LH and FSH are the gonads where they initiate sexual maturation and gametogenesis. FSH stimulates spermatogenic tissues of testes and granulosa cells of the ovarian follicle to enhance the maturation of the sperm and egg. LH stimulates the Leydig cells of the testis to produce testosterone (T) and thecal cells of the ovaries to produce estrogen (E2) and progesterone (P4). In addition to their roles in the development of secondary sexual characteristics, these gonadal steroid hormones feed back on both the hypothalamus and anterior pituitary to regulate GnRH and LH secretion (Pinila et al. 2012, d'Anglemont de Tassigney et al. 2010).

GnRH neurons in the hypothalamus receive a variety of stimulatory and inhibitory inputs but among these, kisspeptin (KISS1) is the most potent positive trigger of GnRH release. Kisspeptin neurons lie upstream of hypothalamic GnRH neurons, specifically in the arcuate nucleus (ARC) and anteroventricular periventricular nucleus (AVPV) in rodents and the infundibular nucleus in primates. While testosterone in males inhibits GnRH and LH secretion by negative feedback on Kiss1 neurons in the ARC, E2 and P4 in females can stimulate Kiss1 neurons in the AVPV while inhibiting the Kiss1 neurons of the ARC. This differential regulation is dependent on the stage of the ovarian cycle. Some other afferent inputs that regulate GnRH secretion are leptin, Neurokinin B (NKB), glutamate, norepinepherine, N-methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Inhibitory inputs include: RF-related peptides (RFRP), gamma-aminobutyric acid (GABA) and Dynorphin.

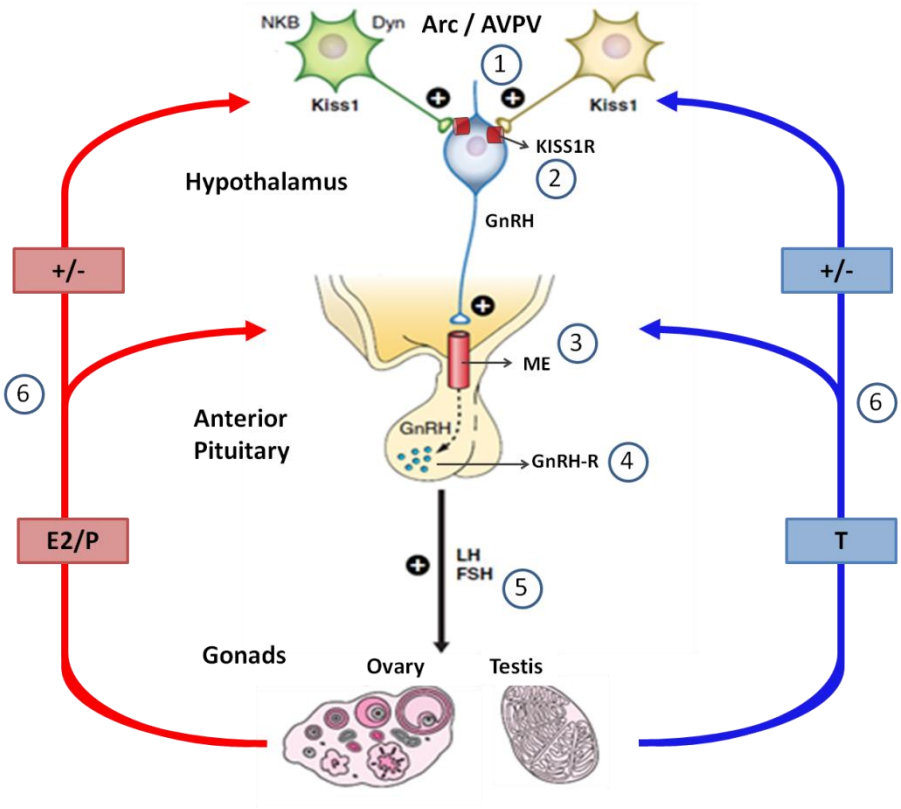

Figure 1.1: Graphic representation of the hypothalamic-pituitary gonadal (HPG) axis.

(1) Kisspeptin neurons in the ARC and AVPV of the hypothalamus secrete KISS-1 which activates KISS1R (2) on the membrane of hypothalamic GnRH neurons. Intracellular signaling cascade downstream of KISS1R results in the pulsatile secretion of GnRH to the ME (3). In the anterior pituitary GnRH activates GnRH-R expressed on gonadotropes (4) leading to the pulsatile secretion of gonadotropins, LH and FSH into systemic circulation (5). LH and FSH act on the gonads to promote gametogenisis and gonadal steroid hormone production; E2, T and P4. These hormones feedback (positively and negatively) on the hypothalamus and pituitary to regulate GnRH, LH and FSH release (6). AVPV, anteroventral periventricular nucleus; ARC, arcuate nucleus; ME, median eminence. Adapted from Pinilla et al. 2012 and d'Anglemont de Tassigny et al. 2010.

GnRH expressing neurons are unique since they originate outside the CNS. During embryonic development GnRH secreting neurons originate from the epithelium cells of the olfactory placode (~ E10.5 in the mouse). (Bless et al. 2004, Limonta et al. 2003, Wray 2002, Schwanzel-Fukuda et al. 1989, Wray et al. 1989, Jasoni et al., 2009) Prior to birth, these neurons co-migrate along the vomeronasal axons with the olfactory neurons across the cribiform plate (Fig. 1.2) (Wireman et al. 2011). The olfactory neurons migrate to the olfactory bulb and the GnRH neurons migrate to the anterior hypothalamus (~ E18.5 in the mouse) (Wray, 2002, Jasoni et al. 2009, Tobet et al. 2003).

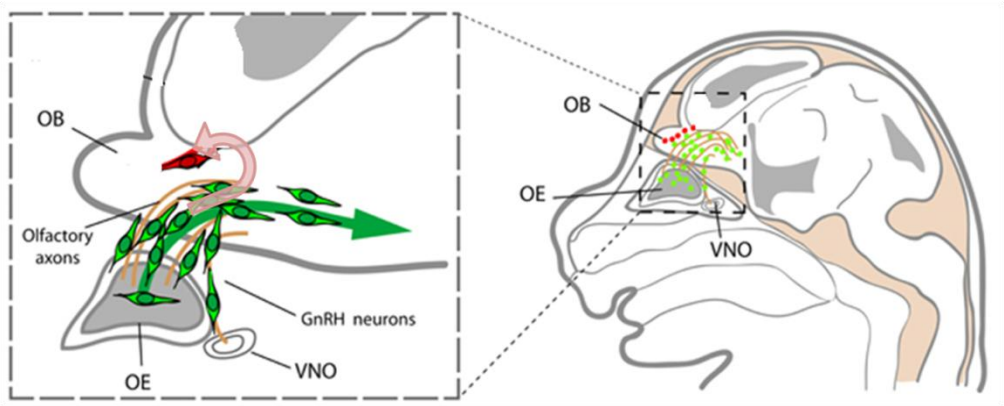

Figure 1.2: Graphic representation of GnRH neuronal migration during development.

Nascent GnRH neurons (green) in the OE co-migrate with olfactory neurons (red) along vomeronasal axons. GnRH neurons migrate towards the anterior hypothalamus and olfactory neurons towards the OB. OE, olfactory epithelium, VNO, vomeronasal organ, OB, olfactory bulb. Adapted from Suarez et al. 2012 and Cariboni et al. 2011.

Disruption in GnRH neuronal migration during development results in a hypogonadotropic condition called Kallmann syndrome (Seminara 1998). This syndrome is characterized by delayed puberty and anosmia due to a defect in the developmental co-migration if the GnRH and olfactory neurons. In humans and primates, GnRH pulse frequency and amplitude increase embryonically as the hypothalamus and hypophyseal portal system develop. Placental steroids provide negative feedback and thereby lower fetal gonadotropin levels. When this negative feedback is removed at birth GnRH, LH and FSH secretion increase in the neonates and remain elevated for 1-2 years in girls and 6 months in boys (Grumbach et al. 2005). Through an unknown mechanism, thought to involve GABA and NPY, GnRH neurons subsequently enter a quiescent stage characterized by low LH pulse amplitude and frequency for the rest of childhood then gradually reawakens in puberty (Plant 2008, Weisz et al. 1980, Ehlers et al. 2013, Waldhauser et al. 1981 and Blogowska et al. 2003).

GnRH neurons vary slightly in anatomical location depending on species. It is unknown if these differences significantly affect their physiological function but should be kept in mind when comparing GnRH activity between species. In rodents, GnRH neurons are mainly located in the preoptic region (POA), scattered from the medial septum to the band of Broca. In humans, most of the GnRH neurons are located in the infundibular nucleus (referred to as arcuate nucleus in other species) with very few in the POA. In monkeys, GnRH neurons are mainly found scattered lateral to the ARC in a region called the mediobasal hypothalamus (MBH). In sheep, GnRH neurons are found in both the POA and ARC (Herbison et al. 2006, Ramaswamy et al. 2008, Hrabovszky et al. 2010, Goodman et al. 2007, Lehman et al. 1986, Schwanzel-Fukuda et al. 1989, Ohkura et al. 2009).

GnRH pulses occur every 30 minutes in rodents and every 1.5 hours in humans (Knobil 1992, 1980, Belchetz et al. 1978, Moenter et al. 1991). Since GnRH release is mirrored in LH pulsatility, for small animal models where direct measurement of GnRH secretion is difficult, serum LH is an acceptable surrogate marker for GnRH secretion (Fig. 1.3) (Messager et al. 2005, Christian and Moenter, 2010).

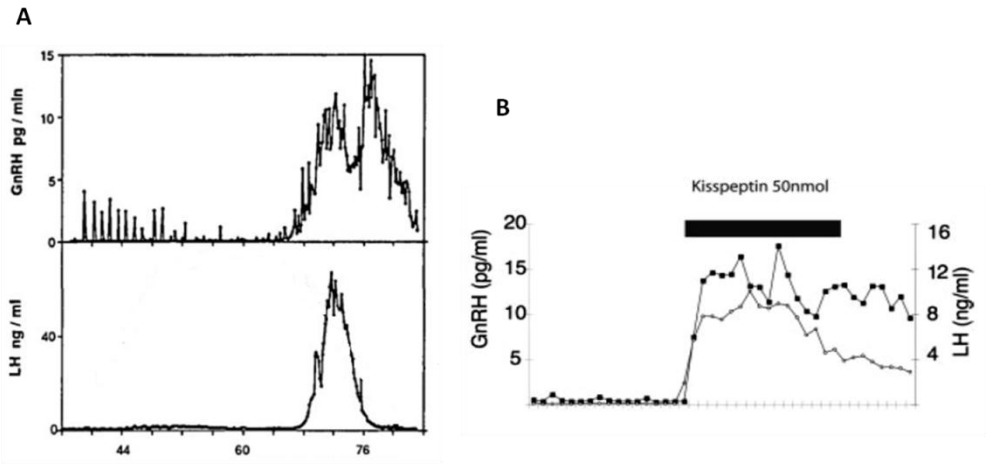

Figure 1.3: GnRH secretion is directly reflected by gonadotropin (LH) release.

(A) Measurement of circulating levels of LH and GnRH in an individual ewe demonstrating a preovulatory surge. (B) Measurement of cerebrospinal GnRH (filled squares) and serum LH (open circles) in an individual ewe after i.c.v. administration of 50 nmol kisspeptin. GnRH pulses closely mirror LH pulses and since GnRH secretion cannot be readily measured in other species such as mice and small rodents, LH pulsatility is used as a surrogate for GnRH. Adapted from Messager et al. 2005, Christian and Moenter, 2010.

1.2.1 Biological systems for studying GnRH neurons

Since GnRH neurons are few in number and scattered throughout the mediobasal hypothalamus they present a technical challenge to obtain and purify for direct analysis of the intracellular mechanisms regulating synthesis and secretion of GnRH. This challenge has been somewhat overcome by the development of immortalized GnRH neuronal cell cultures. (Krsmanovic et al. 1999, Wray 2002, Silverman et al. 1994, Wu et al. 1995, Mayer et al. 2009, Mellon et al. 1990, Liposits et al. 1991). Various strategies to create these cellular models have been employed including; GnRH-neuronal directed tumoregenisis in the hypothalamus and olfactory placode, lentiviral modification of dissociated hypothalamic cells and lastly conditional activation of a GnRH-neuronal specific oncogene (Mellon et al. 1990, Radovick et al. 1991, Salvi et al. 2006, Wolfe et al. 2008, Belsham et al. 2004, Mayer et al. 2009). To date however the GT1-7 line created by Mellon et al. 1990 remains the most well characterized and widely used immortalized GnRH neuronal cell line in the literature (used in over 300 publications) (Liposits et al. 1991, Mayer et al. 2009, Belsham et al. 2004). This line has been shown to express low endogenous levels of KISS1R and respond to kisspeptin treatment by increases in GnRH mRNA and secretion and in serum free media (no cell division) exhibit classical neuronal phenotype with long extended neurites and many interneuronal connections (Mellon et al. 1990, Nunemaker et al. 2001, Novaira et al. 2009). Also, compared to other lines, GT1-7s represent post-migratory, adult GnRH neurons since the line was created by targeted tumorigenesis in the anterior hypothalamus of adult mice. GT1-7s also express estrogen receptors (ERα and ERβ) (Hu et al. 2008 and Krsmanovic et al. 2009). The creation of the GT1-7 line allowed for the investigation at the molecular level in the GnRH neuron however, while GT1-7s are convenient models for GnRH neurons they remain experimentally challenging line to manipulate requiring lipid based transfection techniques and have a relatively slow doubling rate of 3-4 days which can be even slower when under antibiotic selection during the creation of stables for example (Mellon et al. 1990).

While immortalized cultures of GnRH neurons like the GT1-7s are convenient tools they do have some limitations. Firstly, they are transformed cells and might not fully represent

a primary culture of GnRH neurons since the continuous expression of the Simian virus 40 tumor antigen (SV40-TAg) sometimes interferes with regular cell function (Weiner et al. 1991). Secondly, determining how afferent inputs to the GnRH neuron are regulated is not possible in a clonal cell line. For example estradiol feedback regulates KISS1secretion and this in turn affects GnRH secretion, investigating this chain of events would not be possible in a line made purely of immortalized and dissociated GnRH neurons. Lastly, it has been shown that there are sub-populations of GnRH neurons which may serve different functions and secrete GnRH in response to different stimuli. However as a clonal line, where the entire population represents a single GnRH neuron, these differences in responses would not be observed (Kimura et al. 1998). To overcome these experimental limitations, the GnRH-GFP mouse was created where the gene for the green fluorescent protein reporter (GFP) is inserted downstream of the mGnRH promoter (Suter et al. 2000). Because the GFP signal made it easy to locate the scattered GnRH neurons in the hypothalamus this transgenic mouse was used in a wide variety of studies including, electrophysiological analysis of GnRH neuronal pulsatility (Suter et al. 2000), immunoflourescent analysis of protein expression in GnRH neurons directly in brain slices and assessment of changes in gene expression by single cell qPCR on GnRH neurons (Xu et al. 2008).

Another useful tool was the creation of GnRH-Cre transgenic mice which allowed *in vivo* investigation of the functional role of various genes expressed in the GnRH neuron by creating mice with GnRH-neuronal specific genetic ablation of the gene of interest (Yoon et al. 2005, Wintermantel et al. 2006 and Skynner et al. 1999, Kawakowsky et al. 2012, Parkash et al. 2012, Schmid et al. 2013). These mice have been well established and characterized showing more than 97% efficiency of Cre-dependent recombination in GnRH neurons. This is very important since studies have shown that even as little as 10% of the total GnRH neuron population, is sufficient to drive reproductive function of the HPG axis (Mayer and Bhoem, 2011).

1.2.2 Regulation of GnRH secretion

Throughout adulthood a complex and dynamic system of GnRH pulses and surges maintains reproductive function. The nature and mechanisms behind the GnRH pulse

generator as well as the trigger for the onset of puberty is still not fully understood (Dungan et al. 2006 Oakley 2009). In postpubertal men, testosterone formation by the leydig cells is continuous. When levels become high enough they negatively feedback and inhibit the hypothalamus and anterior pituitary (Fig. 1.1) resulting in lower and more infrequent GnRH and LH pulses (Jackson et al. 1991, Steiner et al. 1982, Kelch et al. 1985). In post-pubertal women, the result of feedback regulation by gonadal steroids depends on the phase of menstrual cycle. In the luteal phase of the menstrual cycle, estrogen and progesterone produced by the corpora lutea negatively feedback and suppress GnRH and LH secretion. The resulting lower GnRH pulse frequency promotes preferential FSH secretion over LH secretion which leads to stimulation of follicular development for the next menstrual cycle (Marshall et al. 1993 and 2001). Prior to ovulation, during the follicular phase, the frequency of the GnRH pulses increases but the amplitude remains relatively low. There is a switch in response to estrogen from negative to positive feedback causing the increased GnRH pulse frequency that drives the preovulatory LH surge late in the follicular phase prompting ovulation. While our understanding of the exact mechanisms underlying the switch from positive to negative E2 feedback on the hypothalamus during the menstrual cycle remains incomplete, it is clear that estrogen plays an important role in regulating GnRH secretion (Simerly et al. 1998, Cooke et al. 1998).

It is important to note that while some animals like rats and mice have estrous cycles, primates and humans have menstrual cycles. The overall events are similar but there are some significant differences between these cycles. In mice the estrous cycle is approximately 5 days compared to the 28 day menstrual cycle in women. Also, in the menstrual cycle the endometrium is shed in the absence of conception whereas it is absorbed in estrous cycles. Unlike primates, mice are spontaneous ovulators where ovulation is induced by copulation or male pheromones. While the overall hormonal milieu remains the same between these two types of cycles, the timing is slightly different. In mice estrogen, progesterone and LH surges overlap prior to ovulation compared to women where the estrogen surge slightly precedes the LH surge and ovulation (Staley et al. 2005).

In addition to gonadal steroid feedback many excitatory and inhibitory factors influence GnRH neuronal activity which allows these neurons to integrate external and internal cues in the dynamic control of the reproductive axis. In addition to secreting into the median eminence, GnRH neurons also transmit signals to other neurons involved in odour and pheromone processing, sexual behaviour, appetite and defensive behaviour (Dumalska et al. 2008, Merchenthaler et al. 1984, Jennes et al. 1991, Boehm et al. 2005, Yoon et al. 2005). GnRH neuron activity and secretion are stimulated by excitatory afferent inputs from AMPA, NMDA, glutamate (via mGluR), and GABA (post-puberty) while inhibitory inputs such as endorphins, corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY) suppress it (Terasawa and Fernandez 2001, Plant 2001, Seminara and Kaiser 2005, Han et al. 2005, Howlett et al. 1986, McShane et al. 1992). Other molecules such as dopamine and norepinepherine are thought to act as neuromodulators which fine-tune GnRH pulsatility (Dumalska et al. 2008). Although numerous regulators of GnRH release have since been identified the exact mechanisms underlying pulsatile GnRH secretion are still not fully understood. What is clear however is that a dynamic balance between excitatory and inhibitory signals together regulate GnRH pulses (d' Anglemont de Tassigney 2010, Pinilla et al. 2012). In 2003 a novel neuropeptide called Kisspeptin (KISS1) was discovered to be the most potent positive trigger for GnRH secretion and was critical for the timely onset of puberty (Seminara et al. 2003, de Roux et al. 2003, Pinilla et al. 2012, Popa et al. 2008).

1.3 Kisspeptin and the Kisspeptin receptor

Kisspeptin was initially described as a metastasis suppressor (Lee et al. 1996) and ongoing studies continue to investigate its role in the pathology of various cancers (Harms et al. 2003). In 2001, several independent groups identified KISS-1 as the ligand for the orphan receptor GPR54, now called the kisspeptin receptor (KISS1R) (Kotani et al. 2001, Ohtaki et al. 2001 and Muir et al. 2001, Kirby et al. 2010).

In humans, the *KISS1* gene located on the long arm of chromosome 1 (1q32) encodes a 145 amino acid precursor protein which is cleaved into smaller peptides collectively referred to as kisspeptins (West et al. 1998). These kisspeptins belong to the superfamily

of RF-amide related peptides (RFRP) due to a shared carboxy terminus containing an Arg-Phe-NH2 motif (Fig. 1.4) (Roa et al., 2008, Pinilla et al. 2012).

Early studies describe KISS1 as a potent metastasis suppressor in C8161, a human melanoma cell line (Lee et al., 1996, Lee and Welch 1997). It was soon shown that the 54 amino acid kisspeptin peptide, KP-54, undergoes subsequent cleavage and results in the formation of kisspeptin 14, 13 and 10 corresponding to the number of amino acids they contain (Fig. 1.4). KP-54 -14 -13 -10 are all biologically active, share the same carboxy terminus and bind KISS1R. (Kotani et al. 2001, Ohtaki et al. 2001) KP-10 has been shown to have the greatest receptor affinity but is rapidly degraded in serum, while KP-54 has a lower receptor binding affinity but is more stable in serum (Thompson et al., 2006, Mikkelsen et al. 2009, Asami et al. 2013, Matsui et al. 2013). A recent study demonstrated that a specific proprotein convertase called furin is essential for post-translational processing of KP-54 into the smaller peptides (Harihar et al. 2014).

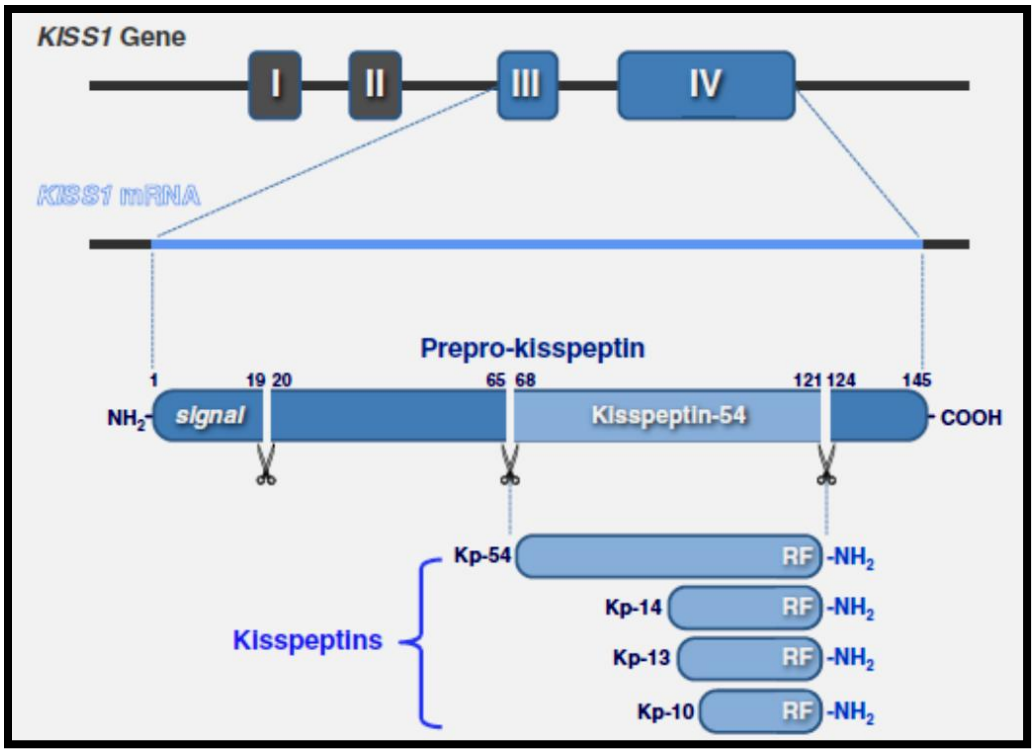

Figure 1.4: Graphic representation of the formation of kisspeptins from the cleavage of a 145 amino acid precursor encoded by the Kiss1 gene.

The prepro-kisspeptin is enzymatically cleaved to 54 amino acid kisspeptin fragment (KP-54, also called metastin). Subsequent proteolytic cleavage yields KP-14, KP-13 and KP-10 all of which share the 10 amino acid sequence of Kp-10 which is shown to be enough to activate KISS1R. (Adapted from Pinilla et al. 2012 and Roa et al. 2008).

KISS1R is a $G\alpha_{q/11}$-coupled (Class A) GPCR and activation by kisspeptin stimulates phospholipase C (PLCβ) catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to the $InsP_3$ and DAG. $InsP_3$ activates its receptor on the endoplasmic reticulum (ER) resulting in the efflux of ER Ca^{2+} into the cytosol (Fig. 1.5). PLCβ mediated hydrolysis of PIP_2, intracellular Ca^{2+} mobilization and $InsP_3$ accumulation are therefore key readouts for KISS1R signaling via $G\alpha_{q/11.}$ Studies showed that pertussis toxin had no effect on $InsP_3$ turnover or Ca^{2+} mobilization which together confirm that KISS1R is coupled to $G\alpha_{q/11.}$ They also showed that kisspeptin did not change cAMP levels indicating that it does not couple to G_s or G_i. Kisspeptin stimulation also resulted in arachidonic acid release, ERK1/2 and p38 MAP kinase phosphorylation (Lee et al. 1999, Wacker et al. 2008, Muir et al. 2001, Liu et al. 2008 and Constantin et al. 2009). ERK1/2 phosphorylation is an important result of KISS1R action since it has been shown to regulate GnRH secretion (Castellano et al. 2006). *In vitro* work in the Babwah laboratory demonstrated that β-arrestin mediates Kp-dependent ERK1/2 phosphorylation using WT KISS1R and β-arrestin deficient MEFs (Pampillo et al. 2009, Szereszewski et al. 2010). In this dissertation we confirmed that KISS1R signals in a β-arrestin-dependent manner to ERK1/2 using a KISS1R mutant, L148S and β-arrestin deficient MEFs and further demonstrated that a functional result of this β-arrestin- dependent cascade is GnRH secretion.

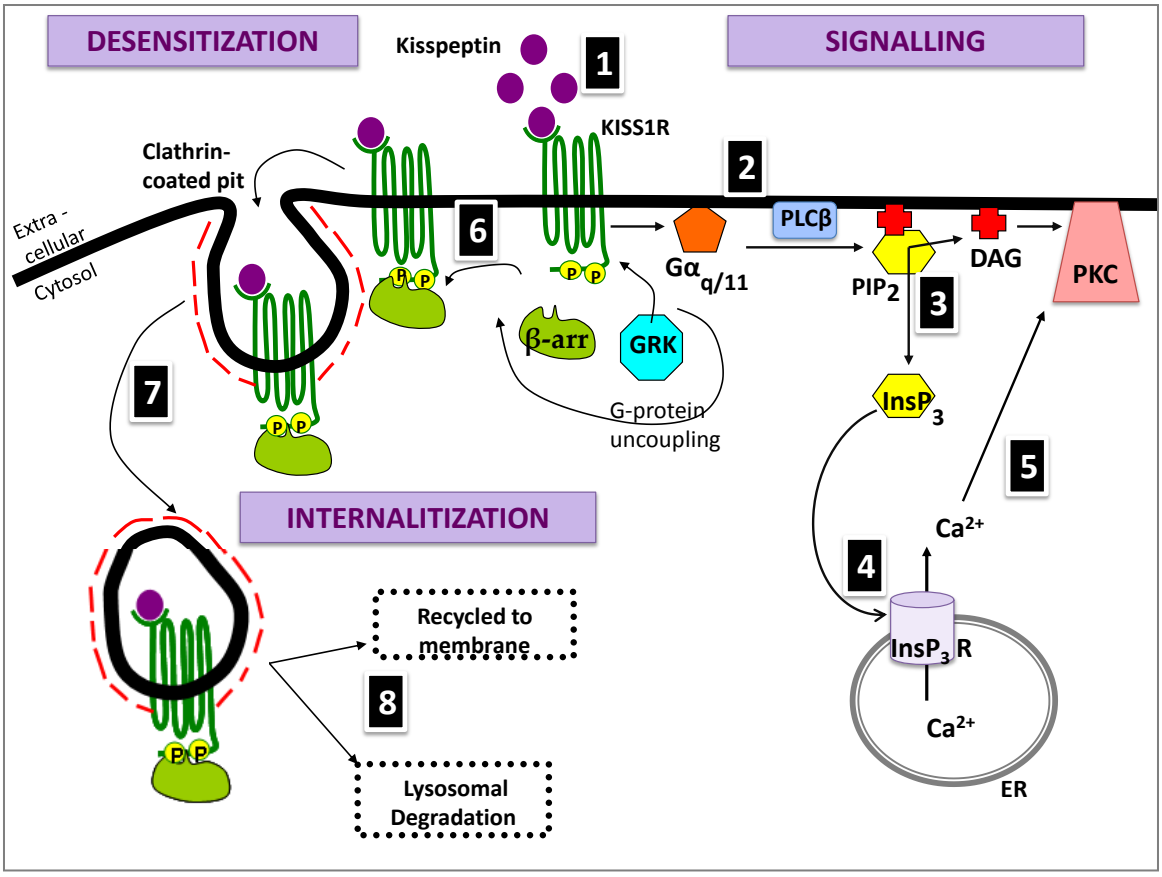

Figure 1.5: Schematic diagram for intracellular KISS1R signaling combining known and proposed mechanisms.

Kisspeptin (1) couples KISS1R to $G\alpha_{q/11}$ (2) and results in the cleavage of PLCβ catalysed hydrolysis of PIP_2 into second messengers, $InsP_3$ and DAG (3). $InsP_3$ binds its receptor and causes release of Ca^{2+} from ER stores (4) DAG and Ca^{2+} together activate the PKC enzyme (5) needed to potentiate the $G\alpha_{q/11}$–dependent KISS1R cascade. In the continued presence of kisspeptin, GRK2 and possibly β-arrestin proteins mediate homologous desensitization of KISS1R (6) and internalization (6-7). Internalized KISS1R may then be either degraded or recycled to the plasma membrane (8) (Ferguson 2001, Shenoy et al. 2003, Kotani et al. 2001, Pampillo et al. 2009, Bianco et al. 2011, Wacker et al. 2008).

Two landmark studies in 2003 highlighted the critical role of kisspeptin and KISS1R in the re-awakening of the HPG axis for the timely onset of puberty (de Roux et al. 2003 and Seminara et al. 2003). These studies examined genetic profiles of patients diagnosed with idiopathic hypogonadotropic hypogonadism (IHH), a syndrome characterized by an underactive HPG axis leading to delayed pubertal onset, low levels of serum gonadotropins and sex steroids and isolated infertility. Patients diagonosed with IHH in these studies were found to also bear homozygous deletions in the *KISS1R* gene. Further evidence that confirmed the importance of KISS1R as a regulator of fertility came from genetically engineered mice completely lacking the KISS1R gene (Lapatto et al. 2007, d'Anglemont de Tassigny et al. 2007, Dungan et al. 2012). These mice recapitulated the infertile hypogonadotropic hypogonadism (HH) phenotype of the human patients. Taken together, these results show that both mice and humans with non-functional KISS1R leads to impeded pubertal onset resulting in infertility in adults combined with low serum gonadotropin and sex steroids as well as abnormal gonadal development.

1.3.1 Kiss1/Kiss1R system directly potentiates GnRH secretion

Regardless of species, route of administration, estrous cycle phase and type of kisspeptin agonist used, application of kisspeptin was demonstrated to be a potent trigger of gonadotropin secretion *in vivo*. Continuous as well as bolus administration of kisspeptin resulted in significant increases in serum gonadotropins in humans (Dhillo et al. 2005 and 2007), monkeys (Plant et al. 2006, Shahab et al. 2005) ruminants (sheep and goats) (Messager et al. 2005, Hashizume et al. 2010) rodents (Gottsch et al. 2004, Irwig et al. 2004, Messager 2005, Matsui et al. 2004, Navarro et al. 2005) and horses (Magee et al. 2009 and Decourt et al. 2014). Kisspeptin regulation of gonadotropin secretion was shown to be upstream of the GnRH neuron since pre-treatment with GnRH antagonists (acyline and antide) result in elimination of the kisspeptin-triggered rise in serum LH and FSH. (Irwig et al. 2004, Plant et al. 2006, Shahab et al. 2005, Gottsch et al. 2004 and Rosewier et al. 2009) Also, kisspeptin treatment of hypothalamic explants stimulates GnRH secretion *ex vivo* (Thompson et al. 2004 and Tovar et al. 2006). Additionally, kisspeptin treatment increases c-Fos activity (marker of neuronal activity) and GnRH transcription in GnRH neurons (Matsui et al. 2004, Irwig et al. 2004, Han et al. 2005,

Novaira et al. 2009 and Oakley, 2009). Three layers of evidence demonstrate that kisspeptin action is not just upstream of GnRH secretion but directly on the GnRH neurons. Firstly, Kiss1 neurons are not just found in close association with GnRH neurons but also make direct contact with GnRH cell bodies and axons on the internal side of the median eminence (ME) in rodents, (Roseweir et al. 2009, Clarkson et al. 2006, Smith et al. 2008, Inoue 2008, Decourt et al. 2008 (horse) Magee et al. 2009 (horse), Pompolo et al. 2006 (sheep) Hrabovsky et al. 2010 (humans)) Secondly, Kiss1R is expressed in the majority of GnRH neurons in many species (Han et al. 2005, Messager et al. 2005 and Irwig et al. 2004, Herbison et al. 2010, Smith et al. 2011). Finally, KISS1 can depolarize and increase firing rates of GnRH neurons *in vitro* in the presence of tetrodotoxin (TTX) and treatment with kisspeptin antagonist (peptide 234) suppresses GnRH neuron firing, pulsatility and secretion. (Liu et al. 2008, Zhang et al. 2008, Han et al. 2005, Pielekla-Fortuna et al. 2008, Quaynor et al. 2007, Dumalska et al. 2008, Rosewier et al. 2009, Millar 2010)

1.3.2 Kisspeptin neuron subpopulations

With their close spatial associations, it is not surprising that both GnRH neurons and Kiss1 neurons are localized mainly to the infundibular nucleus (InF) in humans (ARC in other species) with a second dense population in the rostral preoptic area (Rometo et al. 2007 and Hrabovszky et al., 2010). While the Kiss1 population in the ARC seems to be standard across species, there seems to be a wide variation in the rostral preoptic population (Clarkson et al. 2006, Pompolo et al. 2006, Ramaswamy et al. 2008; Clarkson et al. 2009, Hrabovszky et al.2010). Rodents for example, have a well-defined nucleus of Kiss1 neurons in the anteroventral periventricular nucleus (AVPV) and the periventricular nucleus PeN (Gottsch et al. 2004, Smith et al. 2005 and 2006, Clarkson et al. 2006 and 2009) but humans, sheep, goats have a more scattered kisspeptin neuron distribution in the preoptic area and in the monkey it seems to be completely absent (Pompolo et al. 2006, Ramaswamy et al. 2008, Oakley 2009, Hrabovszky et al. 2010). These anatomically separate populations of Kiss1 neurons also play functionally distinct roles in regulation of the HPG axis (Millar 2010). It is well established that gonadal sex steroids (E2, T, P4) regulate the HPG axis by negative and positive feedback on the

hypothalamus and the pituitary (Karsch et al. 1987, Shaw et al. 2010).However, GnRH neurons do not express significant levels of estrogen receptors (ERα) or androgen receptors (AR). Recent studies show that kisspeptin neurons express high levels of sex steroid receptors (ERα, PR and AR) and mediate the feedback of gonadal steroids on GnRH secretion (Lehman and Karsch 1993, Navarro et al. 2004, Estrada et al. 2006; Franceschiniet al. 2006, Smith et al. 2005, Adachi et al. 2007, Cheng et al. 2010, Smith et al. 2007). GnRH neurons do express ERβ but this receptor is not involved in steroid feedback (Herbison and Pape 2001, Smith 2005, Bateman et al. 2008) It is now established that kisspeptin neurons in the ARC mediate negative steroid feedback and those in the AVPV mediate positive feedback (Smith et al. 2005, Clarkson et al. 2006, Adachi et al. 2007). In sheep, humans and monkeys there is less distinct anatomical separation of the two kisspeptin neuronal populations but it remains entirely possible for two distinct types of kisspeptin neurons to both be localized in the ARC/InF (Rometo et al. 2007, Estrada et al. 2006; Pompolo et al. 2006, Ramaswamy et al. 2008 and Oakley 2009).

Evidence for Kiss1 neurons in ARC mediating negative steroid feedback comes from studies using gonadectomised animals (rodents, sheep, monkeys) to show that in the absence of gonadal steroid feedback Kiss1 mRNA expression in the ARC increases and this result is reversed with estrogen or testosterone replacement (Irwig et al. 2004, Oakley 2009 and Lehman et al. 2010, Smith et al. 2005, Pompolo et al. 2006). Due to the absence of negative steroid feedback, castrates normally experience a post-castration LH surge (McPherson 1982). The administration of a kisspeptin antagonist (peptide 234) in these animals prevents this surge, indicating that increased Kiss1 mRNA expression in the ARC is translated to increased KISS1 secretion resulting in the post-castration LH surge (Roseweir et al. 2009). Further evidence for this is that ovariectomized *Kiss1r*$^{-/-}$ mice show increased Kiss1 mRNA expression in the ARC but fail to show the expected LH surge (Dungan et al. 2007). Taken together, these findings demonstrate that gonadal steroids inhibit kisspeptin neurons in the ARC and reduce their stimulatory input for GnRH/LH release Skorupskaite et al. 2014.

Kiss1 neurons in ARC also mediate negative steroid feedback over the estrus cycle in rodents. Kiss1 mRNA is low during proestrus when estrogen levels are high and high during diestrus when estrogen levels are low (Adachi et al. 2007, Smith et al. 2006). Menopause is another period when estrogen levels are low due to decreased ovarian follicles, and in both humans and monkeys Kiss1 mRNA and LH secretion are increased (Kim et al. 2009, Rance et al. 2008 and Rometo et al. 2007).

1.3.2.1 KNDy neurons in the ARC

While kisspeptin is the most potent trigger for GnRH secretion, it is not the only regulator. This is suggested by the sustained low gonadotropin levels and Gnrh pulses in

Kiss1r $^{-/-}$ mice and variable hypogonadal phenotype with some degree of estrous cyclicity in *Kiss1*$^{-/-}$ mice (Seminara et al. 2003, Colledge 2009, Lapatto et al. 2007, Navarro et al. 2009). Also, in a separate *Kiss1r*$^{-/-}$ mouse, ovariectomy followed by estradiol treatment resulted in a Gnrh/LH surge and Fos activity in Gnrh neurons (Dungan et al. 2007). Kisspeptin neurons in the ARC also express the neuropeptides neurokinin B (NKB) and dynorphin (Dyn) which have been shown to regulate Gnrh secretion (Cheng et al. 2010, Goodman et al. 2007, Wakabayashi et al. 2010, Hrabovszky et al. 2010, Topalaglu et al. 2009). Because almost all Kiss1 neurons in the ARC co-express NKB and Dyn they were called KNDy neurons (Goodman et al. 2007

Similar to Kiss1, NKB has been shown to mediate negative steroid feedback on the hypothalamus (Rance et al. 1991 and 1994). Ovariectomized monkeys showed an increase in NKB mRNA expression in the InF (ARC) which like Kiss1, is reduced with estrogen replacement (Abel et al. 1999, Sandoval-Guzman et al. 2004). Humans bearing NKB or NKB receptor mutations displayed IHH symptoms similar to those caused by KISS1R mutations (Guran et al. 2009 and Topalaglu et al. 2009). NKB effect on gonadotropins seems to vary depending on species and hormonal status. NKB (senktide) stimulates LH release in ruminants that have been ovariectomized but treated with E2 and P4, anestrous ewes, and females in the follicular phase of the estrus cycle (NKB also stimulates LH secretion in juvenile male monkeys and intact male and female mice. LH response decreases when NKB is administered to sheep in the luteal phase or

ovariectomized mice treated with E2. The exact understanding of NKB regulation on GnRH secretion is incomplete but it is clear from patients with NKB or NKBR mutations that it does play a critical role in HPG axis function (Wakabayashi et al. 2010, Navarro et al. 2011, Sandoval-Guzman et al. 2004, Kinsey-Jones et al. 2012).

Dynorphin activates the kappa opioid receptor (KOR) and mediates the inhibitory progesterone feedback on GnRH pulsatility. Dyn or KOR antagonists result in increased LH levels and OVX sheep and postmenopausal women show decreased Dyn expression in the ARC and this result is not reversible with E2 administration. Taken together these results indicate that progesterone and dynorphin inhibit GnRH pulsatility and LH secretion (Foradori et al. 2006, Goodman et al. 2004 and 2007). Overall, gonadal steroids negatively regulate GnRH secretion by acting on the kisspeptin neurons in the ARC (KNDy neurons). Here they suppress kisspeptin and NKB (GnRH stimulators) and promote dynorphin (GnRH inhibitor).

1.3.2.2 Kisspeptin neurons in the AVPV

Kisspeptin neurons in the preoptic area/AVPV do not express NKB or dynorphin but do co-express tyroxine hydroxylase and mediate E2 positive feedback on GnRH neurons (Simerly et al.1989, Smith et al. 2005 and 2006). Evidence for Kiss1 neurons in AVPV mediating positive steroid feedback comes from studies using gonadectomised animals (rodents, sheep, monkeys) to show that in the absence of gonadal steroid feedback Kiss1 mRNA expression in the AVPV decreases. This result is reversed with estrogen or testosterone replacement (Dungan et al. 2007, Smith et al. 2005; Pompolo et al. 2006). During the pre-ovulatory LH surge in the late follicular phase when E2 levels are at their highest, Kiss1 neurons in the AVPV are activated and direct delivery of kisspeptin antagonist to this area suppresses the surge (Kinoshita et al. 2005, Pineda et al. 2010, Adachi et al. 2007, Smith et al. 2006, Clarkson et al. 2008, Teresawa 1980, Wintermantel et al. 2006). In mice during proestrus, when E2 is the highest, Kiss1 mRNA in the AVPV is also at the highest expression level (Smith et al. 2006). In rodents, ERα expressing neurons make direct contact with GnRH neurons and due to their localization these are thought to be Kiss1 neurons in the AVPV (Teresawa 1980, Wintermantel et al. 2006). Ovariectomized mice treated with E2 and P4 are still able to induce GnRH neuron

activation (using Fos as a marker of neuronal activation) and corresponding LH surge. However, *Kiss1r* $^{-/-}$ mice treated in the same manner show no LH surge and no GnRH neuron activation demonstrating a role for Kiss1/Kiss1R mediating E2/P4 positive steroid feedback on GnRH neurons (Clarkson et al. 2008).

While humans and sheep do not have a distinctly separate Kiss1 neuronal population in the AVPV, studies have still demonstrated that Kiss1/Kiss1R signaling in the ARC/InF mediates positive steroid feedback on GnRH neurons. In humans, a study in assisted reproductive therapies utilised KP-54 to generate a LH surge which resulted in successful ovulation and live term birth (Abbara et al. 2013). Healthy women treated with KP-54 twice daily resulted in an advanced pre-ovulatory LH surge during their menstrual cycle (Jayasena et al. 2013). In sheep, kisspeptin treatment advances the pre-ovulatory LH surge which is ablated or reduced by administration of Kiss1 antagonists. (Kinoshita et al. 2005, Caraty et al. 2007, Clarkson et al. 2008, Pineda et al. 2010) Also, since sheep like humans do not have anatomically separate Kiss1 populations, it is not surprising that Kiss1 mRNA is increased in the ARC during their pre-ovulatory LH surge when E2 levels are at the highest (Smith et al. 2008).

In rodents, the suprachiasmatic nucleus (SCN) is the circadian clock and is responsible for the timing of the LH surge. Studies show that Kiss1 neurons in the AVPV receive direct afferent input from the SCN providing further evidence for their role in regulating the pre-ovulatory surge (Christian and Moenter 2010, Khan and Kauffman 2012). Taken together, these studies demonstrate that Kiss1 neurons in the AVPV play an important role in mediating the positive sex steroid feedback to GnRH neurons that is seen during the pre-ovulatory LH surge.

1.3.3 Role of kisspeptin in puberty

In primates, GnRH pulse frequency and amplitude are high in the embryo and neonates. Pulse frequency drops to a quiescent state during childhood referred to as the "juvenile phase", then during puberty pulse frequency gradually increases nocturnally as the HPG axis reawakens (Plant 2008, Terasawa et al. 2001, Weisz et al. 1980, Styne et al. 1994; Plant et al. 2004; Sisk et al. 2004). In the 1980's, studies established that GnRH secretion

increases at the onset of puberty and that pulsatile administration of exogenous GnRH can induce precocious puberty in monkeys (Wildt et al. 1980 and Wantanabe et al. 1989). While the trigger for the reawakening of GnRH pulsatility at puberty is not fully understood, it is thought that it involves a combination of decreased activity from inhibitory inputs and increased activity in excitatory ones (Brann et al. 1994; Sisk and Foster, 2004; Ojeda et al.2006, Terasawa et al. 2001, Cookson et al. 2012). Of these inputs, the Kiss1/Kiss1R signaling system is thought to be the most potent positive trigger for GnRH release at puberty (Irwig et al. 2004, Han et al. 2005 and Pielecka-Fortuna et al. 2008). The following studies serve to highlight the critical role of the KISS1/KISS1R system in regulating reproductive function. Firstly, using linkage analysis and genome sequencing, patients with loss-of-function KISS1R mutations were found to display a condition referred to as IHH where there is a significant delay in the onset of puberty (de Roux et al. 2003, Seminara et al. 2003, d'Anglemont de Tassigny et al. 2007, Topaloglu et al. 2012). While loss-of-function mutations in KISS1R are associated with IHH, patients with gain-of-function mutations in KISS1R show symptoms of precocious puberty (Teles et al. 2008). Recently, children with a gain-of-function mutation in KISS1 have been diagnosed with precocious puberty due to prolonged serum stability of the mutant KISS1 agonist (Silveira et al. 2010). Mice with targeted deletions of *Kiss1* and *Kiss1r* recapitulate the IHH phenotypes in humans (Seminara et al. 2003, Funes et al. 2003, Lapatto et al. 2007). Finally, mice with GnRH neuronal-specific deletion of Kiss1R also display the IHH phenotype indicating that although Kiss1R may be expressed in other tissues, the GnRH neuron is the site for KISS1R regulation of reproduction (Kirlov et al. 2013, Novaira et al. 2014). Kirlov et al. 2013 went further to demonstrate this by rescuing the infertile global Kiss1R knockout mice (Seminara et al. 2003) by specifically re-introducing Kiss1R into GnRH neurons in mice with a global Kiss1R deletion.

Increased hypothalamic Kiss1/Kiss1R signalling is detected prior to puberty using various experimental techniques and across species. Kiss1 and Kiss1R mRNA increase at puberty in rats, mice and monkeys (Navarro et al. 2004, Sun et al. 2007, Han et al. 2005, Shahab et al. 2005). In mice, Kiss1 positive neurons also increase exponentially in the AVPV/PeN between postnatal day 10 (P10) to P64 (Clarkson et al. 2009). Kiss1 neurons start forming close appositions with GnRH neurons in the ME and POA reaching 50%

co-localization at 7 weeks (pubertal age in mice) Clarkson et al. 2006). Using monkeys, Keen et al. (2008) showed that the increase in *Kiss1* mRNA described in previous studies translated to increased pulsatile nocturnal Kiss1 secretion which largely coincided with GnRH pulses at puberty (Shahab et al. 2005). The higher frequency of GnRH pulses at puberty is thought to be due to a combination of increased KISS1 secretion and increased sensitivity of GnRH neurons to KISS1. This is demonstrated by increased *Kiss1r* mRNA expression in the AVPV/PeN and a higher GnRH neuronal depolarization and firing rate in response to KISS1 in pubertal mice compared to juveniles (Han et al. 2005). KISS1 administration in juveniles advances the onset of puberty in rats and monkeys (Navarro et al. 2004 and Plant et al. 2006) and KISS1 antagonist (Peptide 234) inhibits pulsatile GnRH secretion and delays puberty in monkeys (Roseweir et al. 2009 and Pineda et al. 2010). Taken together, these studies provide evidence that at the time of pubertal onset, hypothalamic kisspeptin expression and secretion are increased, GnRH neuronal activity also increases, and this leads to an increase in GnRH release which triggers the onset of puberty.

1.4 Pathophysiological causes of clinical infertility involving KISS1/KISS1R in humans and mice.

The foundation for our present understanding of the role of KISS1/KISS1R signalling in regulating reproductive function is greatly due to *in vivo* work done with *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ mice (Funes et al., 2003, Seminara et al. 2003, Lapatto et al. 2007, d'Anglemont de Tassigny et al. 2007). These mice recapitulated the phenotypes observed in IHH patients bearing inactivating KISS1R mutations. While pubertal onset was significantly delayed and these mice were completely infertile, these mice still displayed evidence of partial sexual maturation and low levels of GnRH release (Seminara et al. 2003, Lapatto et al. 2007, Chan et al. 2009, Dungan et al. 2012). This is a parallel finding in some IHH patients bearing inactivating KISS1R mutations where low level LH pulses are detected and some even experience reversal of symptoms later in life (Seminara et al. 2003, Tenebaum-Rakover et al. 2007, Wahab et al. 2011, Nimiri et al. 2011, Topaloglu et al. 2012, Sidhoum et al. 2014). Overall, these findings indicate that KISS1/KISS1R signaling is essential for triggering GnRH release for the timely onset of puberty but is

not a strict requirement or absolutely needed for GnRH secretion (Lapatto et al. 2007, Chan et al. 2009). This is not very surprising since reproductive capability is essential for the survival of any species so therefore over a period of evolutionary time many redundant systems possibly developed in order to safeguard fertility. Studies have even demonstrated that <3% of KISS1 neurons and <12% GnRH neurons are sufficient to maintain fertility in mice (Mayer and Boehm 2011, Herbison et al. 2008).

1.4.1 *Kiss1r* $^{-/-}$ mice

Like humans with inactivating mutant KISS1R, mice with an absence of KISS1R display hypogonadotropic hypogonadism and experience abnormal and incomplete pubertal maturation and are completely infertile (Seminara et al. 2003, Funes et al. 2003, Lapatto et al. 2007, d'Anglemont de Tassigny et al. 2007). Exogenous GnRH can stimulate gonadotropin release in these mice but exogenous kisspeptin does not. This indicated that the defect was at the hypothalamic level upstream of GnRH secretion and further suggests that kisspeptins act through KISS1R expressed on GnRH neurons (Messager et al. 2005). The females display an ability to undergo spontaneous estrus and folliculogenisis since various stages of follicle development are seen in the mutant ovary, however ovulation is impaired as indicated by the absence of corporea lutea. Female gonadal weights in mutants are significantly less than wild-types (thread-like) but vary between 70 - 90% less than wild-type (Chan et al., 2009, Kirilov et al., 2013, Novaira et al., 2014). Testicular weight varies widely in *Kiss1r*$^{-/-}$ males from significantly decreased to comparable to wild-type males. Onset of female puberty was also variable as indicated by vaginal opening (VO) where some mutant females experienced delayed VO while others never did. Mutant males mirrored the variable phenotype of the mutant females where spermatogenesis was severely affected in some while unaffected in others and similarly preputial separation (indicator of puberty) only occurred in some *Kiss1r*$^{-/-}$ while never occurring in others (Lapatto et al., 2007, d'Anglemont de Tassigny et al., 2007, Chan et al., 2009, Kirilov et al., 2013, Novaira et al., 2014). These findings indicate that in the absence of *Kiss1r*$^{-/-}$ some degree of GnRH secretion is still possible. Authors suggest that this may be possible through the many stimulatory afferent inputs to the GnRH neuron such as NKB, for example, which is secreted by KNDy neurons in the

ARC. Finally, while the GnRH-neuron specific *Kiss1r*$^{-/-}$ mice (Kirilov et al. 2013 and Novaira et al., 2014) were important for showing the importance of KISS1/KISS1R signaling in regulating GnRH secretion, the global *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ mice (Seminara et al., 2003, Lapatto et al., 2007) were essential for other studies demonstrating that this signaling also played a role in diabetes (Hague-Evans et al., 2006, Silvestre et al., 2008), placentation, (Bilban et al. 2004, Janneau et al. 2002, Kotani et al. 2001, Hiden et al., 2007), pregnancy (Jayasena et al. 2009 and Dhillo et al. 2008) and energy homeostasis (Castellano et al. 2005, Smith et al. 2006, Hill et al. 2008).

1.4.2 *Kiss1*$^{-/-}$ mice

Mice with an absence of KISS1 display hypogonadotropic hypogonadism like their receptor counterparts, and experience abnormal and incomplete pubertal maturation and are completely infertile (Seminara et al. 2003, Lapatto et al. 2007, d'Anglemont de Tassigny et al. 2007). Exogenous GnRH as well as kisspeptin can stimulate gonadotropin release in these mice. However, *Kiss1*$^{-/-}$ mice were found to display even more phenotypic variation compared to *Kiss1r*$^{-/-}$ mice (Lapatto et al., 2007, Chan et al., 2009). One possible explanation for this is that these mice express functional and intact KISS1R. Recent studies demonstrated that KISS1R has constitutive (kisspeptin-independent) activity (Babwah et al., 2012). This result taken together with previous findings that GnRH neurons have a basal firing rate (Moenter, 2010) can explain the increased phenotypic variability observed with *Kiss1*$^{-/-}$ mice compared to *Kiss1r*$^{-/-}$ being possibly due to sustained basal GnRH secretion.

1.4.3 KISS1R mutant receptors

Clinically, hypogonadism is characterized by infertility coupled with low sex steroid levels and can be as a result of a defect at any level in the hypothalamic-pituitary gonadal axis. In the absence of CNS lesions, gonadal defects and with normal pituitary function, indicated by a GnRH stimulation test, the cause for hypogonadism would therefore be hypothalamic. These patients are further diagnosed as having hypogonadotropic hypogonadism (HH) where in addition to infertility and low gonadal steroids, serum gonadotropins are also low. In such patients, GnRH deficiency can be due to either

aberrant developmental migration or defective GnRH secretion. Aberrant developmental migration of GnRH neurons causes a condition termed Kallman syndrome (KS) which is characterised by anosmic hypogonadotropic hypogonadism. Main genes that have been found to be associated with Kallman's syndrome are; KAL1, FGFR1/FGF8, PROKR/PROK2 (Noel et al. 2011, Bianco et al. 2009). If patients exhibiting the symptoms of HH are normosmic, it would indicate that GnRH neuron migration was intact along with the co-migration of the olfactory neurons which occurs simultaneously during embryonic development. These patients are diagnosed with normosmic or idiopathic hypogonadotropic hypogonadism (IHH). Main genes associated with IHH are KISS1/KISS1R, NKB/NK3R (Noel et al. 2011, Wahab et al., 2011, Nimiri et al. 2011, George et al. 2012).

Table 1.1: Overview of genes found to be associated with hypogonadotropic hypogonadism in patients.

Gene	Function	Disease and cause
KAL1	Encodes for anosmin-1. An extracellular protein essential for guiding GnRH neurons along axons from the nasal placode to anterior hypothalamus.	HH – abnormal GnRH migration
FGFR1/FGF8	Encodes for the tyrosine kinase receptor; fibroblast growth factor receptor1 and ligand, FGF-8. Resulting MAPK signaling is essential during development for neuronal migration, differentiation, survival and proliferation.	HH – abnormal GnRH migration
PROKR/PROK2	Encodes for the GPCR; prokineticin receptor 2 and cognate ligand. Signalling cascade is essential for development of the neuronal progenitors of the olfactory and GnRH neurons.	HH – abnormal GnRH migration
KISS1/KISS1R	Encodes for GPCR; kisspeptin receptor and cognate ligand. Signalling cascade is essential for the triggering of GnRH release at puberty.	IHH – defective GnRH secretion
NK3R/NKB(mice) *TACR3/TAC3(human)*	Encodes for the GPCR; neurokinin B receptor and cognate ligand. NKB is co-expressed in the KNDy neurons of the ARC. NKB is thought to act synergistically with KISS1 to stimulate GnRH release.	IHH – defective GnRH secretion

Kallman's syndrome (KS) and normosmic hypogonadotropic hypogonadism (IHH). [1]

[1] (Bianco et al., 2009, Legoius et al., 1991, Ford-Perriss et al. 2001, Gill et al. 2004, Li et al., 2001, Lin et al., 2002, Cheng et al., 2002, Prosser et al., 2007, Pitteloud et al. 2007, deRoux et al., 2003, Seminara et al. 2003, Gottsch et al., 2004, Irwig et al., 2004, Matsui et al., 2004, Shahab et al., 2005, Navarro et al., 2004, Topalaglu et al., 2009, Rance et al., 2009, Navarro et al., 2011, Ramaswamy et al., 2010)

While HH is a rare condition, for the cases in which the genetic cause is known, 60% are classified as KS while 40% as IHH. The genetic causes for the majority of HH cases however, have not been discovered (Bianco et al. 2009). Focusing on the genetic causes behind IHH, the kisspeptin and neurokinin B signaling systems play key roles. While patients with inactivating mutations of either neurokinin B or its receptor display IHH, mice lacking NKB or NK3R display preserved fertility (Siuciak et al., 2007). NKB and KISS1 are co-expressed on KNDY neurons and it is thought that NKB modulates estradiol-dependent GnRH secretion along with KISS1 (Wakabayashi et al., 2010, Navarro et al., 2011, Rance et al., 2010).

IHH paients associated with inactivating mutations in KISS1R present with different degrees of severity of hypogonadism but all share the common phenotype of delayed puberty and impaired fertility. Some present with micropenis and cryptorchidism indicating that the KISS1/KISS1R cascade is important during the "mini-puberty" during fetal development (Wahab et al. 2011, Seminara et al., 2003). All these patients can be treated with a regimen of pulsatile exogenous GnRH and gonadotropins to stimulate gonadal development and sex steroid production (Lanfranco et al., 2005 and Pallais et al., 2006). When treated, one female patient bearing the p.L148S mutation was reported to conceive and carry a full-term pregnancy with the delivery of a healthy baby (Pallais et al. 2006). These results in humans parallel findings in *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ mutant mice where KISS1/KISS1R signaling is important for the onset of puberty but is not absolutely necessary for GnRH release (Wahab et al. 2011, Chan et al. 2009). Patients with IHH corresponding to KISS1R inactivating mutation occurs with a frequency of less than 5% however there are no population studies to date which give estimates as to the prevalence of this in the general population (Cerrato et al., 2007).

Table 1.2: Summary of KISS1R mutations found in patients diagnosed with IHH or CPP.

KISS1R mutation[2]	Phenotype	Reference
L148S	IHH	Seminara et al 2003 and Wacker et al. 2008
L102P	IHH	Tenebaum-Rakover et al. 2007
R331X & X399R	IHH	Seminara et al. 2003
R297L	IHH	Semple et al. 2005
155 nuc. Deletion	IHH	de Roux et al. 2003
C223R	IHH	Semple et al. 2005
1001ins1002C	IHH	Lanfranco et al. 2005
F272S	IHH	Nimiri et al. 2011
R386P	CPP	Teles et al. 2008, Bianco et al. 2011

[2] Mutations include point, deletion and insertion mutations of the *KISS1R* gene.

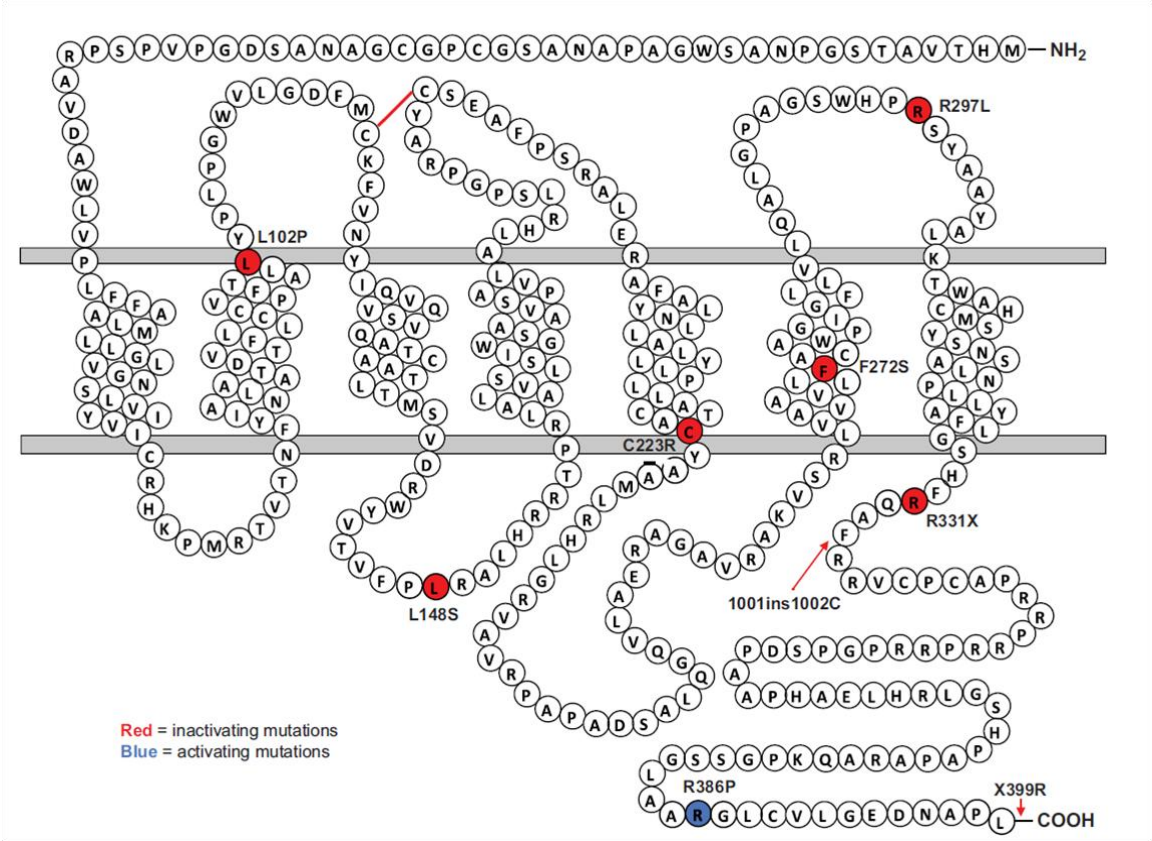

Figure 1.6: Schematic representations of the known KISS1R mutations reported in humans with impaired reproductive function.

Mutations in KISS1R are rare and as they are found throughout the length of the gene so there are no specific "hot-spots" of biological significance. Adapted from Pinilla et al. 2012.

Functional studies performed with some of the inactivating KISS1R mutations associated with IHH demonstrate that the receptor is impaired at one or more of the following stages of expression or function; trafficking through the cytosol resulting in low expression at the plasma membrane, impaired ligand binding capacity or impaired ability to activate effectors in the signaling cascade. Detailed functional studies have not been done for all inactivating KISS1R mutations found in clinical studies so here I will discuss further the ones that have been well characterised in the literature.

1.4.3.1 Loss-of-function KISS1R mutation - L148S

In 2003 a study described 6 siblings from a consanguineous Saudi-Arabian family bearing the homozygous mutation of KISS1R, p.L148S. These patients presented with delayed puberty, primary amenorrhea and low gonadotropins (Seminara et al. 2003). This mutation affects the second intracellular loop (ICL2) of KISS1R, an important site for G-protein and β-arrestin association with GPCRs (Fig. 1.6) (Wacker et al. 2008). Functional studies showed that the L148S mutant receptor is expressed at the plasma membrane and binds kisspetin with equivalent levels and efficiency as the wild-type KISS1R. However, it is significantly impaired in stimulating $InsP_3$ formation with kisspeptin treatment and further molecular analysis revealed that while L148S binds kisspeptin and interacts with $G\alpha_{q/11}$ it is unable to catalyse the dissociation of Gα from Gβγ necessary for the activation of the G-protein signalling cascade (Wacker et al. 2008). Another female patient in 2006 bearing the homozygous L148S mutation was able to be treated with exogenous pulsatile GnRH and gonadotropins resulting in successful assisted conception full term uncomplicated pregnancy and normal vaginal delivery of a healthy baby (Pallais et al., 2006).

1.4.3.2 Loss-of-function KISS1R mutation - L102P

In 2003, a female patient bearing homozygous mutation L102P in KISS1R was diagnosed with IHH (de Roux et al. 2003). She presented with delayed puberty, primary amenorrhea low gonadotropins and low sex steroid levels. Treatment with exogenous pulsatile GnRH and estrogen rescued the phenotype sufficiently to allow the patient to carry two normal term pregnancies (de Roux et al 2003, Tenebaum-Rakover et al. 2007

and Noel et al. 2011). In 2007, an Arab-Muslim male patient bearing the same homozygous L102P mutation was described. He presented with micropenis and bilateral cryptorchidism and had two female siblings with primary amenorrhea. This patient was examined from 12.5 – 21 years which allowed for a rare long-term study of humans carrying an inactivating KISS1R mutation (Tenebaum-Rakover et al 2007). From 12.5 – 18 years this patient had only basal gonadotropin response to GnRH stimulation but after 18 years there was a pattern switch leading to significant gonadotropin response to GnRH stimulation. This result is unexplained but suggests that while KISS1R function regulates timely onset of puberty, it is not strictly required for GnRH release and that there is a slow maturation of the HPG axis over time. This is important since "loss-of –function" KISS1R mutations were previously thought to be a permanently infertile diagnosis, this finding with L102P suggests that this may not always be the case but long-term follow up of patients is necessary to further determine this. Functional studies show that the L102P mutation affects the ICL1 of KISS1R (Fig. 1.6). While L102P has equal binding affinity for kisspeptin as the wild-type KISS1R, it does result in a slight decrease in cell surface expression. This slight reduction of the receptor at the plasma membrane does not correlate to the 95% reduction in $InsP_3$ formation relative to wild-type KISS1R however the molecular explanation for this $G\alpha_{q/11}$ uncoupling is still unknown (Tenebaum-Rakover et al 2007).

1.4.3.3 Gain-of-function KISS1R mutation - R386P

First reported in 2008, p.R386P is the only activating KISS1R mutation discovered to date. A Brazilian female was diagnosed with central precocious puberty displaying progressive breast development from birth. At 8 years she had pubic hair and elevated serum estrogen and gonadotropin levels (Teles et al., 2008). Functional studies revealed that R386P affects the cytoplasmic tail (C-tail) of KISS1R, which is critical for desensitization and internalization of many GPCR's (Fig. 1.6) (Teles et al., 2008, Luttrell et al. 2002). R386P was not significantly different from wild-type receptor in terms of binding affinity for kisspeptin, plasma membrane expression, and dose response to kisspeptin. However a prolonged response to kisspeptin was observed by increased InsP3 accumulation and ERK1/2 phosphorylation (Teles et al. 2008). Further functional assays

with R386P revealed that this mutation causes significantly reduced rates of degradation of the receptor and as a result allows for rapid re-sensitization to the membrane following kisspeptin-induced internalization (Bianco et al. 2011). Overall, reduced degradation of R386P leads to the prolonged signaling and excessive GnRH release causing the CPP observed in affected patients.

Major limitations of these human studies include a relatively small pool of affected patients which are sometimes at young (pre-pubertal) ages and also a lack of long-term follow-up of the fertility of these patients. Since KISS1/KISS1R has been proposed to be essential for timely onset of puberty but not an absolute requirement for GnRH secretion, it would be interesting to see if patients with these inactivating mutations can regain some form of reproductive capability later in life.

1.5 KISS1R as a pharmacological target

Depending on the desired outcome of the treatment, GnRH agonists, antagonists and sex steroids are used clinically to manipulate the HPG axis either by upregulating it in patients with a pathological gonadotropin deficit (IHH, delayed puberty, hypothalamic amenorrhoea, diabetes associated hypogonadism) or downregulating it in cases with pathological gonadotropin excess (PCOS, precocious puberty, menopause, assisted reproductive technologies, prostate cancer, breast cancer, endometriosis, uterine fibroids). Currently GnRH analogues are used where they either activate or suppress GnRH-R function on pituitary gonadotropes resulting in increased or decreased gonadal function respectively (Skorupskaite et al. 2014, Harms et al. 2003, Niida et al. 2006, Seminara et al. 2006, Tomita et al. 2008).

1.5.1 Therapies designed to upregulate the HPG axis

Disease conditions with low level HPG axis function:

a) Hypothalamic amenorrhoea
b) IHH
c) Delayed puberty
d) Diabetes associated hypogonadism

In patients with a pathological deficit in HPG axis activation, as listed above, GnRH agonists are administered in a pulsatile fashion so as to stimulate GnRH-R on pituitary gonadotropes to trigger endogenous LH and FSH secretion. Some of the GnRH agonists currently used are; Leuprolide, Buserelin, Nafarelin, Histerelin and Triptorelin. With the novel discovery of KISS1/KISS1R being a potent positive trigger for GnRH secretion it may be possible to develop more potent and efficacious treatments using kisspeptin agonists. Kisspeptin has been shown to advance puberty in monkeys and mice and therefore may be useful as a treatment in children with delayed onset of puberty (Navarro et al. 2004 and Plant et al. 2006).

In healthy men and women kisspeptin treatment has been demonstrated to stimulate gonadotropin secretion. These studies revealed that kisspeptin-triggered LH pulses are very similar to endogenous LH pulses (Dhillo et al. 2005 and 2007, George et al. 2011, Jayasena et al. 2011, Chan et al. 2011and 2012). Studies were also done in hypogonadotropic patients and again kisspeptin was able to potently stimulate GnRH release and gonadotropin secretion. Women with hypothalamic amenorrhea had a sustained gonadotropin response with twice weekly kisspeptin treatment but this response was lost when treatment was increased to twice daily due to the rapid desensitization of KISS1R. Obesity and diabetes are correlated to hypogonadism and in a study of affected male patients kisspeptin was able to stimulate gonadotropin levels to that of healthy patients (Jayasena et al. 2010, George et al. 2010 and 2013). A recent study even showed that kisspeptin was able to restore normal gonadotropin pulsatility to a patient with TAC/TAC3R mutations (Young et al. 2013). Kisspeptin is also being tested for a role in fine-tuning the LH surge required for ovarian stimulation in assisted reproductive technologies and in one study lead to a normal pregnancy and healthy live birth (Abbara et al. 2013). By being able to stimulate a more endogenous pattern of LH pulses, kisspeptin may be a more effective alternative in assisted reproductive technologies for avoiding the risk of ovarian hyperstimulation syndrome (Skorupskatie et al. 2014).

New kisspeptin analogues are being designed and tested, primarily due to the antimetastatic properties of kisspeptin initially described (Kotani et al. 2001). However it is possible that these analogues can also be used to modulate the HPG axis to treat the disease states discussed above (Thompson et al. 2004). Initially, it was shown that all forms of circulating kisspeptin (KP-54, 14, 13 and 10) were able to bind KISS1R with the same affinity and had the same potency in mice (Kotani et al. 2001 and Mikkelsen et al. 2009) However it was later revealed that KP-10 had greater receptor binding affinity but poor biological stability in serum since it was prone to faster degradation. Conversely, KP-54 was shown to be less susceptible to degradation and was reported at being more efficient at triggering gonadotropin release in rats compared to KP-10 (Thompson et al. 2004, Asami et al. 2013). Pharmacological kisspeptin analogues have been created using the knowledge that KP-10 as the minimal structure capable of activating KISS1R and the RF-amidated COOH-terminal moiety is essential for KISS1R binding (Clements et al. 2001, Orsini et al. 2007 and Tomita et al. 2006). TAK 488 and TAK 693 are currently in clinical trials after tested for increased potency and greater bioavailability due to reduced susceptibility to degradation (Curtis et al. 2010, Matsui et al. 2013 and 2014 and Asami et al. 2013).

1.5.2 Therapies for the down-regulation of the HPG axis

Conditions where clinical downregulation of the HPG axis can be useful:

a) Polycystic ovary syndrome (PCOS)
b) Central precocious puberty (CPP)
c) *In vitro*-fertilization (IVF) - preventing premature LH surge during controlled ovarian hyperstimulation in A.R.T.
d) prostate cancer
e) breast cancer
f) endometriosis
g) uterine fibroids
h) female contraception

When used for downregulating sex-steroid production, GnRH agonists administered in a continuous manner cause the desensitization and internalization of pituitary GnRH-R.

However due to the unique structure of GnRH-R, there is a long desensitization period of 3 - 4 weeks during which symptoms get severely exacerbated known as the "flare-up" phenomenon (Millar et al. 2010, George et al. 2012). In certain conditions such as malignant prostate and breast cancers therefore, use of GnRH agonists are contraindicated. In such cases GnRH antagonists may be an alternative treatment where they competitively inhibit pituitary GnRH-R from activation by endogenous GnRH. Examples of GnRH antagonists include; Cetrorelix, Ganirelix, Abarelix, Degarelix and Elagolix. Currently GnRH antagonists are not approved for use in Canada but in the past were efficacious as prostate cancer treatments (Akaza et al. 2010 and Matsui et al. 2012). While they are effective in reducing serum LH levels and do not provoke a flare-up of symptoms, GnRH antagonists as well as the agonists tend to lead to a drastic reduction of LH below castration levels (Fig. 1.7). When levels of sex steroids drop to such a low level, the rate of bone loss greatly exceeds the rate of bone regeneration and bone thinning results. Therefore, with both GnRH agonists and antagonists, levels of sex steroids have to be titrated and "add-back" therapy of exogenous estrogens and progesterones must be co-prescribed to avoid onset of osteoporosis. They have been associated with an acute histamine response however but more modern versions if GnRH antagonists seem to have overcome this issue.

Similar to GnRH agonists, continuous administration of kisspeptin agonists causes desensitization of hypothalamic KISS1R leading to potent decreases in LH secretion (Seminara et al. 2006, Ramaswamy et al. 2007, Thompson et al 2006). While a single bolus application of kisspeptin can potently stimulate GnRH release, continuous application can lead to homologous desensitization and internalization of KISS1R so that it is no longer available to respond to increasing amounts of agonist.

In vivo evidence for KISS1R desensitization was demonstrated in monkeys when castrated juvenile male monkeys exposed to continuous IV adminstration of KP-10, showed an initial rise in LH in the first 3 hours followed by basal levels for the remainder of the experiment. To further prove that the decreased response was not just due to GnRH depletion, NMDA, another trigger for GnRH secretion, was administered and evoked a GnRH response (Seminara et al. 2006). Another study using intact adult male monkeys

produced similar results (Ramasawmy et al. 2007). Continuous kisspeptin exposure in rats also demonstrated KISS1R desensitization using LH and testosterone as surrogate markers for GnRH secretion (Thompson et al. 2006). These *in vivo* studies demonstrate that KISS1R desensitizes rapidly compared to GnRHR which can take weeks to desensitize with continuous agonist treatment (Pinilla et al. 2012 and Millar, 2010). Studies in the Babwah laboratory demonstrated that GRK2 mediates KISS1R homologous desensitization and inferred that β-arrestin may also be involved (Pampillo et al. 2009) and here in this dissertation we confirm that β-arrestin does in fact mediate the homologous desensitization of KISS1R thereby revealing the mechanism behind this process. Finally, women treated with kispeptin twice weekly displayed a rise in serum gonadotropins, however when the frequency was increased to twice daily administration, gonadotropins dropped to basal levels (Jayasena et al. 2010). With further studies to elucidate the mechanism, the rapid rate of KISS1R desensitization (hours) compared to the known prolonged rate for GnRH-R (weeks) (McArdle et al. 1999, Tello et al. 2012), demonstrates that the continuous infusion of kisspeptin agonists can be a potentially more effective treatment than the continuous GnRH agonists currently being used. Furthermore, the "flare-up" period is significantly contracted and since kisspeptin agonists do not reduce LH levels below basal, there will not be the associated risk of osteoporosis (Fig. 1.7) (Matsui et al. 2014). While some conditions like malignant prostate and breast cancer require complete ablation of gonadotropin and sex steroids, benign prostatic hyperplasia, endometriosis and uterine fibroids do not require such a complete suppression and so kisspeptin agonists may be a better therapeutic alternative (Matsui et al. 2014).

In addition to using kisspeptin agonists to desensitize hypothalamic KISS1R, kisspeptin antagonists have also been developed. Similar to kisspeptin agonists they have been shown to rapidly and effectively reduce LH secretion while not reducing it lower than basal levels (Fig. 1.7) (Roseweir et al. 2009). One kisspeptin antagonist called Peptide 234 has been tested in mice, rats, monkeys and sheep and conclusively demonstrated that it inhibited kisspeptin neuron firing rate, reduced GnRH neuron firing rate, delays pubertal onset in rodents, inhibits LH surge post-castration and the pre-ovulatory LH surge (Roseweir et al. 2009).

Other potential applications of kisspeptin antagonists or continuous treatment with kisspeptin agonists to downregulate the HPG axis include; as an alternative female contraception and reducing the pre-ovulatory LH surge in ART procedures. Currently female contraception is largely based on oral doses of supraphysiological levels of sex steroids are used to negatively feedback and reduce gonadotropins needed for ovulation. Kisspeptin agonists or antagonists can be used to reduce LH levels and prevent the ovulatory LH surge without lowering it below basal levels so follicular development and estrogen secretion would still continue but ovulation would not occur (Pinilla et al. 2012 and Millar 2010, Jayasena et al. 2014). IVF procedures involve a period of controlled ovarian hyperstimulation where continuous GnRH agonists are given to inhibit ovulation while follicular development is stimulated then exogenous gonadotropins are applied to trigger timed ovulation for ovum transfer. Kisspeptins antagonists may be better alternatives here since they effectively ablate the ovulatory LH surge yet maintain basal levels so lower levels of stimulatory gonadotropins need to be applied for timed ovum collection (Durnerin et al. 2008 and Drakakis et al. 2009). The supraphysiological hormone levels used in current IVF treatment has been shown negatively impact endometrial receptivity to implantation and early gestational success (Hayden, 2008, Marchini et al. 1991, Ubaldi et al. 1997, Kolibianakis et al. 2002, Devroey et al. 2004).

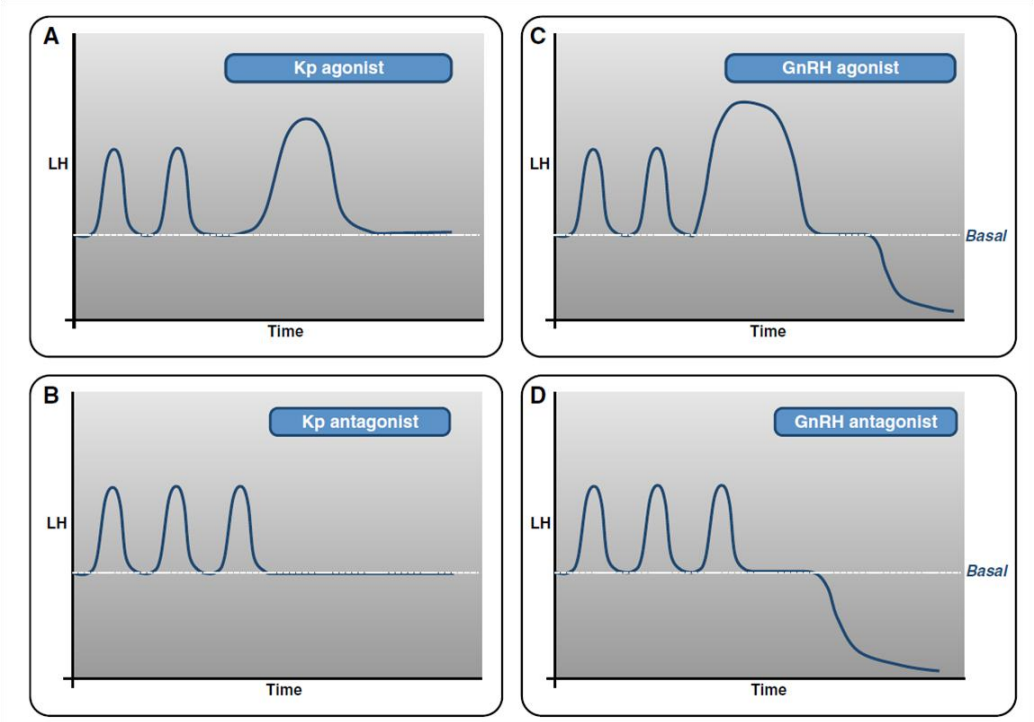

Figure 1.7: Schematic diagram showing differences in LH response to kisspeptin analogues compared to GnRH analogues.

Treatment with kisspeptin agonists (A) or kisspeptin antagonists (B) do not lower LH levels below basal. Unlike GnRH agonists (C) and antagonists (D) which not only reduce LH levels to castration levels but with GnRH agonists (C) a significantly long period of initial flare-up occurs. Adapted from Pinilla et al. 2012 with data from Seminara et al. 2006, Ramaswamy et al. 2007, Thompson et al 2006 and Roseweir et al. 2009.

1.6 KISS1R and G-protein signaling

Guanine nucleotide binding proteins (G-proteins) are the most widely evolved intracellular signal transducers in biological systems (Pierce et al. 2002 and Luttrell, 2008). Heterotrimeric G-proteins consist of three subunits, α, β and γ and exert their effects by coupling to seven transmembrane receptors (7TMRs) also referred to as G-protein coupled receptors (GPCRs) (Offermanns, 2003). The Gα-subunit is bound to GDP in the inactive state and has a high affinity for the βγ-subunit dimer. When agonists activate GPCRs it causes a conformational change allowing for the activation of the Gα-subunit through the exchange of GDP with GTP. The activated Gα-subunit dissociates from the Gβγ- complex and activates associated G-protein signalling cascades (Helper et al. 1992, Neer et al. 1995, Gudermann et al. 1996 and Hamm, 1998). The Gα-subunit has intrinsic GTPase activity which catalyses the hydrolysis of GTP back to GDP causing the inactivation of Gα and re-association to the Gβγ- complex in the basal inactive state. Many types of G-protein subunits have been discovered and are associated with specific effectors and signalling events (Table 3). The exact mechanism that regulates the specificity of the GPCR-G-protein-Effector interaction is still unknown but conformational change is thought to play an important role (Gudermann et al. 1997, Schoneberg et al. 1999, Palczewski et al. 2000, Luttrell, 2008). G_{α} subunits have two main domains; the GTP-GDP binding domain and the α-helical domain that binds effectors and Gβγ subunits. While the central guanine nucleotide-binding pocket is highly conserved among Gα subunits (sharing 40% amino acid sequence homology), the C- and N-termini are more unique and play important roles in receptor and effector association as well as Gβγ dimer binding respectively (Luttrell, 2008, Kohout et al. 2004 and Holloway et al. 2002).

KISS1R is a GPCR that signals primarily through $G\alpha_{q/11}$ proteins (Figs. 1.5 and 1.6) (Kotani et al. 2001, Ohtaki et al. 2001 and Muir et al. 2001). The $G\alpha_q$ family stimulates pertussis toxin insensitive PLCβ hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) (Table 3). Many receptors that regulate the reproductive axis are $G\alpha_q$ –coupled GPCRs including hypothalamic KISS1R and NK_3B as well as GnRH-RI expressed in

pituitary gonadotropes. Of the four members of the $G\alpha_q$ family, $G\alpha_q$ and $G\alpha_{11}$ are ubiquitously expressed whereas $G_{\alpha 14}$ is primarily expressed in kidney, testis and lung and $G\alpha_{15/16}$ is only expressed in some hematopoietic cells (Exton, 1996, Rhee, 2001, Amatruda et al. 1991, Wilkie et al. 1991, Tanka et al. 2000). KISS1R is a $G_{\alpha q/11}$ –coupled receptor, where the term $G\alpha_{q/11}$ indicates the level of functional redundancy and receptor affinity between $G\alpha_q$ and $G\alpha_{11}$. In addition to sharing 88% amino acid sequence, receptors such as α_1-adrenergic and vasopressin that couple to $G\alpha_{q/11}$ show no preference for association or activation between $G\alpha_q$ and $G\alpha_{11}$ and both G-protein subtypes are able to effectively activate PLCβ1,3 and 4 and poorly activate PLCβ2 (Wange et al. 1991, Wu et al. 1992, Jiang et al. 1994, Offermanns et al.1994, Xu et al. 1998 and Rhee, 2001).

Table 1.3: Summary of the various classes of Gα-subunits.

G-protein family[3]	Subtypes	Cognate GPCR example	Primary effector molecules	Main intracellular signalling events
$G\alpha_s$	$G\alpha_s$, $G\alpha_{sXL}$, $G\alpha_{olf}$	FSH-R LH-R β_2AR	Adenylate cyclase	Stimulates cAMP production
$G\alpha_{i/o}$	$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{io}$, $G\alpha_o$, $G\alpha_{gust}$	NPY-R	Adenylate cyclase	Inhibits cAMP production
$G\alpha_q$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$ (15= mouse, 16 = human)	GnRH-RI KISS1R NK_3B $AT_{1A}R$	Phospholipase C-β (PLCβ)	Stimulates $InsP_3$ and DAG production
$G\alpha_{12}$	$G\alpha_{12}$, $G\alpha_{13}$	$AT_{1A}R$	Rho GTPase activation	Stimulates stress fibre formation

[3] Four distinct Gα-subunit families exist which signal through discrete effectors and second messengers. See list of receptor abbreviations. ($InsP_3$ – inositol 1,4,5-triphosphate; DAG – diacylglycerol) (Lambert, 2008 and Offermans, 1999 and Offermans 2003).

1.6.1 $G\alpha_q$ (*Gnaq*) and $G\alpha_{11}$ (*Gna11*) mouse models

To further explore the biological significance of the functional redundancy and ubiquitous expression of $G\alpha_q$ and $G\alpha_{11}$ mice with inactivating (null) mutations for each were created. It was surprising to discover that of all the mice generated with inactivating mutations in each Gα-subunit gene (*Gnaq*$^{-/-}$, *Gna11*$^{-/-}$, *Gna14*$^{-/-}$ *and Gna15/16*$^{-/-}$) only those lacking $G\alpha_q$ (*Gnaq*$^{-/-}$) demonstrated phenotypic defects (Davignon et al. 2000, Offermanns et al. 1998). This indicates that while $G\alpha_q$ may be able to compensate for the other ubiquitous $G\alpha_{11}$ *in vivo*, the converse does not hold true since mice with intact *Gna11* in the absence of *Gnaq* display an affected phenotype (Table 1.4) . While *Gnaq*$^{-/-}$ mice are viable they have obvious defects in motor coordination and ataxia due to disrupted cerebellar development. Studies in the *Gnaq*$^{-/-}$ mice demonstrated that $G\alpha_q$ is essential during the third postnatal week for the elimination of multiple climbing fibers innervations to cerebellar purkinje cells (Offermanns et al. 1997). *Gnaq*$^{-/-}$ mice also display impaired platelet-dependent thromboembolism and hemorrhagic diathesis. Platelets unlike most mammalian cells only express $G\alpha_q$ and not $G\alpha_{11}$ so a loss of $G\alpha_q$ results in a lack of PLCβ stimulation, Ca^{2+} intracellular increase and an overall lack of platelet aggregation and degranulation (Offermanns et al. 1997). Furthermore, mice lacking various combinations of $G\alpha_q$ and $G\alpha_{11}$ alleles demonstrate gene dosage effects on phenotype and this is summarised in Table 4 below (Offermanns, 1999).

Table 1.4: Gene dosage effects of $G\alpha_q$ and $G\alpha_{11}$ expression on phenotypic abnormalities.[4]

	#$G\alpha_q$ alleles	**#$G\alpha_{11}$ alleles**	***Gnaq***	***Gna11***	**Phenotype**
A	1	1	-/+	-/+	None
B	2	0	+/+	-/-	None
C	0	2	-/-	+/+	Viable with cerebellar ataxia and platelet aggregation defect
D	1	0	-/+	-/-	Not viable – die at P0 due to cardiac defects
E	0	1	-/-	-/+	Not viable – die at P0 due to cardiac and craniofacial defects
F	0	0	-/-	-/-	Not viable – die at E11 due to myocardial hypoplasia

[4] While $G\alpha_q$ seems to be able to functionally compensate for $G\alpha_{11}$ *in vivo*, the converse does not hold true and total allelic contribution from both genes are critical in determining the functional state of the $G\alpha_{q/11}$ signalling cascade. (Adapted from Offermans, 1999).

When both *Gna11* alleles are inactivated in the presence of functional *Gnaq* (Table 4 row B) there is no obvious defect but when both *Gnaq* alleles are inactivated in the presence of functional *Gna11* (Table 4 row C) cerebellar ataxia and platelet hemostasis defects are observed (Offermanns et al. 1998 and 1999). Also while at least one functional allele of the four is needed to allow full-term birth (Table 4 rows D and E), at least two functional alleles are required for postnatal viability (Table 4 rows A, B and C). Double homozygous loss of $G\alpha_q$ and $G\alpha_{11}$ (*Gnaq*$^{-/-}$;*Gna11*$^{-/-}$) (Table 4 row F) develop normally until E9.5 then growth is retarded and evidence of a thinning of the myocardial layer of the heart results in death at about E11 (Offermanns et al. 1998).

To circumvent embryonic lethality and enable further study of the roles of $G\alpha_q$ and $G\alpha_{11}$ at the molecular and physiological level, two strategies were employed. Firstly, MEFs from E10.5 mouse embryos were used to create *Gnaq*$^{-/-}$;*Gna11*$^{-/-}$ MEFs for *in vitro* analysis of intracellular mechanisms and secondly, Cre-LoxP-mediated recombination was used to create conditional *Gnaq*$^{-/-}$;*Gna11*$^{-/-}$ mouse mutants. Since *Gna11*$^{-/-}$ mice were fertile, viable and lacked obvious phenotypic defects, the conditional *Gnaq*$^{-/-}$;*Gna11*$^{-/-}$ mice were created by Cre-mediated inactivation of *Gnaq* in the background of *Gna11* deficiency (Zywietz et al. 2001 and Wettschureck et al. 2001). Numerous studies have used the *Gnaq*$^{-/-}$;*Gna11*$^{-/-}$ MEFs to study the role of $G\alpha_{q/11}$ signalling downstream of GPCRs. We have also used them previously to demonstrate that KISS1R was capable of activating ERK1/2 in a $G\alpha_{q/11}$- independent manner (Szereszewski et al. 2010, Blaukat et al. 2000 and Adjobo-Hermana et al. 2008). While they are a very useful tool for elucidating molecular mechanisms, they are still a cellular model made from embryonic mouse fibroblasts and do not provide information about the physiological response associated with $G\alpha_{q/11}$- independent signalling; to achieve this the conditional *Gnaq*$^{-/-}$;*Gna11*$^{-/-}$ mouse models are required. Wettschureck et al. (2001), created and characterized mice in which allowed for conditional inactivation of *Gnaq* in *Gna11* deficient mice by Cre-mediated recombination caused the excision of exon 6 in the *Gnaq* gene (*Gnaq*$^{fl/fl}$; *Gna11*$^{-/-}$). Various Cre expressing lines under the regulation of tissue-specific promoters have been used in conjunction with the *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ mice such as; *MLC2a-Cre* (cardiomyocyte-specific), *Nestin-Cre* (neuron and glial-specific), *P0-Cre* (neural-crest specific), *Camkcre4* (specific to the principal neurons in the forebrain) and

all these studies have demonstrated almost complete efficiency of $G\alpha_{q/11}$ ablation in target tissues (Kero et al. 2007, Wettschureck et al. 2001, 2005, 2006, 2007, Dagmara et al. 2005). In our studies described later, we used *Gnrh-Cre* and these well characterised *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ mice to create mice in which $G\alpha_{q/11}$was inactivated only in hypothalamic GnRH neurons (Yoon et al. 2005, Skynner et al 1999 and Wintermantel et al. 2001).

1.6.2 $G\alpha_{q/11}$ signaling through extracellular-signal regulated kinases (ERK 1/2)

Mitogen-activated protein kinase (MAPK) is a collective term for three families of intracellular signaling cascades namely, the extracellular signal-regulated 1 and 2 kinases (ERK 1/2), p38 kinases and c-Jun N-terminal kinases (JNKs). These cascades regulate intracellular signaling by phosphorylating specific substrates in the cytosol and nucleus (Gutkind, 2000 and Rozengurt, 2007). Not only is it the best characterized MAPK, but ERK 1/2 phosphorylation has been demonstrated to be a critical step preceeding GnRH secretion (Castellano et al. 2006) therefore we focused on the activity of this pathway in our *in vivo* studies. When $G\alpha_{q/11}$ is activated by the receptor it triggers a series of events (Figure 1.5) that eventually leads to the activation of PKC. One consequence of this is the activation of Ras (a GTPase) and Raf (a MAP3 kinase). Raf phosphorylates MEK (a MAPK kinase) which in turn phosphorylates and activates ERK 1 (p44) and ERK 2 (p42) (Gutkind, 2000). One substrate for ERK 1/2 is ribosomal protein kinase (RSK) which phosphorylates ribosomal protein S6 and therefore regulated transcription and gene expression (Caunt et al. 2006)

1.7 KISS1R and β-arrestin interaction and consequences

1.7.1 β-arrestin structure

There are four arrestin proteins divided into two subfamilies; visual arrestins (arrestin-1 and -4) and β-arrestins (arrestin-2 and -3) (Attramadal et al. 1992, Lohse et al. 1990). Arrestin-1 is expressed in both rods and cones of the retina while arrestin-4 is solely expressed in the cones (Murakami et al. 1993 and Craft et al. 1994). β-arrestin-1 and -2 also referred to as arrestin-2 and -3 respectively are ubiquitously expressed and share

over 78% amino acid sequence homology (Lohse et al. 1992). Based on the known crystal structure of visual arrestin in combination with mutagenesis studies, the putative structure of the β-arrestins has been suggested (Fig. 1.8) (Gurevich et al. 1995). The N-terminal domain (A) is thought to be involved in recognition of agonist bound GPCRs and the C-terminal domain (B) contains secondary receptor binding sites. The N- and C-domains are separated by a phosphate sensor region in the polar core of the protein and in the inactive state the C-terminus associates with this region. Upon binding a phosphorylated agonist-bound GPCR, the C-terminus is released from this association and the β-arrestin protein adopts an active conformation (Gurevich et al. 1995 and Hirsch et al. 1999). Both visual arrestins and β-arrestins contain regulatory regions, R1 and R2 but in β-arrestins only the R2 domains contain the necessary phosphorylation sites to allow them to scaffold to members of the endocytotic pathway. Specifically, the R2 domain has been shown to contain a LIEF binding motif that allows for the recruitment of clathrin and the β2-adaptin subunit of the AP-2 complex, both clathrin and AP-2 are key molecules involved in the endocytotic internalization of plasma membrane GPCRs (Krupnick et al. 1997 and Laporte et al. 2000).

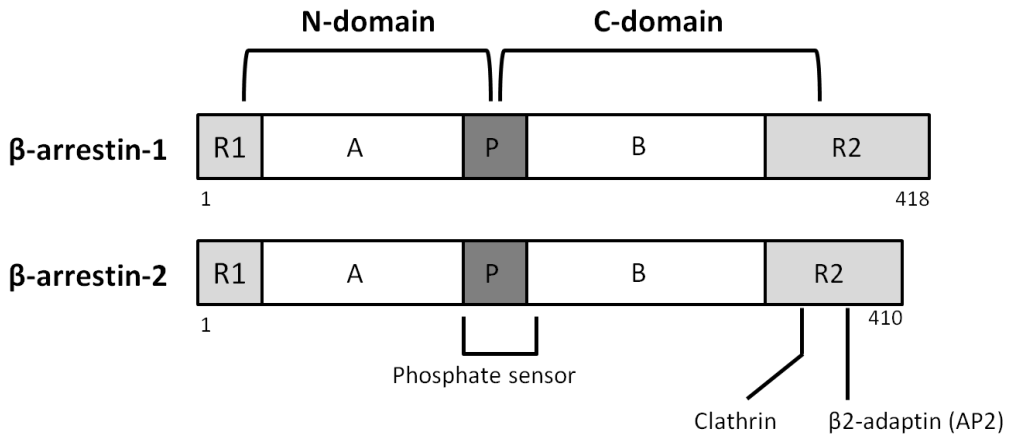

Figure 1.8: Graphical representation of the structure of β-arrestins.

The N-terminal domain (A) is involved in the recognition of phosphorylated GPCRs and the C-terminal domain (B) contains secondary GPCR binding sites. P is a phosphate sensor region to which the C-terminus binds in the inactive state. R1 and R2 are regulatory domains involved with β-arrestin mediated GPCR internalization via clathrin-coated pits (Adapted from Luttrell and Lefkowitz, 2002 and Ferguson, 2001).

1.7.2 β-arrestin function

β-arrestins have been best described for their critical role in mediating GPCR signal termination by homologous desensitization but they have also been shown to be involved in GPCR internalization by endocytosis, receptor resensitization and down regulation as well as secondary signal transducers.

As described for the prototypical receptor, **homologous desensitization** involves the action of two protein families; GPCR serine/threonine kinases (GRKs) and β-arrestins (Freedman and Lefkowitz, 1996 and Krupnick and Benovic, 1998). Briefly, there are seven GRKs identified to date, GRK1 and -7 are expressed in the retina and GRK4 is expressed in testes, kidney and cerebellum (Ribas et al. 2007 and Sallese et al 2000). GRK2, -3, -5 and -6 are ubiquitously expressed but while GRK5 and -6 are constitutively associated with the plasma membrane, GRK2 and -3 are not (Pitcher et al. 1996, Premont et al. 1996, Stoffel et al. 1997). GRKs are recruited to agonist bound GPCRs and have been shown to phosphorylate the intracellular loops (IL) 2, IL3 or carboxy-terminal C-tail and demonstrate receptor specificity (Dhami et al. 2005 and Dhami and Ferguson, 2006). After prolonged exposure to the agonist, GRKs are recruited and phosphorylate the receptor which disrupts the interaction with the associated G-protein (Ferguson, 2001 and Luttrell and Lefkowitz, 2002). GRK-mediated receptor phosphorylation promotes high-affinity binding of β-arrestins which sterically uncouples the receptor from its G-protein and interdicts further signaling (Ferguson, 2001 and Luttrell and Lefkowitz, 2002).

In fact, studies with β2AR demonstrate that β-arrestins have a ten to thirty fold greater affinity for the GRK-phosphorylated receptor compared to the unphosphorylated (Lohse et al. 1992). Using MEFs lacking either or both β-arrestins, recent studies demonstrated that β2AR and $AT_{1a}R$ desensitization was mediated by both β-arrestin isoforms (Kohout et al. 2001).

Subsequent to homologous desensitization, GPCRs are commonly targeted for **internalization** or sequestration by clathrin-mediated endocytosis (Luttrell et al. 2010). After β-arrestins bind phosphorylated GPCRs, the C-terminus is freed from binding to the internal polar core. The C-terminus of β-arrestin contains binding sites for clathrin and

β2-adaptin (Fig. 1.8) (Goodman et al. 1996, Krupnick et al. 1997). β2-adaptin is a subunit in an AP-2 complex which coordinates GPCR internalization by binding to the various molecules necessary including clathrin, dynamin and EPS-15 (Kirchhausen, 1999, Fergusson et al. 1996, Zhang et al. 1996, Laporte et al. 2000). While most GPCRs undergo β-arrestin-mediated sequestration, not all do such as PAR-1, SST2A and N-formyl peptide receptor (Paing et al. 2002, Vines et al. 2003 and Brasselet et al. 2002). These particular receptors also demonstrate that GPCRs may undergo β-arrestin-mediated desensitization and not necessarily be sequestered in a β-arrestin-dependent manner as well. *In vitro* studies using MEFs lacking either or both β-arrestins showed that β-arrestin-2 was more potent than β-arrestin-1 in mediating β2AR internalization while either β-arrestin isoform was sufficient for $AT_{1a}R$ internalization (Kohout et al. 2001). GPCRs have been categorized based on the degree of association with β-arrestins into Class A or Class B (Barak et al. 1997, Oakley et al. 2001 and 2000). This is not to be confused with the structural scheme of GPCR classification however which also uses the terminology of Class A, B, C etc. (Bockaert et al. 1999, Sharman et al. 2011). Overall, class A receptors transiently bind β-arrestin-2 with higher affinity than β-arrestin-1 so that after internalization the receptor- β-arrestin complex dissociates while class B receptors stably bind both β-arrestin isoforms with equal affinity so that it remains associated with the receptor post-internalization (Luttrell, 2008). μ-opioid, endothelin A and dopamins D1A are examples of class A receptors and $AT_{1A}R$, neurotensin 1, vasopressin V2, neurokinin NK-1 receptors are examples of those in class B. Studies demonstrated that GRK phosphorylation sites on the C-tail of the GPCR were responsible for determining the stability of their association with β-arrestin (Oakley et al. 2000 and Tohgo et al. 2003). Furthermore, the stability of this association also determines the intracellular fate of the internalized GPCRs; whether they are **recycled** as is the case for most class A receptors or whether like most class B receptors, are targeted for lysosomal **degradation** (Oakley et al. 1999, 2000, Shenoy and Lefkowitz, 2011, Kang et al. 2013). In order for a GPCR to be recycled or resensitized to the plasma membrane it often requires agonist dissociation and GPCR dephosphorylation. This is commonly achieved in acidified vesicles associated with GPCR-specific protein phosphatase (PP2A) (Sibley et al. 1986, Pitcher et al. 1995, Krueger et al. 1997, Ferguson, 2001). Alternatively,

internalized β-arrestin bound GPCRs may be targeted for degredation in lysosomes which results in a downregulation of receptor expression at the plasma membrane (Dery et al. 1999 and Anborgh et al. 2000).

1.7.3 β-arrestin-dependent signaling and biased signaling

β-arrestins also have been shown to be able to bind various signalling molecules and thereby act as an adapter or scaffolding protein facilitating G-protein independent signaling downstream of GPCRs (Defea, 2008 and Kovacs et al. 2009, Luttrell et al. 2010). *In vitro* studies with β_2AR, NK-1R and CXCR-1R demonstrate that after internalization of these β-arrestin bound receptors, members of the Src family of tyrosine kinases were able to bind β-arrestin and directly interact with the receptors (Luttrell et al. 1999, De Fea et al. 2000, Barlic et al. 2000, Imamura et al. 2001). These findings revealed that β-arrestin played critical roles in termination of G-protein dependent signaling and also mediating G-protein independent signaling. Further studies demonstrated that β-arrestins served as critical scaffolding agents for members of the mitogen-activated protein kinase (MAPK) cascade. In this role β-arrestins serve three important molecular functions; to increase efficiency by binding sequential agents in close proximity, to allow for intracellular compartmentalization of signaling and to ensure specificity response by preventing crosstalk with other signaling cascades (Luttrell and Lefkowitz, 2002). Briefly the MAPKs consist of three families which phosphorylate cytosolic and nuclear targets to regulate responses including gene expression, cell proliferation and apoptosis. The three families are; extracellular-signal regulated 1 and 2 kinases (ERK1/2), p38 kinases and c-Jun N-terminal kinases (JNKs) (Gutkind, 2000, Rozengurt, 2007). While GPCRs activate many intracellular signalling systems, ERK1/2 phosphorylation has been the best characterised and widely established in the literature. Additionally, both G-protein and β-arrestin pathways seem to be able to signal to ERK1/2 demonstrating that it is a crucial cascade capable of integrating and regulating cellular and biological responses (Luttrell and Lefkowitz, 2002).

While both pathways activate ERK1/2 the G-protein mediated ERK1/2 response tends to be rapid but transient and for some receptors (PAR1 and $AT_{1a}R$) is capable of translocating to the nucleus where it can regulate gene transcription. The β-arrestin mediated ERK1/2 response occurs later after agonist exposure but is more persistent however β-arrestin-activated ERK1/2 remains sequestered in the cytosol (Tohgo et al. 2002, Ahn et al. 2004, Shenoy et al. 2006, Kobayashi et al. 2005, DeFea, 2008). The two β-arrestin isoforms have been shown to sometimes differentially regulate ERK1/2 phosphorylation. For some receptors, β-arrestin-1 and -2 act synergistically to trigger ERK1/2 activation (PAR2, PTH1R and β_2AR) while for others such as $AT_{1a}R$ the isoforms have reciprocal effects (Ahn et al. 2004, DeFea et al. 2000, Shenoy et al. 2006, Getsy-Palmer et al. 2006).

With the discovery that GPCRs can signal via both G-protein and β-arrestin-dependent pathways came the emergence of a novel paradigm referred to as **biased signalling**. A biased agonist is one which stabilizes receptor conformation so that it would preferentially interact with certain signaling molecules over others (Violin and Lefkowitz, 2007, Kenakin, 2007). Ligands for various GPCRs have since been described which stimulate the β-arrestin pathway and antagonize the G-protein cascade for example. *In vivo* however, these pathways may not only be antagonistic but can also be cooperative as explained later. To tease apart the G-protein-dependent from the β-arrestin-dependent responses various strategies have been employed such as; G-protein uncoupled mutant receptors, G-protein pathway chemical inhibitors, β-arrestin RNA interference (siRNA/shRNA), β-arrestin null MEFs and mice (Bohn et al. 1998, Conner et al. 1997, Kohout et al. 2001, Ahn et al. 2004, Wei et al. 2003, Drake et al. 2008).

For $AT_{1A}R$, a combination of techniques has been used to demonstrate that this receptor is capable of signaling to ERK1/2 in the absence of G-protein. $AT_{1A}R$-DRY/AAY; a $G\alpha_{q/11}$ uncoupled $AT_{1A}R$ mutant receptor was used to show that while the receptor is incapable of activating the $G\alpha_{q/11}$ pathway, it was still able to activate ERK1/2 via a β-arrestin-2-dependent mechanism (Wei et al. 2003). To confirm this finding, SII, a mutated $AT_{1A}R$ agonist that binds $AT_{1A}R$ but is incapable of activating the $G\alpha_{q/11}$ pathway was shown to activate β-arrestin-2-dependent ERK1/2 phosphorylation (Wei et

al. 2003). Taken together, these findings indicate that $AT_{1A}R$ is capable of signaling to ERK1/2 via both $G\alpha_{q/11}$ and β-arrestin mediated mechanisms and also that SII is an example of a β-arrestin biased agonist.

Biased signaling was also demonstrated for β_2AR by *in vitro* experiments using a $G\alpha_s$-uncoupled receptor mutant, β_2AR^{TYY} and a β_2AR inverse agonists, ICI 118,551and carvedilol (Shenoy et al. 2006, Azzi et al. 2003, Wisler et al 2007 and Drake et al 2008). These experiments revealed that while the G_s pathway was inactive, the receptor was still capable of signaling to ERK1/2 in a β-arrestin-dependent manner.

In the previous examples, the G-protein pathways are independent from the β-arrestin-mediated pathways however for some GPCRs, these pathways may act cooperatively as with CCR7. When stimulated with CCL19, CCR7 is capable of activating both $G\alpha_{i/o}$ and β-arrestin signaling but administration of pertussis toxin, a potent $G\alpha_{i/o}$ inhibitor, abolishes all ERK1/2 activation (Lefkowitz and Shenoy, 2005, Kohout et al. 2004, Zidar et al. 2009). Therefore, for CCR7, the $G\alpha_{i/o}$ and β-arrestin pathways cooperatively act to stimulate ERK1/2 phosphorylation.

1.7.4 Physiological relevance of biased signaling

With the recognition that GPCRs are capable of having multiple active states and that signaling can be G-protein dependent or independent (and frequently β-arrestin-dependent), the potential for developing novel drugs to manipulate GPCR response has greatly increased. The following are some examples of the biological consequences of biased signaling and some examples of how it can be used to improve existing therapies.

One of the roles of $AT_{1A}R$ *in vivo* is the regulation of vascular cell growth in the heart and associated blood vessels. $AT_{1A}R$ can be activated by the endogenous ligand, angiotensin II, but can also be put into an active state after the cell experiences mechanical stretch as occurs in patients with hypertension (Rakesh et al. 2010). Angiotensin II stimulates $G\alpha_{q/11}$ signalling which leads to myocyte hypertrophy in response to short-term increases in cardiac load as is experienced with exercise for example. Recent studies have shown that in the absence of agonist mechanical stress

induces signalling via the β-arrestin pathway, independent of $G\alpha_{q/11}$ activation, which mediates cardio-protective mechanisms including, ERK1/2 phosphorylation, EGFR transactivation and reduced myocyte apoptosis (Rakesh et al. 2010, Wei et al. 2003). Current drugs used are angiotensin receptor blockers (ARBs) and while they effectively block the hypertropic effects of the $G\alpha_{q/11}$ pathway they unfortunately also block the potential cardioprotective response from the β-arrestin pathway. Work is currently underway to develop novel β-arrestin-biased agonists for $AT_{1A}R$ which would both lower blood pressure by abolishing $G\alpha_{q/11}$ signalling while promoting the β-arrestin pathway that will assist in protecting the cells from potential damage due to the increased blood pressure (Rakesh et al. 2010).

Currently, β-blockers are commonly prescribed to treat hypertension, glaucoma, congestive heart failure and myocardial infarction (Patel et al. 2009, Andresen, 2011). These drugs act by inhibiting β2AR, the receptor for adrenaline. One of these β-blockers was carvedilol and was considered to simply be a β2AR antagonist until studies in 2007 revealed that it was in fact a β-arrestin-biased agonist (Packer et al. 1996, Vanderhoff et al. 1998, Kim et al. 2008, Wisler et al. 2007, Drake at al. 2008). While blocking the harmful effects of adrenaline-stimulated G_s signaling downstream of β2AR, carvedilol was shown to promote β-arrestin transactivation of EGFR and ERK1/2 phosphorylation important for cardioprotective function (Noma et al. 2007, Kim et al. 2008, Drake et al. 2008). EGFR activation is associated with antihypertensive properties such as nitric oxide production that is important for vascular muscle relaxation (Namiki et al. 1990 and Kurosaki et al. 2000). Although the mechanisms behind biased signalling are not fully understood yet, carvedilol is an example of a biased agonist that has been used for years with excellent clinical results and further demonstrates that they may provide additional benefits than conventional GPCR agonists and antagonists currently used.

1.7.5 Mice bearing genetic deletions for β-arrestin and derived MEF cell lines

As mentioned previously, various tools are used for teasing apart the G-protein and β-arrestin pathways downstream of GPCRs. Two of the tools used to characterise the physiological roles of β-arrestin *in vivo* are the mouse models that were developed lacking either β-arrestin-1 or β-arrestin-2 (Conner et al. 1997 and Bohn et al. 1999). Both *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice are viable, fertile, producing normal sized litters and show no obvious phenotypic defects until challenged with certain GPCR agonists. This indicated that while the β-arrestin isoforms shared 78% amino acid sequence homology and were mostly functionally redundant there were still certain functions that were distinctly regulated by each isoform. The role of β-arrestin-1 in β2AR desensitization is evident in the challenged phenotype of the *Arrb1*$^{-/-}$ mice (Table 5 row B). This is not surprising given the importance of β_2AR in cardiac function and when *Arrb1*$^{-/-}$ mice were challenged with isoproternol, a potent β_2AR agonist, they exhibited an exaggerated hemodynamic response due to prolonged signaling and reduced β2AR desensitization (Conner et al. 1997). Similarly, the role of β-arrestin-2 in μOR desensitization is demonstrated by prolonged morphine analgesic effect in *Arrb2*$^{-/-}$ mice (Table 5 row A). The potentiation of μOR signaling in response to morphine is due to reduced μOR desensitization in the central nervous system resulting in tachyphylaxis or increased drug tolerance (Bohn et al. 1999). While the mice lacking single isoforms of β-arrestin are useful in identifying differential functions of *Arrb1*$^{-/-}$ or *Arrb2*$^{-/-}$ *in vivo,* they are not as useful for separating β-arrestin-dependent vs. G-protein dependent responses *in vivo*. Ideally, a model lacking both β-arrestin isoforms would be applicable for these studies but *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ mice die at birth due to pulmonary hypoplasia (Table 5 row E) (Zhang et al. 2010).

Table 1.5: Phenotypes associated with lack of *Arrb* expression in mice.

	***#Arrb1* alleles**	***#Arrb2* alleles**	***Arrb1***	***Arrb2***	**Phenotype**[5]
A	2	0	+/+	-/-	Prolonged morphine analgesia (μOR)
B	0	2	-/-	+/+	Hyperactive cardiac response (β_2AR)
C	0	1	-/-	-/+	No reported defects
D	1	0	-/+	-/-	No reported defects
E	0	0	-/-	-/-	Die at birth - Pulmonary hyperplasia

[5] *Arrb1* and *Arrb2* seem to be able to functionally compensate for each other during development since mice lacking either isoform are viable and fertile and only demonstrate a phenotype when pharmacologically challenged (row A and B). Homozygous loss of both *Arrb* isoforms results in mice that die at birth due to pulmonary hyperplasia (Conner et al. 1997, Bohn et al. 1999, Zhang et al. 2010).

To circumvent the experimental limitation of perinatal lethality in *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ mice, mouse embryonic fibroblasts (MEFs) were generated from mice between E10.5 – E 13.5 (Kohout et al. 2001). As such five cell lines were established for further *in vitro* investigation:

- β-arrestin-1 knockout (KO): *Arrb1*$^{-/-}$
- β-arrestin-1 wild-type (WT): *Arrb1*$^{+/+}$
- β-arrestin-2 knockout (KO): *Arrb2*$^{-/-}$
- β-arrestin-2 wild-type (WT): *Arrb2*$^{+/+}$
- β-arrestin-1-2 double knockout (DKO): *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$

The wild-type parental line for the β-arrestin DKO line is also β-arrestin-2 WT (*Arrb2*$^{+/+}$). These cell lines have since been used extensively in studies to elucidate the role for β-arrestin in regulating GPCR function. One of the initial studies demonstrated that while β-arrestin-1 and -2 equally regulated β_2AR desensitization and $AT_{1A}R$ desensitization and internalization, they differentially regulated β2AR internalization (Kohout et al. 2001). Desensitization for PAR1 was also shown to be differentially regulated by the isoforms where β-arrestin-1 plays a critical role *in vitro*. Overall, these studies show that while the mouse models bearing genetic deletions of β-arrestin are useful for examining physiological relevance of β-arrestin function, until conditional inactivating models are created the true biological contribution of β-arrestin cannot be evaluated. The MEFs therefore facilitate the understanding of the molecular mechanisms regulating β-arrestin function *in vitro*.

1.7.6 KISS1R and β-arrestins

As mentioned earlier, KISS1/ KISS1R signalling has extensive physiological function with respect to reproduction and fertility *in vivo*. However, the underlying intracellular mechanisms governing KISS1R mediated-GnRH secretion is still not fully understood and even less is known about the molecular mechanisms involved in terminating this response. While KISS1R has been described as a $G\alpha_{q/11}$ coupled GPCR that signals to ERK1/2 and triggers GnRH secretion (Kotani et al. 2001, Ohtaki et al. 2001, Seminara et al. 2003, Castellano et al. 2006), recent *in vitro* studies demonstrated that in the absence of G-protein signaling, KISS1R can activate ERK1/2 through a β-arrestin mediated

mechanism (Pampillo et al. 2009, Szereszewski et al. 2010). The physiological relevance of this alternative signaling pathway has not yet been investigated until these current studies described later, where GnRH and gonadotropin secretion was measured. The *in vivo* results from these studies potentially have huge therapeutic value for certain patients such as those diagnosed with IHH bearing inactivating KISS1R mutations, women suffering from hypothalamic amenorrhea or men suffering from hypogonadism. With further characterization of this pathway it is even possible that the β-arrestin biased agonists may also mediate previously unknown fertility-enhancing mechanisms similar to those discovered for carvedilol and β2AR.

The underlying mechanism behind KISS1R signal termination has not been fully elucidated but they are best understood for the prototypical GPCR, β_2AR, (Ferguson, 2001, Luttrell and Lefkowitz, 2002) and a similar series of intracellular events have been proposed for KISS1R (Fig. 1.5). Animal and human *in vivo* studies demonstrated evidence for KISS1R desensitization as a result of continuous kisspeptin exposure (Seminara et al. 2006, Plant et al. 2006, Thompson et al. 2006, Ramaswamy et al. 2007, Messager et al. 2005, Jayasena et al. 2010, George et al 2010, Chan et al. 2011). Using HEK 293 cells our lab provided evidence for GRK2 mediation of KISS1R desensitization following prolonged kisspeptin exposure in addition to rapid ligand-induced KISS1R internalization (Pampillo et al. 2009). Current studies described later, provide strong evidence for the role of β-arrestin in KISS1R desensitization. Other studies using ^{125}I-labelled kisspeptin in CHO cells confirmed that KISS1R underwent desensitization, ligand-induced internalization and further demonstrated that KISS1R was also rapidly resensitized to the plasma membrane from an intracellular receptor pool (Bianco et al. 2011). This study also suggested that unlike most GPCRs, KISS1R does not undergo lysosomal degradation upon internalization but is degraded by a proteasome mediated process. While uncommon, proteasomal degredation has also been described for β2AR, μOR and δOR (Chaturvedi et al. 2001 and Shenoy et al. 2001). The physiological basis for better understanding KISS1R desensitization is as an alternative treatment to the GnRH-R desensitization therapies currently used to treat a range of disorders including CPP, endometriosis, uterine fibroids, sex-steroid dependent cancers (prostate and breast for example), PCOS. Current therapies involve an extended period of symptom "flare-

up" which is contraindicated in some cancer patients for example. Also GnRH analogues lower sex-steroid levels below basal to castration level which affects bone structure. KISS1R desensitization has been shown to occur rapidly in vivo and KISS1 analogues do not lower sex steroid levels below basal levels. KISS1 analogues have also been proposed as alternatives for GnRH analogues currently used in fertility IVF and for the supraphysiological androgen treatment currently used in female contraceptives.

1.8 Current study overview

While KISS1/KISS1R signaling has been shown to critically regulate GnRH secretion and by extension HPG axis function and fertility, only a small number of studies have provided mechanistic insight into the KISS1R intracellular signaling cascades which regulate this physiological function. The overall goal of this study is to identify novel KISS1R-dependent mechanisms that regulate GnRH secretion and thereby fertility. It is our hope that these studies would lay the foundation on which future mechanistic and pharmacological investigations with KISS1R can build towards the development of more effective treatment strategies in patients. We hypothesized that KISS1R can signal through both $G\alpha_{q/11}$ and $G\alpha_{q/11}$ – independent pathways, the latter involving β-arrestins and investigated the following aims:

a) KISS1R triggers LH secretion in mice that is in part, β-arrestin-dependent.
b) The naturally occurring $G\alpha_{q/11}$ uncoupled KISS1R mutant, L148S is capable of $G\alpha_{q/11}$ – independent signaling measured by ERK1/2 phosphorylation.
c) L148S-dependent $G\alpha_{q/11}$ – independent ERK1/2 phosphorylation is partially β-arrestin-dependent.
d) β-arrestin mediates KISS1R homologous desensitization, as measured by $InsP_3$ accumulation.
e) Endogenous $G\alpha_{q/11}$-independent signaling in the Gnrh neuron is able to trigger puberty and sustain reproductive function in mice.
f) KISS1R can signal through the $G\alpha_{q/11}$ – independent pathway in the Gnrh neuron to stimulate LH, FSH and testosterone secretion.

1.9 References

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Chapter 2: Experimental Details

A version of this chapter has been published in Endocrinology.

Ahow M.*, Min L.* Pampillo M., Nash C., Wen J., Soltis K., Carroll R.S., Glidewell-Kenney C.A., Mellon P.L., Bhattacharya M., Tobet S.A., Kaiser U.B. and Babwah A.V. (2014). KISS1R Signals Independently of $G\alpha_{q/11}$ and Triggers LH Secretion via the β-arrestin Pathway in the Male Mouse. Endocrinology 155:1-14. *Authors contributed equally to this study.

2.1 Introduction

Gonadotropin-releasing hormone (GnRH)-containing neurons originate in the olfactory placode during embryonic development and migrate along olfactory/vomeronasal nerves into the forebrain (Schwanzel-Fukuda et al. 1989, Wray 2001). They migrate caudally to final sites within the hypothalamus where they send projections to and secrete GnRH into the median eminence. GnRH is transported by the hypophysial portal vessels to reach anterior pituitary gonadotropes where it stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and secretion and thereby reproduction (Belchetz et al. 1978, Clayton, 1987 and 1988, Savoy-Moore et al. 1987, Wildt et al. 1981, Kaiser et al. 1997, Thompson et al 2013 and 2014). In the past decade, hypothalamic kisspeptin (KISS1) and the kisspeptin receptor (KISS1R) have been established as the most potent positive regulators of GnRH secretion and are critical for the timely onset of puberty (Kotani et al 2001, Ohtaki et al. 2001, Muir et al. 2001, de Roux et al 2003, Seminara et al. 2003). Kisspeptin neurons in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) synapse directly on and activate KISS1R on the membrane of hypothalamic GnRH neurons (Messager et al. 2005, d'Anglemont de Tassigny et al. 2008, Gottsch et al. 2004, Smith et al. 2005, Han et al. 2005). This triggers GnRH secretion which in turn stimulates pituitary gonadotropin release that is necessary for driving gametogenisis and gonadal sex steroid production. (Irwig et al. 2004, Seminara et al. 2003, deRoux et al. 2003, Novaira et al. 2014).

Pubertal onset requires the re-activation of GnRH secretion from a basally active state to increase the secretion of gonadal hormones responsible of the secondary sexual developments observed in adolescents (Seminara et al. 2003, Funes et al. 2003, deRoux et al. 2003, Han et al. 2005, Lapatto et al. 2007, Kirilov et al. 2013, Novaira er al. 2014). This role of KISS1/KISS1R in regulating reproduction is highly conserved in many species; mice, rats, pigs, sheep, goats, hamsters, horses, monkeys and humans. (Ramaswamy et al. 2007, Navarro et al. 2004, Tenenbaum-Rakover et al. 2007, Shahab et al. 2005, Keen et al. 2008, Roseweir et al. 2009). Humans bearing *KISS1* or *KISS1R* mutations display impaired fertility and reproductive development and mice with genetic ablations of either *Kiss1* or *Kiss1r* are infertile and recapitulate the hypogonadotropic

phenotype of affected humans, further demonstrating the critical importance of kisspeptin signalling for the proper functioning of the hypothalamic-pituitary-gonadal (HPG) axis. (Seminara et al. 2003, Funes et al. 2003, deRoux et al. 2003, Lapatto et al. 2007, Wahab et al. 2011 and Noel 2011). Kisspeptin administration in both humans and rodents has been used to stimulate or restore fertility thus revealing the therapeutic potential of this novel pathway (Dhillo et al. 2005, Jayasena et al. 2009, Jayasena et al. 2010, Navarro et al. 2004).

When activated by KISS1, KISS1R couples to two G-proteins encoded by *Gnaq* ($G\alpha_q$) and *Gna11* ($G\alpha_{11}$) and triggers the activation of phospholipase C (PLC) which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) and results in inositol 1,4,5 trisphosphate ($InsP_3$) and diacylglycerol formation, calcium release, protein kinase C (PKC) mobilization, ERK1/2 activation, membrane depolarization and GnRH secretion (Kotani et al. 2001, Muir et al. 2001, Ohtaki et al. 2001, Castellano et al. 2006, Wacker et al. 2008, Liu et al. 2008, Szereszewski et al. 2010 and Babwah et al. 2012). Current pharmacological drugs target GPCRs to not only potentiate their signaling but also to terminate it. One process by which GPCR signal termination occurs naturally is referred to as homologous desensitization. Once a GPCR undergoes homologous desensitization it is incapable of further G-protein signaling even in the constant presence of agonist (Ferguson, 2001, Ganietdinov et al. 2004). Members of two main protein families regulate homologous desensitization, namely, G-protein-coupled receptor kinases (GRKs) and β-arrestins (Ferguson, 2001 and Luttrell et al. 2002). While *in vivo* studies in humans and animals indicate that continuous kisspeptin exposure causes KISS1R desensitization, there are limited reports which investigate the underlying mechanism. (Belchetz et al. 1978, Seminara et al. 2006, Ramaswamy et al 2007, Thompson et al 2006, Tovar et al 2006, d'Anglemont de Tassigny et al. 2008) To date we have identified a role for G protein-coupled receptor kinase 2 (GRK2) in mediating $G\alpha_{q/11}$-dependent KISS1R signal termination and provided evidence to suggest β-arrestin involvement (Pampillo et al. 2009). We hypothesize and demonstrate here for the first time that β-arrestins mediate KISS1R homologous desensitization. Since inositol phosphate ($InsP_3$) is a secondary messenger formed in the $G\alpha_{q/11}$-dependent KISS1R signaling cascade, we used $InsP_3$ levels as an inverse indicator for KISS1R desensitization. Firstly we

investigated the effect of the loss or absence of β-arrestin *in vitro,* on $InsP_3$ formation. We used two separate cellular systems and approaches, firstly, we used shRNA to stably downregulate β-arrestin expression in a well established GnRH neuronal cell line; GT1-7 (Mellon et al. 1990) and secondly we used mouse embryonic fibroblasts (MEFs) derived from genetically modified mice completely lacking expression of either or both β-arrestin isoforms (Kohout et al. 2001, Bohn et al. 1998, Conner et al. 1997). Results from these experiments demonstrated that diminished β-arrestin expression corresponded to higher $InsP_3$ levels over a range of kisspeptin concentrations which is interpreted as reduced KISS1R desensitization. To complement and further validate these experiments we used a reciprocal approach in a COS-7 cell line with low endogenous β-arrestin levels. Over-expression of β-arrestin in COS-7 corresponded to lower $InsP_3$ formation interpreted as increased KISS1R desensitization. Taken together these results demonstrated that β-arrestin mediates KISS1R homologous desensitization.

In addition to their classical role in GPCR desensitization, β-arrestins also act as signal transducers in their own right. β-arrestin-dependent signaling has been described for many GPCRs including β_2 adrenergic receptor, angiotensin II type 1A receptor and the vasopressin (V_2) receptor (DeFea 2008, Kovacs et al. 2009 and Luttrell et al. 2010). β-arrestins have been shown to bind a number of signaling molecules including ERK1/2, Ras and Raf1 thereby acting as a molecular scaffold to facilitate G-protein independent interactions between a specific GPCR and these major signaling molecules (Luttrell et al. 1999, DeFea et al. 2000 and Luttrell and Lefkowitz 2002). Previous studies in the Babwah laboratory using *in vitro* cell systems demonstrated that KISS1R can bind β-arrestin and signal to ERK1/2 in a β-arrestin dependent manner (Szereszewski et al. 2010 and Pampillo et al. 2009). Since ERK1/2 mediates GnRH secretion we hypothesized that β-arrestin would also mediate KISS1R triggered GnRH secretion (Castellano et al. 2006). To test this hypothesis we measured LH secretion, an established surrogate marker for GnRH secretion in mice lacking either β-arrestin-1 or β-arrestin-2 (Clarke et al. 1982, Messanger et al. 2005, Novaira et al. 2014). The results revealed that kisspeptin-dependent LH secretion was significantly diminished relative to wild-type mice thus supporting the hypothesis that β-arrestin mediates kisspeptin-induced GnRH secretion.

To date, a small but growing number of naturally occurring KISS1R "loss-of-function" or "inactivating" mutations (Nimiri et al. 2011, Noel et al 2011 and Wahab et al. 2011) and one "gain-of-function" mutation (Teles et al. 2008) have been identified. At the physiological level, the term "loss-of-function" reflects the association with idiopathic hypogonadotropic hypogonadism (IHH) (Seminara et al. 2003); a disorder of the hypothalamus or pituitary that is characterized by the absence of spontaneous sexual maturation in the presence of low or inappropriately normal gonadotropin levels in humans. At the cellular level, KISS1R "loss-of-function" is characterized by its diminished capacity to activate the $G\alpha_{q/11}$ signaling pathway. Based on our finding that, in addition to using $G\alpha_{q/11}$, Kiss1r also employs the β-arrestin pathway to mediate LH secretion, patients bearing some KISS1R mutations might continue to secrete LH, albeit at diminished levels. If that is so, such patients might display a less severe form of IHH and may even experience a reversal of IHH. One of the first loss-of-function KISS1R mutants identified was L148S. L148S was isolated from a consanguineous Saudi Arabian family in whom members bearing the mutated receptor gene in the homozygous state were diagnosed with IHH and infertility (Bo-Abbas et al. 2003, Seminara et al. 2003). In an *in vitro* assay, L148S was shown to be strongly defective in triggering $InsP_3$ formation and therefore "functionally-uncoupled" from $G\alpha_{q/11}$ at kisspeptin concentrations that triggered a maximum response with WT KISS1R (Seminara et al. 2003). A subsequent study showed that, while L148S was unable to trigger the ligand-induced catalytic activation of $G\alpha_{q/11}$ and signal via this pathway, it remained physically coupled to $G\alpha_{q/11}$ (Wacker et al. 2008). That study also showed that L148S had a similar intracellular activity to WT KISS1R in terms of expression level, kisspeptin binding, membrane localization and interaction with various intracellular proteins. Consistent with this finding was our observation that, like WT KISS1R, L148S also physically associated with β-arrestins (Szereszewski et al. 2010). Another naturally occurring KISS1R loss-of-function mutant associated with IHH and infertility, L102P, was discovered but based on a detailed phenotypic and endocrine profiling of five patients bearing the mutation, the authors observed that gonadotropic deficiency was highly variable, ranging from partial to complete (Tenebaum-Rakover et al. 2007). L102P was shown to be strongly uncoupled from $InsP_3$ formation at the supra-physiological concentration of 1 μM Kp (Horikoshi et

al. 2003), leading the authors to conclude that KISS1R loss-of-function mutations do not necessarily cause complete gonadotropic deficiency (deRoux et al. 2003, Tenebaum-Rakover et al. 2007, Nimiri et al. 2011). While it was clearly recognized that, based on $InsP_3$ formation, L102P was a loss-of-function mutant that only delayed pubertal development, the mechanism by which this was possible was not understood. We hypothesize that some $G\alpha_{q/11}$-uncoupled KISS1R mutants like L148S and L102P retain some ability to signal, although such signaling would be diminished. Such signaling should be sufficient to trigger some degree of GnRH secretion and activation of the gonadotropic axis, albeit to a lesser extent. To test this hypothesis, L148S was expressed in human embryonic kidney (HEK) 293 cells and it was confirmed that, while it was strongly uncoupled from the $G\alpha_{q/11}$ pathway, it retained the ability to trigger significant ERK1/2 phosphorylation following kisspeptin treatment (Ahow et al. 2014). The Babwah lab previously demonstrated that wild-type KISS1R can bind and signal to ERK1/2 in a β-arrestin dependent manner and here we showed that KISS1R stimulation of LH secretion is in part, β-arrestin-dependent (Szereszewski et al. 2010 and Ahow et al. 2014). We therefore hypothesized that β-arrestin also mediated the kisspeptin triggered ERK1/2 phosphorylation observed with the $G\alpha_{q/11}$-uncoupled mutant, L148S. When expressed in MEFs completely lacking β-arrestin expression, L148S lost the ability to activate ERK1/2 following kisspeptin stimulation confirming that this response is β-arrestin-dependent (Ahow et al. 2014). Taken together with the *in vivo* results we can conclude that KISS1R signals via both $G\alpha_{q/11}$ and β-arrestin to regulate GnRH secretion and that some KISS1R mutants that are uncoupled from $G\alpha_{q/11}$ retain the ability to signal independently of $G\alpha_{q/11}$, likely via β-arrestin, to mediate GnRH secretion.

So far, our *in vitro* results demonstrated that KISS1R signals strongly through β-arrestin and while absence of either β-arrestin isoform *in vivo* reduced kisspeptin-dependent LH secretion, overall fertility of these mice was unaffected (Ahow et al. 2014). This can be attributed to isoform compensation where the expressed β-arrestin isoform functionally compensates for the one that is absent (Lohse et al. 1992, Kohout et al. 2001, Zhang et al. 2010). Therefore a mouse model completely lacking both β-arrestin isoforms would be useful for investigating the importance of β-arrestins on kisspeptin-dependent LH secretion however these mice are neonatal lethal and conditional models are not available

(Zhang et al. 2010, Kohout et al. 2001). For this reason instead of looking at the role of only β-arrestin signaling, we took a slightly different approach to investigate the significance of $G\alpha_{q/11}$-independent signaling downstream of KISS1R on LH secretion and fertility. In order to accomplish this we created a conditional GnRH-neuron specific $G\alpha_{q/11}$ double knockout mouse (*Gnaq*$^{d/d}$) by interbreeding a *Gnaq*$^{fl/fl}$*;Gna11*$^{-/-}$ mouse line with a well established *Gnrh-Cre* line (Offermanns et al. 1997 and Yoon et al. 2001). While the $G\alpha_{q/11}$ pathway downstream of the KISS1/KISS1R system is the most characterised cascade for GnRH release, based on the combination of *in vivo* and *in vitro* results described above, we hypothesize that in the *Gnaq*$^{d/d}$ mouse, KISS1R would still be able to trigger kisspeptin-dependent LH secretion via $G\alpha_{q/11}$ independent mechanisms, albeit at a diminished level. We further hypothesize that in the absence of exogenous stimulation *Gnaq*$^{d/d}$ mice would be sub-fertile and not completely infertile due in part to endogenous KISS1R mediated GnRH secretion via $G\alpha_{q/11}$ independent pathways. After creating the *Gnaq*$^{d/d}$ mice we first ensured that loss of $G\alpha_q$ and $G\alpha_{11}$ did not affect GnRH neuronal development and migration. After creating *Gnaq*$^{d/d}$ and littermate control mice we performed extensive characterization of the spatial and temporal expression of Cre recombinase activity. We confirmed that recombination occurred well before puberty in e18 mice and that it was specific to hypothalamic GnRH neurons. To investigate if $G\alpha_{q/11}$ independent mechanisms were sufficient to maintain some level of fertility we assessed various phenotypic and anatomic indicators of puberty and reproductive function. While there was no significant difference in body weight between *Gnaq*$^{d/d}$ mice and their littermate controls, both male and female *Gnaq*$^{d/d}$ mice displayed delayed onset of puberty and significantly smaller reproductive organs. *Gnaq*$^{d/d}$ females demonstrated irregular estrous cycles and were completely infertile while *Gnaq*$^{d/d}$ males were able to impregnate and sire litters with 60% of wild-type female partners. These results indicate that endogenous $G\alpha_{q/11}$- independent mechanisms were sufficient to sustain some degree of GnRH neuron function and secretion. Next we wanted to investigate if kisspeptin-stimulated GnRH release was also still possible in the *Gnaq*$^{d/d}$ mice. We found that while kisspeptin triggered diminished levels of LH, FSH and testosterone in *Gnaq*$^{d/d}$ mice compared to littermate controls (hypogonadotropic), this response was still significantly greater than vehicle treatment. We propose that this diminished $G\alpha_{q/11}$ independent

response is sufficient for partial functioning of the HPG axis allowing for delayed puberty and sub-fertility observed in the *Gnaq*$^{d/d}$ mice. Furthermore, when pre-treated with a GnRH inhibitor, antide, (Gottsch et al. 2004 and Dansforth et al. 2005) kisspeptin is no longer able to trigger gonadotropin secretion in *Gnaq*$^{d/d}$ and control littermates confirming that the response observed in *Gnaq*$^{d/d}$ mice could only be due to upstream kisspeptin-triggered GnRH secretion.

Overall, we have demonstrated that KISS1R is able to signal independently of $G\alpha_{q/11}$ proteins to regulate reproduction and fertility. This has great clinical significance for patients suffering from idiopathic hypogonadotropic hypogonadism (IHH), a condition characterized by the absence of the timely onset of pubertal maturation in the presence of low or inappropriately normal gonadotropin levels (Seminara et al 2003, deRoux et al. 2003, Nimiri et al. 2011, Wahab et al. 2011, Noel et al 2011). Some of these IHH patients carry inactivating KISS1R mutations which completely impair the receptor's ability to activate $G\alpha_{q/11}$ signaling in the GnRH neurons and as such disrupts normal HPG axis function (Tenebaum-Rakover et al. 2007 and Seminara et al 2003). Studies of these patients revealed that while they were infertile, gonadotropic deficiency was highly variable, ranging from partial to complete and that for some, a reversal of the IHH symptoms occurred later in life. These studies therefore suggested that KISS1R inactivation does not impede sexual maturation, rather just delays the onset however the underlying mechanism was not understood (Tenebaum-Rakover et al. 2007). Here we describe a novel $G\alpha_{q/11}$-independent pathway downstream of KISS1R in the GnRH neuron where kisspeptin is still able to trigger significant, albeit diminished, gonadotropin secretion and that this pathway is regulated by β-arrestins to some extent. We therefore propose that some patients bearing KISS1R mutations that uncouple the receptor from $G\alpha_{q/11}$ still retain some capacity for $G\alpha_{q/11}$-independent GnRH secretion, through β-arrestins. We suggest this as a potential molecular explanation for the variable gonadotropin levels and symptom reversal reported for some patients diagnosed with IHH. This is an important finding that not only provides novel insight at the molecular level of KISS1R signaling in the GnRH neuron but also identifies a potentially new therapeutic approach by which specific cases of clinical infertility can be treated.

2.2 Materials and Methods

2.2.1 Materials

Kisspeptin 10 (KP-10, #445888, human metastin 45-54) was purchased from Calbiochem (La Jolla, USA). KP-54 (#SCP0186, human metastin 68-121), Buserelin (#B3303, GnRH-R agonist) and Antide (#A8802, GnRH antagonist) were all purchased from Sigma (St. Louis, MO, USA). Unless stated, all other biochemical reagents were acquired from Sigma, Fisher, and VWR Scientific. The following plasmid constructs were described previously: untagged WT KISS1R (formerly referred to as GPR54) and untagged L148S (Seminara et al. 2003); FLAG-KISS1R (WT) (formerly referred to as FLAG-GPR54) (Pampillo et al. 2009); WT KISS1R-EYFP (Szereszewski et al. 2010); GFP-PH-PLC-δ1 (Babwah et al. 2012) and myc-KISS1R (WT) (Min et al. 2014). FLAG-L148S and L148S-EYFP were created by conducting site-directed mutagenesis (QuickChange kit, Stratagene, CA, USA) to introduce the mutation in the corresponding epitope-tagged WT receptor. Using the previously constructed myc-L148S-PCS2+ (Gill et al. 2008), myc-L148S was subcloned into the pcDNA3 expression vector. Proteome Profiler kit (#ARY003B) was purchased from R & D systems (Minneapolis, MN, USA) and was used according to manufacturer's instructions to detect an array of human-phospho-kinases. Human *Gnaq* and *Gna14* were purchased from Origene, (SC_125360 and SC_122675 respectively). All constructs were subjected to automated DNA sequencing to confirm sequence integrity.

2.2.2 Animal Husbandry and Genotyping

Animals were housed at the London Regional Cancer Program Animal Facility (London, Ontario, Canada) at controlled temperature and a 12 h light-dark cycle. Animal care and handling were done according to the guidelines of the University of Western Ontario (Canada) Animal Care Committee approved by the Canadian Council on Animal Care (See Appendix 3).

2.2.3 *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice

Genetically modified mice lacking β-arrestin-1 and -2 (*Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ respectively) were generated as previously described (Conner et al. 1997 and Bohn et al. 1999). Homozygous *Arrb1*$^{-/-}$ and heterozygous *Arrb2*$^{+/-}$ mice were obtained from R. J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). The *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ lines were in the C57BL/6J and 129/S1/SvImJ backgrounds, respectively. *Arrb2*$^{-/-}$ mice and their wild-type littermates (*Arrb2*$^{+/+}$) were generated by breeding heterozygous animals. Mouse genotyping was performed by PCR using Arrb2-WT-forward GATCAAAGCCCTCGATGATC (intron 2) and Arrb2-KO-forward GCTAAAGCGCATGCTCCAGA (Neo), which are specific for wild type (WT) and knockout (KO), respectively, and the primer Arrb-2-reverse ACAGGGTCCACTTTGTCCA (exon 3), which is in common for both WT and KO. PCR products are 605 bp and 300 bp for *Arrb2*$^{+/+}$ and *Arrb2*$^{-/-}$ alleles, respectively. Homozygous *Arrb1*$^{-/-}$ mice were crossed to WT C57BL/6J mice to generate a segregating colony. Due to the design of the targeting sequences, *Arrb1*$^{-/-}$ mice could not be distinguished from *Arrb1*$^{+/-}$ mice by PCR analysis as was done for *Arrb2* segregating populations. Instead, segregating *Arrb1* colonies were genotyped at Transnetyx, Inc. (Cordova, TN) based on the determination of *Neo* copy numbers (Conner et al. 1997) and data were verified by western blotting. All experiments were performed using 8 week-old post-pubertal male mice to preclude the effects of the estrous cycle on GnRH secretion in females.

2.2.4 Generation of mice lacking both $G\alpha_q$ and $G\alpha_{11}$ in GnRH neurons: (*Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$;*Gnrh-Cre*)

Gnaq$^{fl/fl}$;*Gna11*$^{-/-}$ and *Gnrh-Cre* mice were generated as previously described Offermans et al. 1997, Wintermantel et al. 2006, Yoon et al. 2005 and Skynner et al. 1999). *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ mice were obtained from Dr. S. Offermanns (University of Heidelberg, Heidelberg, Germany) and *Gnrh-Cre* mice were obtained from Dr. C. Dulac (Harvard University, Cambridge, MA, USA). The *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ and *Gnrh-Cre* lines were in the C57BL/6J background. The parental line was created by crossing the *Gnrh-Cre* mice (Yoon et al. 2005) to *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ mice (Offermans et al. 1997). Resulting progeny

of genotype *Gnaq*$^{fl/+}$;*Gna11*$^{-/-}$;*Gnrh-Cre* were crossed with *Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$ to generate a segregating population with both experimental and control genotypes (Pampillo and Babwah, unpublished data). The experimental mice contained a GnRH neuron specific deletion of *Gnaq* in the background of full body deletion of *Gna11: Gnaq*$^{fl/fl}$;*Gna11*$^{-/-}$; *Gnrh-Cre* (hereafter referred to as dKO or *Gnaq*$^{d/d}$). The littermate controls were *Gnaq*$^{fl/fl}$; *Gna11*$^{-/-}$ (hereafter referred to as control or *Gnaq*$^{fl/fl}$).

Mouse genotyping was performed by four PCR reactions using the following primers (listed 5'-3') ; *Gnaq* forward GCATGCGTGTCCTTTATGTGAG and *Gnaq* reverse AGCTTAGTCTGGTGACAGAAGC amplify a 600 bp or 700 bp band corresponding to the wild-type (*Gnaq*$^{+/+}$) or floxed allele (*Gnaq*$^{fl/fl}$) respectively. *Gna11*-WT forward AGCATG CTGTAAGACCGTAG and *Gna11*-WT reverse GCCCCTTGTACAGATGGCAG amplify a 820 bp band corresponding to the wild-type allele for *Gna11*$^{+/+}$. *Gna11*-KO forward CAGGGGTAGGTGATGATTGTGC and *Gna11*-WT reverse GACTAGTGAGACGTGCTACTTCC amplify a 450 bp band corresponding to the deleted allele for *Gna11*$^{-/-}$. *Gnrh-Cre* forward CTGGTGTAGCTGAT GATCCG and *Gnrh-Cre* reverse ATGGCTAATCGCCATCTTCC amplify a 354 bp band corresponding to the inserted *Cre* cassette under the regulation of the *Gnrh* promoter.

Analysis of Cre recombination was done by PCR amplification of genomic DNA from hypothalamic tissues using primers (listed 5'-3') GCATGCGTGTCCTTTATGTGAG and TGGATATTCAAAGTATCACACTCAC which gave a 2.3 kb product that corresponded to the floxed allele (*Gnaq*$^{fl/fl}$) and a 0.5 kb product that corresponded to the recombined allele (*Gnaq*$^{\triangle}$). Because recombination occurs in the GnRH neurons only and hypothalamic tissue contains a mix of GnRH and non-GnRH neurons, *Gnaq*$^{d/d}$ mice would have both the 2.3 kb and 0.5 kb bands (Wettschureck et al. 2001). RT-PCR analysis of *Gnrh* gene expression was done to show that the hypothalamic tissue collected contained GnRH expressing neurons. The following primers (listed 5'-3') were used; Gnrh forward; GCATTCTACTGCTGACTGTGTGTT and Gnrh reverse; GTTCTGCCATTTGATCCACCT and a resulting 144 bp product corresponded with the Gnrh allele. To further assess whether Cre recombinase was expressed, RNA extracted

from the hypothalamus, pituitary and ovaries of experimental and control mice was used in reverse transcription-PCR (RT-PCR) with the primers 5'-GCATTACCGGTCG ATGCAACGAGTGATGAG-3' and 5'- GAGTGAAC GAACCTGGTCGAAATCAG TGCG-3' and a 300 bp product corresponded with Cre expression.

2.2.5 Developmental Characterization of *Gnrh-Cre* expression in mice

The well-characterized GnRH-Cre line targets essentially all GnRH neurons (Yoon et al. 2005 and Wintermantel et al. 2006). Further characterization was necessary however, since it was essential in this study for Cre recombination occur before puberty which coincided with approximately post-natal day 35 in the wild-type strain used in this study. To achieve this, *Gnrh-Cre* mice were crossed with a ROSA26::LoxP-Stop-LoxP-Green Flourescent Protein (GFP) reporter line (Soriano1999). The resulting progeny of genotype ROSA26::LoxPStop-LoxP-GFP; *Gnrh-Cre* were sacrificed at E18, 1,2 and 3 weeks of age. Mice were transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed and post-fixed for 3 h in 8% PFA; then infiltrated with 30% sucrose. Coronal sections, 30 µm thick, were cut on a Leica freezing microtome and blocked in 10% normal donkey serum for 1 h at room temperature and incubated overnight at 4 °C in a rabbit anti-GnRH primary antibody (HU60, donated by Dr. H. Urbanski). The primary antibody was used at a concentration of 1:1000.

Following primary antisera, tissue sections were washed in PBS and blocked in 10% normal donkey serum then incubated at room temperature in secondary antisera for 2 h (AlexaFlour 568, 1:2000 goat anti-rabbit, Invitrogen). Sections were subsequently stained with Hoechst (Invitrogen) 1:10,000 for 3 min to detect nuclei and then washed in PBS and mounted on positively charged microscope slides and allowed to dry before being cover-slipped with Immunomount (Fisher Scientific, USA). Sections were viewed and images captured using Olympus Fluoview 1000 laser scanning confocal microscope 20X and 40X objectives were used. Colocalization studies were performed using multiple excitation (405, 488, 559) and emission (band pass 425-475, 500-545 nm and 575-675 nm for Hoechst, endogenous ROSA26::GFP, and AlexaFluor 568 respectively) filter sets. Multi-colour images were acquired in the sequential acquisition mode to avoid cross-

excitation. Ten consecutive coronal sections per brain, spanning the rostral-caudal hypothalamic axis, were analyzed by confocal microscopy where neuronal soma (**S**) were scored for green (GFP) and red (GnRH) signals.

2.2.6 Analysis of GnRH neurons by immunohistochemistry in *Arrb1*$^{-/-}$, *Arrb2*$^{-/-}$, *Gnaq*$^{d/d}$ mice and their respective littermate controls.

Following CO_2 euthanasia, mice were transcardially perfused with 0.08 % heparinized saline followed by 4 % paraformaldehyde (PFA). Brains were removed and post-fixed overnight in 4% PFA; then embedded in 5 % agarose and cut into 50 µm thick coronal sections using a vibrating microtome (Leica VT1000S). Tissue sections were then blocked in 5 % normal goat serum for 1 h at 4 °C and incubated over two nights at 4 °C in primary antisera with antibodies directed against the GnRH peptide. The following anti-GnRH primary antiserum were used, polyclonal EL14 (Ellinwood et al. 1985) at a 1:5,000 dilution, polyclonal PA1-122, (Affinity BioReagents, Golden, CO) at a 1:300 dilution and monoclonal QED, (19304, QED bioscience, San Diego, CA) at a 1:5,000 dilution. For eight week old *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice primary antiserum EL14 was used and alternate sections were incubated in either PA1-122 or QED. For E18 *Arrb1*$^{-/-}$*;Arrb2*$^{-/-}$ mice, and for eight week old *Gnaq*$^{d/d}$ and *Gnaq*$^{fl/fl}$ mice, only primary antiserum EL14 was used.

Following primary antisera, tissue sections were incubated at room temperature in secondary antisera for 2 h. For sections incubated in EL14 or PA1-122, a biotin conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) was used at 1:2,500. For sections incubated in the QED, a biotin conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA) was used at 1:5,000. Secondary antibody labeling was amplified using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) for 1h at room temperature. Reaction product was visualized after 5 min incubation in Tris-buffered saline (TBS, pH 7.5) containing 0.025% diaminobenzidine, 0.02% nickel and 0.02% H_2O_2. Free-floating tissue sections were washed in TBS mounted on gelatin-coated glass microscope slides and allowed to dry before being cover-slipped with Permount (Fisher Scientific, USA). Sections were

viewed and images were captured using an Olympus BH2 microscope with an Insight QE digital camera with Spot Advanced Software. Coronal brain slices along the rostral-caudal axis were ordered and aligned relative to the organum vasculosum of the lamina terminalis (OVLT) and an investigator blind to genotype manually counted GnRH neuronal cell bodies using a 40x objective (Gill et al. 2008). The effect of genotype on the number and location of GnRH neurons was determined by 2-way ANOVA as a repeated measure using SPSS software (SPSS Inc., Chicago, IL). Values are reported as mean ± SEM and $P < 0.05$ was considered statistically significant.

2.2.7 Buserelin (GnRH-agonist) and KP-54 treatment in *Arrb1*$^{-/-}$, *Arrb2*$^{-/-}$ mice and respective littermate controls.

Eight week-old male mice (*Arrb1*$^{-/-}$, *Arrb2*$^{-/-}$ and their respective WT littermate controls) were administered 3 ng Buserelin/g body weight (for the Buserelin-challenge) or 100 nmol KP-54/Kg body weight (for the Kp-challenge). Both KP-54 and Buserelin were delivered into the abdominal peritoneal cavity referred to as an intraperitoneal (i.p.) injection. For the Buserelin- and Kp-challenged mice, 20 and 60 min later, respectively, mice were anesthetized by CO_2 exposure followed by cervical dislocation and blood was collected immediately after by cardiac puncture and serum extracted for hormone assays as described later.

2.2.8 KP-54 and antide (GnRH-antagonist) treatment in *Gnaq*$^{d/d}$ mice and littermate controls.

To avoid cycle-dependent LH surges from interfering with kisspeptin-induced LH secretion, seven week old virgin *Gnaq*$^{d/d}$ and *Gnaq*$^{fl/fl}$ female mice were anesthetized using 1.5% isoflurane (4% for induction) in an oxygen/nitrous oxide 30% /70% mixture and an ovariectomy was performed by dorsal incision as described by Strom et al. 2012. Females were allowed to recover for 14 days post-surgery. Ovariectomized females and males 9 weeks of age were administered a single i.p. injection of 100 µl of vehicle (saline) or 100 µl of 100 nmol Kp-54/Kg body weight (d'Anglemont de Tassigny et al. 2008 and Kirilov et al. 2013). 1 h later, mice were anesthetized by CO_2 exposure followed by cervical dislocation and blood was collected immediately thereafter by terminal cardiac puncture (Hoff 2000).

In a separate cohort of nine week old ovariectomized females and males, mice received two 100 µl subcutaneous injections of the GnRH antagonist, antide (Sigma A8802) (1.25 ug/g body weight dissolved in sterile saline). Antide was administered at 24 h and 1 h before i.p. injection of 100 µl of vehicle (saline) or 100 µl of 100 nmol KP-54/Kg body weight (Barkan et al. 2004, Gottsch et al. 2004 and Dansforth et al. 2005). 1 h after the final injection blood was collected by terminal cardiac puncture.

2.2.9 Serum hormone Assays.

Blood was allowed to clot at room temperature for 120 min. The clot was then removed with a wooden applicator stick and the remaining serum centrifuged at 2000 X g for 20 min at RT. Serum was stored at -80 ^{o}C and analyzed for LH, FSH and testosterone by the Endocrine Technology and Support Lab (ETSL), Oregon National Primate Research Center (Beaverton, OR, USA). Mouse serum LH was analyzed by a traditional double-antibody RIA procedure similar to that described previously (Pau et al. 1986). The detection limit of the assay was 0.1-0.2 ng/ml. The interassay variation was <12% and the intra-assay variation was <8%. Mouse serum FSH was analyzed by a traditional double-antibody RIA procedure similar to that described previously (Pau et al. 1986). The detection limit of the assay was 0.01-0.02 ng/tube, or 0.1-0.2 ng/ml. The interassay and intrassay variation was less than 12 % and 8%, respectively. Mouse serum testosterone (T) was measured by ether extraction and radioimmunoassay (RIA) as described previously (Rasmussen et al. 1984). Hormonal values were corrected for extraction losses determined by radioactive trace recovery at the same time with sample extraction; hot recovery usually is better than 90%. The sensitivity was 5 pg/tube for T RIA. The effect of administered drugs on serum hormone levels was determined by Student's t-test using GraphPad Prism software (Graph Pad, San Diego, CA). Values are reported as ± SEM and $P < 0.05$ was considered statistically significant.

2.2.10 Pubertal onset, estrous cycle and fertility assessment of *Gnaq*$^{d/d}$ mice and littermate controls.

Mice were genotyped and weaned by 3 weeks of age. Various phenotypic measurements were taken from male and female mice from about 2 weeks to 16 weeks of age. Body weight (g) was recorded weekly while mice were examined daily from 2 weeks until day of vaginal opening in females and preputial separation in males. Anogenital distance (mm) was recorded every 3 -7 days in males. Vaginal opening, preputial separation and anogenital distance are established indices for assessing sexual maturation in mice (Lapatto et al 2007, Herbison et al. 2008 and Chan et al. 2009). Vaginal opening occurs with increased estrogen secretion (Caligioni 2009, Herbison et al. 2008, Ojeda and Urbanski 1994 and Nelson et al. 1982). Preputial separation and anogenital distance are both dependent on testosterone exposure during puberty (Lapatto et al. 2007, Parakarainen et al. 2005, Novaira 2014). Assessment of preputial separation consisted of the application of gentle pressure to manually retract the prepuce (Noviara et al. 2014 and Korenbrot et al. 1977).

Estrous cycle assessment was performed using a combination of visual observation of vulva appearance and examination of vaginal cytology following vaginal lavage and crystal violet staining. The visual method has been fully described in Byers et al. (2012) and Champlin et al. (1973) and was used to confirm cytological assessment. Vaginal smears were collected between 08:00 – 10:00 daily for 17 days as described by Caligioni (2009) using a vaginal lavage technique where a small volume of saline (0.9%) was used to gently flush the vagina then placed on a slide and stained with 0.1% crystal violet solution. Care was taken to not penetrate the vaginal orifice since pseudopregnancy can result (McLean et al. 2012 and Adler et al. 1970). Estrous cycle stage, classified as proestrus, estrus and metestrus/diestrus, was determined based on the proportion of the following three cell types found in the smear; cornified epithelial, nucleated epithelial and leukocytes (Novaira et al. 2014, Byers et al. 2012, McLean et al. 2012 and Caligioni 2009). Estrous cycle was examined in the same female mice over two post-pubertal periods, 8-11 weeks of age and 13-16 weeks of age to assess if a regular pattern of cyclicity developed at a later age in the experimental females. Number of oestrus phases

during the 17 day period was compared between experimental and control mice with unpaired two-tailed t-tests. All phenotyping experiments were done without the knowledge of the genotype.

Intact 7 week old male and female *Gnaq*$^{d/d}$ mice were placed in a cage with a wild-type mouse of the opposite gender for 18 – 21 days and proven fertility and pregnancy in females was noted (Lapatto et al. 2007).

2.2.11 Anatomy and histology of reproductive organs for *Gnaq*$^{d/d}$ mice and littermate controls

All mice used were 8-16 weeks of age and were intact and previously untreated and unmated. Ovaries and uteri were taken from females in the metestrus stage since this stage is the clearest to identify by vaginal cytology and testes were taken from the males. Total body weight (g) and tissue appearance and fresh weight (mg) were noted. Tissues were fixed in 4% paraformaldehyde-PBS overnight then dehydrated in 70% ethanol for 24 h prior to paraffin embedding, sectioning (5 µm thick) and staining for hematoxylin and eosin by the London Health Science Centre Pathology Core (London, ON, Canada). Ovarian and testicular sections were examined and photographed with an Aperio ScanScope XT in conjunction with ImageScope software.

2.2.12 Quantitative real-time PCR for *Gnaq*$^{d/d}$ mice and littermate controls

Real-time qPCR was used to investigate the expression of genes in the hypothalamus and pituitary of control and dKO mice. The genes chosen for investigation are major regulators of the neuroendocrine reproductive axis. RNA was isolated from mouse tissues using the Qiagen RNeasy mini kit according to manufacturer's instructions (Qiagen, Missassauga, ON, Canada). RNA concentration was determined by optical density (260/280 nm) on an N-1000 spectrophotometer (NanoDrop, Wilmington, DE). 1.0 µg RNA was reverse-transcribed using SuperScript II (Invitrogen, Burlington, ON, Canada). Reactions were performed according to manufacturer's recommendations using random hexamer primers (Amersham, Piscataway, NJ). Real-time qPCR was performed in duplicate for each sample and done a total of three independent times using IQ SYBR

Green Master Mix (Bio-Rad Laboratories). The primers used are listed in the following Table 2.1.

To determine PCR efficiency, a 10-fold serial dilution of cDNA was performed as described previously (Wong et al. 2005). All sample reactions were performed as five independent trials with accompanying serial dilution standard curves and water blanks for each gene and treatment time. Each gene was normalized to *Actb* as an internal control and dKO values were presented relative to WT using the Pfaffl method (Pfaffl, 2001). Reaction efficiencies used in these calculations were derived from the standard curves and PCR conditions were optimized to generate >95% efficiency.

Table 2.1: Primers used for qPCR analysis in *Gnaq*$^{d/d}$ mice and littermate controls.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected product size (bp)
Kiss1r	GCCACAGACGTCACTTTCCTAC	CGGGAACACAGTCACATACCA	186
Kiss1	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATACC	140
Gnrhr1	GCTCTCAAGGATGAAGGTGCTT	CCAGGCTAATCACCACCATCAT	197
Gnrh1	CCTGGGGGAAAGAGAAACACT	TCACAAGCCTCAGGGTCAATG	246
Lhb (LH β)	TGTCCTAGCATGGTCCGAGT	AGGAAAGGAGACTATGGGGTCTA	138
Fshb (FSH β)	AGAGAAGGAAGAGTGCCGTTTCTG	ACATACTTTCTGGGTATTGGGCCG	118
Gnaq	GGTGTCTGCTTTTGAGAATCCATATG	ATTCGCTAAGCGCTACTAGA	344
Gna11	GGAGAACATCATCTTCAGGATGG	CAGGGGTAGGTGATGATTGTGC	201
Gna14	TCACTGCACTCTCTAGAGACC	GAAAGAGGGCATGGCGATAC	155
Actb (β-actin)	CTGTCGAGTCGCGTCCACCC	ACATGCCGGAGCCGTTGTCG	128

2.2.13 Cell culture and Transfection

Human embryonic kidney cells (HEK 293) and African Green Monkey embryonic kidney cells (COS-7) cells were from the American Tissue Culture Collection. HEK 293 cells were cultured in MEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acids and gentamicin (5 µg/ml). The immortalized mouse GnRH-neuronal cell line (GT1-7) was obtained from Pamela Mellon (University of California, San Diego) (Mellon et al. 1990). COS-7 and GT1-7 cells were grown in monolayer in DMEM supplemented with 10% FBS, 4.5 mg/ml glucose, 1% penicillin/streptomycin (v/v) and 1% (v/v) sodium pyruvate. Mouse embryonic fibroblast (MEF) cell lines derived from the β-arrestin-1 knockout, β-arrestin-2 knockout, β -arrestin-1/2 double knockout mice (Kohout et al. 2001). The β-arrestin-2 single and β-arrestin-1/2 double knockout mice were derived from the same WT strain and accordingly, the same WT MEFs (referred to as WT2) serve as their WT parents in this study. MEF β-arrestin-1 knockout, β-arrestin-2 knockout, β -arrestin-1/2 double knockout and their corresponding wild-type parental cell line were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (v/v) and 1%(v/v) non-essential amino acids. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. FBS was purchased from Sigma (St. Louis, MO, USA); all other reagents were from Life Technologies (Burlington, ON, Canada). All cells were transfected with plasmid DNA constructs as detailed in the results and figures.

HEK 293 and COS-7 cells were transiently transfected with plasmid DNA using a modified calcium phosphate method as previously described (Bhattacharya et al. 2004). When confluent, all MEF cell lines were transiently transfected with either 20 µg of KISS1R-EYFP or L148S-EYFP cDNA by electroporation using the Bio-Rad Gene Pulser Xcell System (exponential decay protocol: 230 V, 950 mF) with Bio-Rad 0.4 cm electroporation cuvettes. Following electroporation (16–18 h), cells were split to petri dishes. The cells were then allowed to recover for approximately 6 h prior to overnight serum starvation in serum-free media (Szereszewski et al. 2010). GT1-7 neurons were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Arrb1 and *Arrb2* were stably downregulated in GT1-7 neurons using shRNA cloned in the pGFP-V-RS vector (OriGene Technologies). The following are the *Arrb1* and *Arrb2* and scrambled control shRNA sequences that were used (listed 5'- 3'); GACTCCAGTAGACACCAATCTCATAGAGC, GTGGCTCAGCTAGAACAAGAT GACCAGGT and TGACCACCCTGACCTACGTCGTGCAGTGC respectively. Heterogeneous populations of stable transfectants were selected in media containing 0.5 µg/ml of puromycin and maintained on media containing 0.25 µg/ml of puromycin. β-arrestin knockdown was confirmed by western blot analysis. These cell lines were also co-transfected with FLAG-KISS1R and receptor expression was selected for and maintained with 400 µg/ml and 300 µg/ml respectively Gentamicin (G418), a selective antibiotic for the neomycin resistance gene expressed by the FLAG-KISS1R plasmid.

2.2.14 Western Blot Analysis

Non-transfected and transfected HEK 293 cells expressing untagged and tagged WT KISS1R and L148S constructs were used in ERK1/2 and Stat3 phosphorylation assays. The tags used were FLAG, EYFP and myc where position of the tag or epitope in the nomenclature of the construct used indicated if attached to the C- or N- terminus of the receptor sequence. KISS1-YFP- and L148S-YFP-expressing cells were assessed visually for transfection efficiency and only dishes where 75% of the cells displayed YFP expression were used. HEK 293 cells were also transiently co-transfected with 10 µg of receptor DNA (KISS1R-YFP or YFP-L148S) and a range of 0, 0.5, 2.5 or 5 µg of the cDNA encoding for either $G\alpha_q$ or $G\alpha_{14}$. Prior to each assay, cells were serum-starved for 30 mins in Hanks' balanced salt solution (HBSS: 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4). Assays were then conducted by treating these cells with one of the following concentrations of KP-10 (0, 0.1 and 3.0 µM) for 10 min. Following KP treatment, cells were placed on ice then solubilized in lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 1 mM AEBSF, 60 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µg/mL pepstatin) and then clarified

by centrifugation for 20 min. at 23 000 RCF. 50 µg of protein was subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes for immunoblotting.

Immunoblots were then analyzed for phosphorylated ERK1/2 using rabbit monoclonal antibody Phospho p44/42 MAPK (Thr202/Tyr204) (1:2,000) (Cell Signaling #4370), while total ERK1/2 was analyzed using a rabbit monoclonal antibody p44/42 MAPK (1:1,000) (Cell Signaling #4695). Immunoblots were also analyzed for β-arrestin-1 and -2 using mouse anti-β-arrestin-1 and -2 antibodies (1: 300) (BD Biosciences, Mississauga, ON, Canada, rabbit anti-Phospho-Stat3 (Ser727) (1:1000) (Cell Signaling #9134) and mouse monoclonal anti-β-actin (1:2000) (AbCam#8224). Chemiluminescent detection was performed using an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2500) and developed with ECL (GE Healthcare, Piscataway, NJ). Immunoblots were scanned and quantified by densitometry using the Versadoc Imaging System 5000 MP and Image Lab 3.0 software (Bio-Rad, Hercules, CA). The means ± S.E are shown for values obtained for the number of independent experiments indicated in the Figure Legends. GraphPad Prism software (Graph Pad, San Diego, CA) was used to analyze data for statistical significance. Statistical significance was determined by one-way analysis of variance followed by Dunnet's ad hoc test.

2.2.15 PLCβ translocation assay by confocal microscopy

HEK 293 cells co-transfected with (FLAG-, EYFP- or myc-tagged) WT KISS1R or L148S as well as GFP-PH-PLC-δ1 were reseeded on collagen-coated 35-mm glass-bottomed culture dishes. Prior to visualization, the cells were washed three times with HBSS (HEPES-buffered salt solution: 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) and incubated for 1 h at 37 C in HBSS. Confocal analysis was performed on an Olympus Fluoview 1000 laser scanning confocal microscope using the 60X Plan Apochromat 1.42 oil objective. Enhanced GFP fluorescence was visualized with excitation at 488 nm and emission 500-545 nm emission filter set. Regions of interest were identified in cells and the fluorescence intensity (arbitrary units) at a given region was monitored over time. The change in intensity over time was then presented

graphically. Each experiment was conducted five independent times and representative images from one independent experiment are presented.

2.2.16 Inositol Phosphate ($InsP_3$) accumulation assay

Total inositol phosphates were measured using two slightly different methods as previously described (Bianco et al. 2011 and Pampillo et al. 2009). Firstly, for investigating whether β-arrestin regulates KISS1R desensitization, COS-7, MEFs and GT1-7 cells were transfected as described in the figure legends and cultured for 24 h. Secondly, for investigating whether L148S is uncoupled from the $G\alpha_{q/11}$pathway, COS-7 cells transiently expressing 100 ng of receptor (WT KISS1R or L148S) were cultured for 24 h (collaboration with Dr. Kaiser).

For the analysis of the role of β-arrestin in KISS1R desensitization, cells grown in 24-well plates were incubated overnight at 37 ^{o}C in 400 µl of inositol-free DMEM containing 1 µCi of myo-inositol [2-^{3}H] (PerkinElmer, Waltham, MA). The following day unincorporated myo-[3H]-inositol was removed by washing the cells with HBSS. Cells were then pre-incubated for 1 h in 500 µl HBSS at 37 ^{o}C followed by a 15 min incubation in 500 µl HBSS containing 10 mM LiCl. Cells were then incubated for 4 h at 37 ^{o}C in 500 µl of HBSS-LiCl in the absence (basal) or presence of KP-10. The following dilution series of KP-10 concentrations was used; $3x10^{-11}$, $3x10^{-10}$, $3x10^{-9}$, $3x10^{-8}$, $3x10^{-7}$, $3x10^{-6}$ and $3x10^{-5}$M. The reaction was stopped on ice by adding 500 µl of 0.8 M perchloric acid and then neutralized with 400 µl of neutralization buffer (0.72 M KOH, 0.6 M $KHCO_3$) and stored at 4 ^{o}C overnight to allow for the precipitation of perchlorate particles . The following day total and purified [3H]-$InsP_3$ are collected for radioactive counting in liquid scintillation using a Wallac LKB 1211 RackBeta liquid scintillation counter (Perkin Elmer). Purified readings were normalized against total [3H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 µl of the cell lysate in 5ml scintillation buffer. Purified [3H]-$InsP_3$ formation was determined by anion exchange chromatography of the cell lysates using AG-1-X8(formate form) 200–400 mesh anion exchange resin (Bio-Rad, Hercules, CA). 900 µl of the cell lysate was applied to the anion exchange columns and washed twice with 5 ml H2O to remove free [3H]-inositol that was not incorporated into [3H]-inositol

phosphates. Columns were then washed twice with 60 mM ammonium formate. [3H]-$InsP_3$ products were eluted with 4 ml elution solution containing 1 M ammonium formate and 0.1 M formic acid. The means ± SE are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data by nonlinear regression using a sigmoidal curve fit with a variable slope, and to compare curves for statistical significance.

For the analysis of L148S stimulation of $InsP_3$ formation in COS-7 cells, the day after myo-[3H]-inositol labelling, the cells were pretreated with 10 mM LiCl for 15 min and then stimulated with increasing concentrations of KP-10 ($1x10^{-9}$ to $3x10^{-6}$ M) for 1 h. After stimulation, the cells were lysed in ice-cold 20 mM formic acid. The lysates were collected and neutralized to pH 7.5 with 300 µl solution containing 7.5 mM HEPES and 150 mM KOH. [3H]-$InsP_3$ products were isolated through prepared AG 1-X8 resin (Bio-Rad, Hercules, CA) anion exchange columns by washing with 5 ml of H_2O, and then 5 ml solution containing 5 mM borax and 60 mM sodium formate. [3H]-$InsP_3$ products were eluted by 3 ml elution solution containing 0.9 M ammonium formate and 0.1 M formic acid. The means ± S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data by nonlinear regression using a sigmoidal curve fit with a variable slope, and to compare curves for statistical significance.

2.2.17 Statistical Analysis

Data were analyzed and graphed using the GraphPad Prism4 program (GraphPad Software, Inc). Statistical significance was validated when $P < 0.05$. The specific analysis performed is discussed in the relevant sections of the methods.

2.3 Results

2.3.1 KISS1R triggers LH secretion via β-arrestin.

We have previously reported that WT KISS1R signals via the $G\alpha_{q/11}$- and β-arrestin-dependent pathways to phosphorylate ERK1/2 (Pampillo et al. 2009 and Szereszewski et al. 2010). Since ERK1/2 regulate Kp-dependent GnRH secretion (Castellano et al. 2006), we hypothesized that KISS1R stimulates GnRH secretion via the β-arrestin pathway. While a mouse model completely lacking both *Arrb1* (encodes for β-arrestin-1) and *Arrb2* (encodes for β-arrestin-2) would be ideal for testing this hypothesis, full body *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice are perinatal lethal due to pulmonary hypoplasia (Zhang et al. 2010, Conner et al. 1997, Bohn et al. 1998). Floxed β-arrestin mice have not been created yet thereby precluding the creation of Gnrh-neuron specific *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice. Furthermore, while it may have been possible to measure Kp-stimulated Gnrh secretion from E18 hypothalamic explants using *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice (d'Anglemont de Tassigny et al. 2007), our preliminary studies showed that we could not use this approach since loss of both isoforms significantly affected the size and distribution of the hypothalamic Gnrh neuronal population in the anterior hypothalamus. We therefore used the single KO mice lacking either *Arrb1* (*Arrb1*$^{-/-}$) or *Arrb2* (*Arrb2*$^{-/-}$) to test our hypothesis (Conner et al. 1997 and Bohn et al. 1999). Based on brood size, while the single KO mice are as fertile as their WT littermates, we reasoned that since the reproductive axis can withstand significant insults without having an effect on fertility, again as measured by brood size (Popa et al. 2013), we might instead be able to detect differences at the endocrine level that would support our hypothesis. We therefore proposed measuring LH secretion, a well-established surrogate for GnRH secretion in several vertebrate species (Seminara et al. 2003, Gottsch et al. 2004, Ramaswamy et al. 2013), to test whether KISS1R stimulates GnRH secretion via the β-arrestin pathway. While this strategy is possible because Gnrh receptor activity on the anterior pituitary is not regulated by β-arrestin (Vrecl et al. 1998, McArdle et al. 1999, Heding et al. 2000 Hislop et al 2001 and Pawson et al. 2008), it still depends on first demonstrating that the loss of *Arrb1* or *Arrb2* did not affect the size and distribution of the hypothalamic Gnrh neuronal population in the anterior hypothalamus nor did it affect LH secretion from the

pituitary gonadotropes due to developmental or other defects resulting from the whole body loss of *Arrb1* and *Arrb2*.

2.3.1.1 Loss of both β-arrestin-1 and -2 significantly affects GnRH immunoreactive neuronal population size and distribution in the hypothalamus.

We generated a segregating progeny with WT control (*Arrb1*$^{-/-}$;*Arrb2*$^{+/+}$), heterozygous (*Arrb1*$^{-/-}$;*Arrb2*$^{+/-}$) and experimental dKO (*Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$) littermates by interbreeding parents with the genotype, *Arrb1*$^{-/-}$;*Arrb2*$^{+/-}$. The effect of the complete loss of β-arrestin on the number and location of the adult GnRH cell population was analyzed by comparing dKO, WT and heterozygous littermates of 18 day old embryos (E18). Representative sagittal images from sections in the nasal compartment, olfactory bulb, dorsal forebrain and ventral forebrain are shown (Fig. 2.1). The mean number of GnRH immunoreactive neurons in each genotype was plotted in the adjoining bar graph. Based on the EL14 GnRH antiserum, *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice (n = 6) had significantly less GnRH immunoreactive cell counts ($P < 0.05$) when compared to either WT (*Arrb1*$^{+/+}$;*Arrb2*$^{+/+}$) (n=3) or heterozygous (*Arrb1*$^{-/-}$;*Arrb2*$^{+/-}$) (n = 3) littermates. This finding indicates that GnRH neuronal development is significantly affected in *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$dKO embryos and would therefore not be suitable models for this study. This analysis was done in collaboration with Dr. Stuart Tobet.

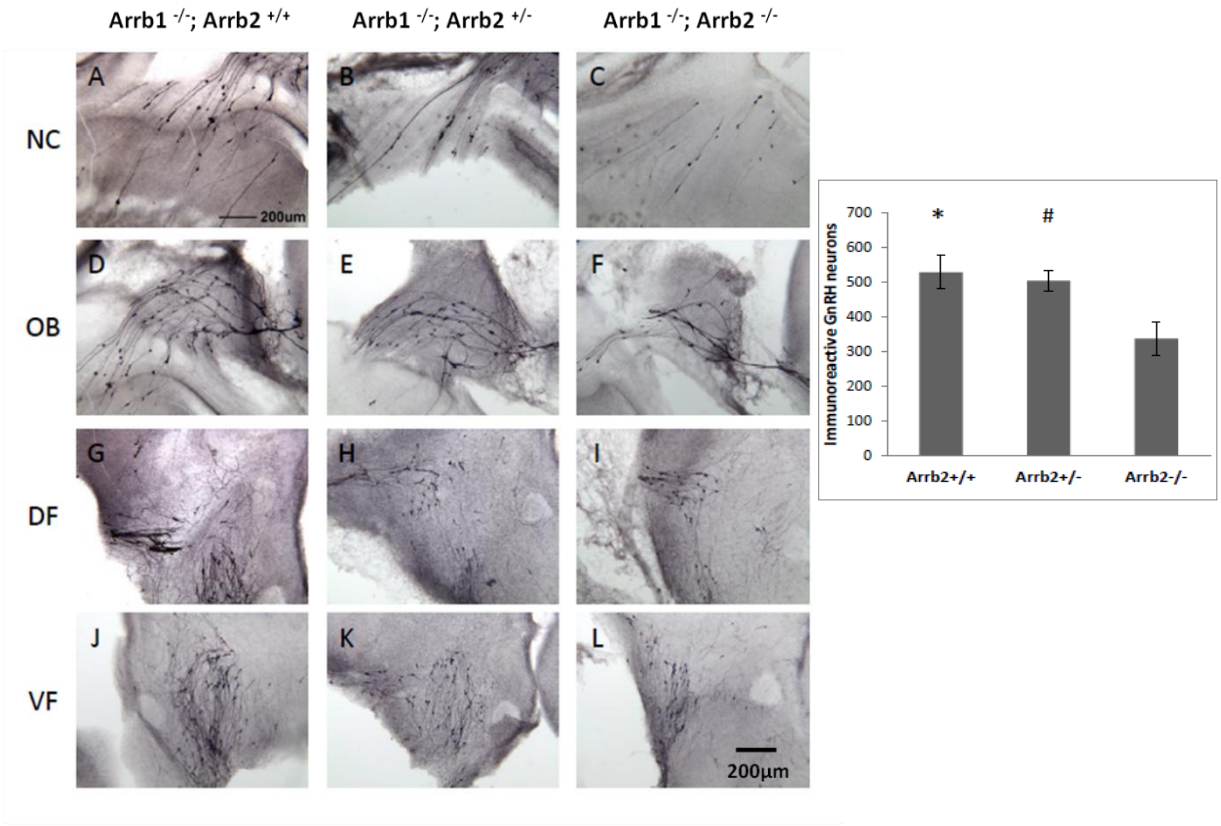

Figure 2.1: Loss of both *Arrb1* and *Arrb2* significantly affects GnRH immunoreactive population size and distribution in brain of E18 mice.

All embryos were generated in the background of *Arrb1* deletion and either *Arrb2* WT control (*Arrb1*$^{-/-}$; *Arrb2*$^{+/+}$), *Arrb2* heterozygous (*Arrb1*$^{-/-}$;*Arrb2*$^{+/-}$) or *Arrb2* KO (*Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$) (dKO) genotype. Number of embryos used in this analysis were WT n = 3 and Het and dKO n = 6. A-L. Photomicrographs of sagittal brain sections from E18 mice where GnRH neurons were revealed with IHC using the EL14 antibody. Each section was divided into four regions for analysis; Nasal Compartment (NC), Olfactory Bulb (OB); Dorsal Forebrain (DF) and Ventral Forebrain (VF). WT; (A,D,G,J) Het; (B,E,H,K) and dKO embryos (C,F,I,L). Inset: Bar chart showing total GnRH immunoreactive neuronal cell count in dKO embryos was significantly lower than for WT or Het embryos. Error bars represent SEM. ***** and # represent $P<0.05$ when WT and Het are compared to dKO respectively.

2.3.1.2 Loss of either β-arrestin-1 or -2 has no effect on the GnRH immunoreactive neuronal population size and distribution in the hypothalamus.

The effect of β-arrestin-1 or -2 on the number and location of the adult GnRH cell population was analyzed by comparing eight week-old male KOs to their respective WT littermates. Representative coronal images from sections at the level of the OVLT are shown (Fig. 2.2A and E). The mean number of GnRH immunoreactive neurons in each section was plotted as a histogram [*Arrb1*$^{-/-}$ (n = 6) and *Arrb1*$^{+/+}$ littermates (n = 6); *Arrb2*$^{-/-}$ (n = 5) and *Arrb2*$^{+/+}$ littermates (n = 5)] (Fig. 2.2B and F). As expected, for both *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ the highest number of GnRH immunoreactive neurons was generally found at or near the OVLT with fewer cells in sections rostral and caudal to the OVLT (Fig. 2.2 B, D, F and G) (Gill et al. 2008). Genotype (KO vs. WT) also had no significant effect on the total GnRH immunoreactive cell counts for β-arrestin-1 (Fig. 2.2C) and β-arrestin-2 (Fig. 2.2G) ($P > 0.1$). This finding, based on the EL14 GnRH antiserum, was corroborated using two other GnRH antisera, PA1-122 and QED. PAI-122 data are shown for the β-arrestin-1 WT and KO mice (n = 3 for WT and KO) (Fig. 2.2C and D) and QED data are shown for the β-arrestin-2 WT and KO mice (n = 5 for WT and KO) (Fig. 2.2G and H). With respect to the location of GnRH immunoreactive neurons, genotype (KO vs. WT) also had no significant effect in the *Arrb1*$^{-/-}$ vs. *Arrb1*$^{+/+}$ mice ($P > 0.1$) (Fig. 2.2B, D, F and H) and *Arrb2*$^{-/-}$ vs. *Arrb2*$^{+/+}$ mice ($P > 0.1$) (Fig. 2.2B, D, F and H). Overall, these findings suggest that the β-arrestin-1 and -2 single KO mice could be used to test whether KISS1R stimulates GnRH secretion via the β-arrestin pathway because the GnRH neuronal population was fundamentally “intact”.

Figure 2.2:

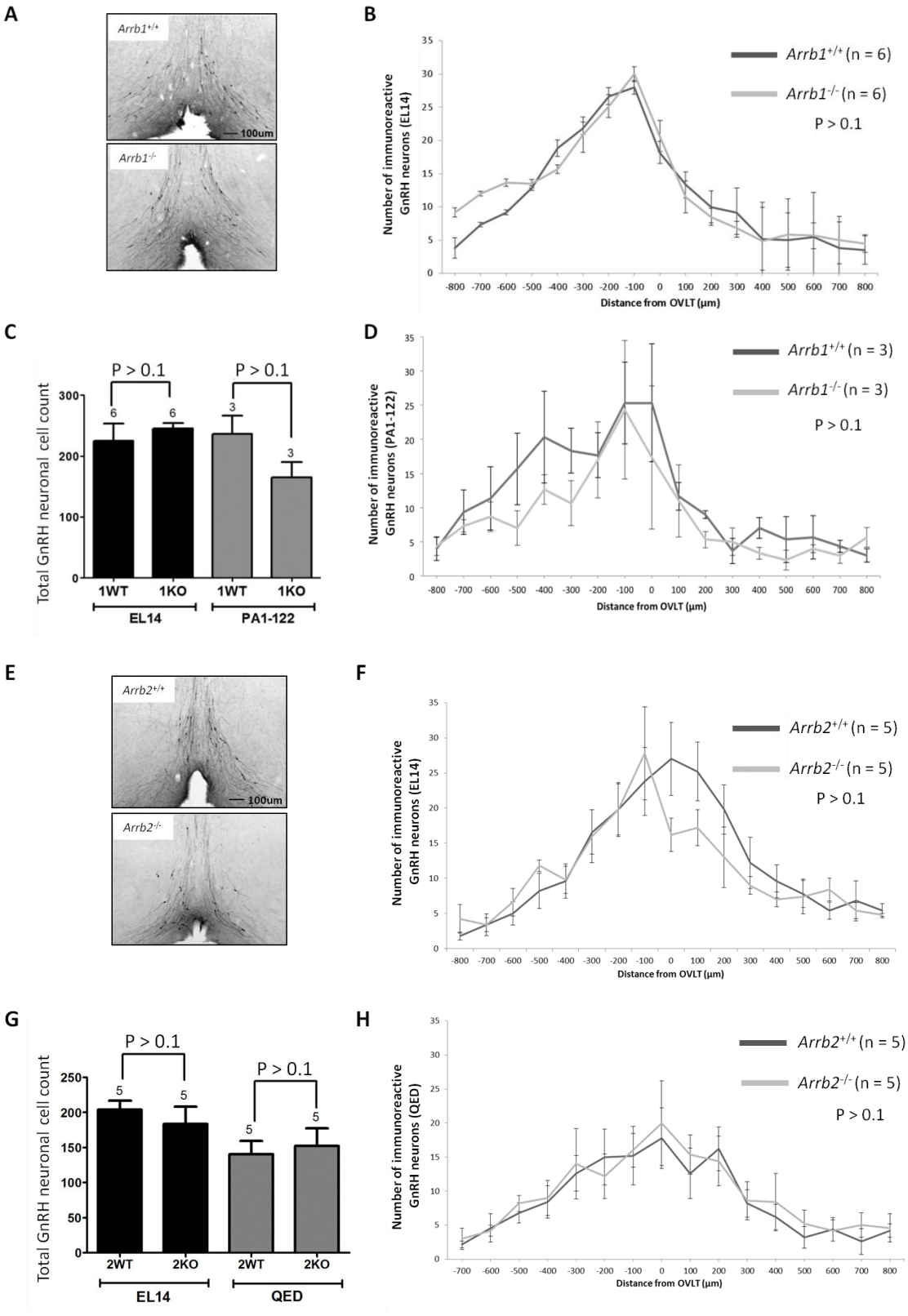
A
Arrb1+/+
100um
Arrb1-/-
B
Arrb1+/+ (n = 6)
Arrb1-/- (n = 6)
P > 0.1
Number of immunoreactive GnRH neurons (EL14)
Distance from OVLT (μm)
C
P > 0.1
P > 0.1
Total GnRH neuronal cell count
1WT
1KO
1WT
1KO
EL14
PA1-122
D
Arrb1+/+ (n = 3)
Arrb1-/- (n = 3)
P > 0.1
Number of immunoreactive GnRH neurons (PA1-122)
Distance from OVLT (μm)
E
Arrb2+/+
100um
Arrb2-/-
F
Arrb2+/+ (n = 5)
Arrb2-/- (n = 5)
P > 0.1
Number of immunoreactive GnRH neurons (EL14)
Distance from OVLT (μm)
G
P > 0.1
P > 0.1
Total GnRH neuronal cell count
2WT
2KO
2WT
2KO
EL14
QED
H
Arrb2+/+ (n = 5)
Arrb2-/- (n = 5)
P > 0.1
Number of immunoreactive GnRH neurons (QED)
Distance from OVLT (μm)

Figure 2.2: Loss of either *Arrb1* or *Arrb2* has no effect on the Gnrh immunoreactive neuronal population size and distribution in the hypothalamus.

(A and E): Photomicrographs of coronal brain sections at the level of the OVLT from 8 week old *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice and their WT littermates. In photomicrographs, GnRH neurons were revealed in the brain slices by IHC using the EL14 antibody and the OVLT was used as a neuroanatomical reference point for rostral to caudal alignment of coronal sections. (B, D, F and H): Histograms displaying the rostral-caudal distribution of GnRH immunoreactive neurons from WT and KO mice aligned at the OVLT. Plot represents average GnRH immunoreactive neurons per 50 µm coronal section. GnRH immunoreactive neurons were identified with the EL14 antibody (B: *Arrb1*$^{-/-}$ mice; F: *Arrb2*$^{-/-}$ mice), PA1-122 antibody (D: *Arrb1*$^{-/-}$ mice) and QED antibody (H: *Arrb2*$^{-/-}$ mice). Total number of GnRH immunoreactive neurons identified with the EL14 and PA1-122 antibody (C: *Arrb1*$^{-/-}$ mice) and El14 and QED antibody (G: *Arrb2*$^{-/-}$ mice). Error bars represent SEM.

2.3.1.3 Loss of either β-arrestin-1 or -2 has no effect on Buserelin-triggered LH secretion.

Next, we determined whether the loss of β-arrestin in the pituitary gonadotropes affected LH secretion due to developmental or other defects along the hypothalamic-pituitary axis. To do this, eight week-old male *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice and their WT littermates were administered Buserelin (GnRH agonist) i.p. and serum LH was measured 20 min later. There was no difference in Buserelin-triggered LH secretion in the single KO mice relative to their WT littermates [P > 0.2 for *Arrb1*$^{-/-}$ (3.8 ± 0.2 ng/ml LH; n = 11) vs. WT (3.3 ± 0.4 ng/ml LH; n = 13); P > 0.2 for *Arrb2*$^{-/-}$ (2.7 ± 10.3 ng/ml LH; n = 15) vs. WT (2.7 ± 0.1 ng/ml LH; n = 9)] (Fig. 2.3A and B). Thus, the loss of either β-arrestin isoform in pituitary gonadotropes did not affect Buserelin-stimulated LH secretion and these single KO mice could be used to test whether KISS1R stimulates GnRH secretion via the β-arrestin pathway.

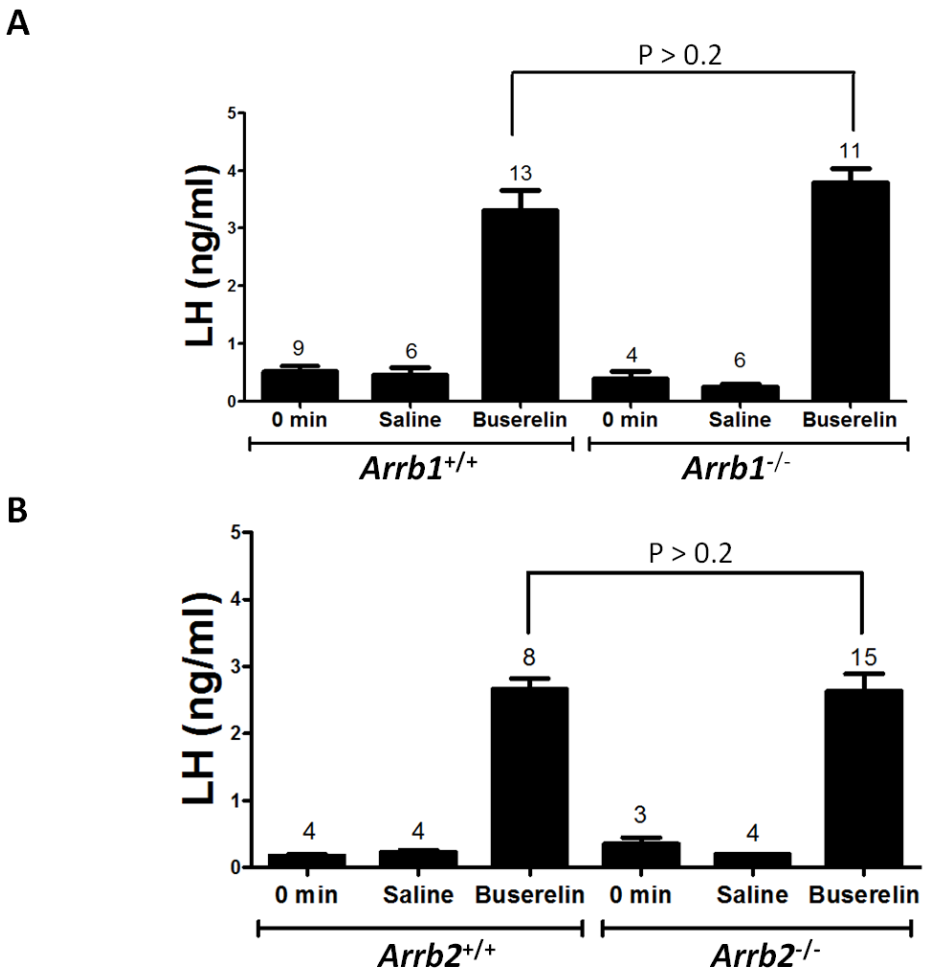

Figure 2.3: Loss of either *Arrb1* or *Arrb2* does not affect Buserelin-triggered LH secretion

Serum LH levels were measured by RIA. Mice were either left untreated or treated with saline or 3 ng Buserelin/g body weight. Twenty min later blood was collected and serum prepared. Buserelin-triggered LH levels in (A) *Arrb1*$^{-/-}$ and (B) *Arrb2*$^{-/-}$ mice were significantly higher ($P < 0.001$) than levels from their respective untreated and saline treated controls. Buserelin-triggered LH levels were not different ($P > 0.2$) between *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice and their WT littermate controls, respectively. Numbers above each bar represent n value for each treatment. Only male mice were used to preclude the effects of estrous cycle variation on GnRH secretion in females. Error bars represent SEM.

2.3.1.4 Loss of either β-arrestin-1 or -2 significantly reduces kisspeptin-triggered LH secretion.

To determine what effect a loss of either β-arrestin-1 or -2 has on LH secretion, eight week-old male *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice and their WT littermates were administered KP-54 i.p. and serum LH was measured 1h later. The 1h time point was chosen based on previous publications (Seminara et al. 2003, Lapatto et al. 2007, Kirilov et al. 2013) as well as our own time course assay that demonstrated robust serum LH elevation after KP-54 administration (Appendix 1 Fig. 1). There was significant reduction in Kp-triggered LH secretion in each of the single KO mice relative to their WT littermates [$P < 0.001$ for *Arrb1*$^{-/-}$ (12.1 ± 0.4 ng/ml LH; n = 14) vs. WT (15.4 ± 0.8 ng/ml LH; n = 10); $P < 0.001$ for *Arrb2*$^{-/-}$ (13.2 ± 0.8 ng/ml LH; n = 14) vs. WT (18.4 ± 1.1 ng/ml LH; n = 12)] (Fig. 2.4A and B). Thus, the loss of either β-arrestin isoform diminished Kp-stimulated LH secretion and β-arrestin-1 and -2 regulate LH secretion in young adult male mice.

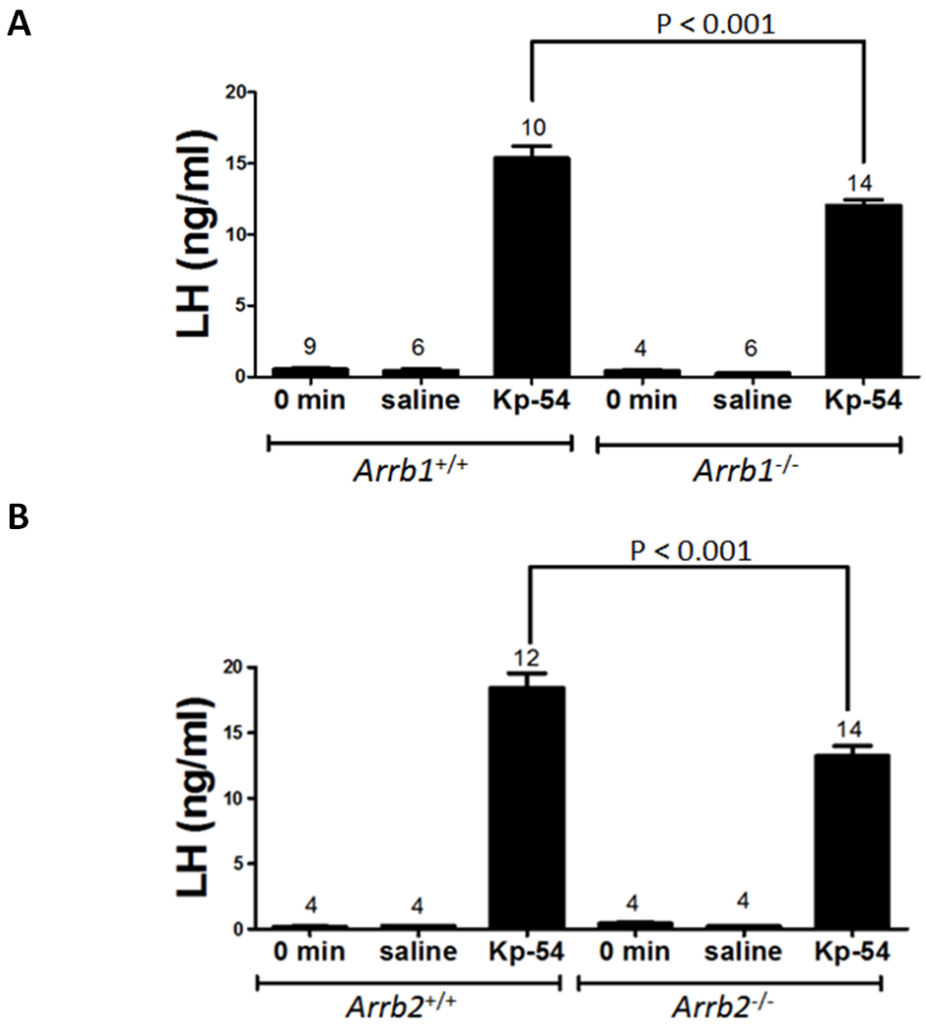

Figure 2.4: Loss of either *Arrb1* or *Arrb2* significantly diminishes KP-54 triggered LH secretion.

Serum LH levels were measured by RIA. Mice were either left untreated or treated with saline or 100 nmol KP-54/kg body weight. 1h later, blood was collected and serum prepared. KP-54-triggered LH levels in *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice were significantly higher ($P < 0.001$) than levels from their respective untreated and saline treated controls. Kp-54-triggered LH levels were significantly lower ($P < 0.001$) in *Arrb1*$^{-/-}$ and *Arrb2*$^{-/-}$ mice compared to their respective WT littermate controls. Only male mice were used to preclude the effects of estrous cycle variation on GnRH secretion in females. Error bars represent SEM.

2.3.2 L148S displays $G\alpha_{q/11}$-independent signaling

WT KISS1R displays $G\alpha_{q/11}$-independent signaling via β-arrestin (Pampillo et al. 2009 and Szereszewski et al. 2010). L148S is a naturally occurring $G\alpha_{q/11}$-uncoupled KISS1R mutant associated with infertility and IHH in patients (Seminara et al. 2003 and Wacker et al. 2008). We also determined that like WT KISS1R, L148S also physically associates with β-arrestin-1 and -2 (Szereszewski et al. 2010). Additionally, it was reported that L148S is expressed at WT levels, localizes to the cell surface, binds Kp and continues to interact with many signaling and regulatory molecules identical to WT KISS1R (Wacker et al. 2008). These observations lead to the hypothesis that while L148S is uncoupled from $InsP_3$ formation it retains the capacity to undergo kisspeptin-triggered $G\alpha_{q/11}$-independent signaling via β-arrestin. We further propose that these KISS1R $G\alpha_{q/11}$-independent pathways may regulate functionally distinct intracellular responses from the $G\alpha_{q/11}$-dependent cascade.

2.3.2.1 L148S triggers significant $G\alpha_{q/11}$-independent ERK1/2 phosphorylation

Relative to kisspeptin-treated WT KISS1R-expressing cells, kisspeptin-treated L148S-expressing cells triggered about 2.5-fold less ERK1/2 phosphorylation ($P < 0.05$) (Fig. 2.5A, B and D). However, following 0.1 and 3.0 μM Kp-10 stimulation, L148S-triggered ERK1/2 phosphorylation was approximately 2-fold greater ($P < 0.05$) than unstimulated L148S-expressing cells (Fig. 2.5B and D) and both unstimulated and stimulated non-transfected cells (Fig. 2.5B, C and D). All these studies were conducted with the untagged WT and L148S receptor.

Since we routinely use tagged receptors to study KISS1R signaling, we determined how tagged-WT and -L148S receptors compared to the untagged receptors with respect to ERK activation. Tagged (YFP, FLAG and myc) WT and L148S receptors triggered similar levels ($P > 0.05$) of ERK activation compared to the untagged WT and L148S receptors, respectively, following 0.1 and 3.0 μM KP-10 stimulation (Fig. 2.5 vs. Figs. 2.6 and 2.7). These results indicate that the presence of tags or epitopes (YFP, FLAG and myc) did not alter the signaling potential of WT and mutant KISS1R. kisspeptin-treated

L148S-expressing cells triggered significant ($P < 0.05$) ERK1/2 activation compared to both unstimulated L148S-expressing cells and stimulated non-transfected cells (Figs. 2.5, 2.6 and 2.7). These findings were verified independently in both the Babwah and Kaiser laboratories and expression constructs were routinely sequenced to verify their nucleotide composition.

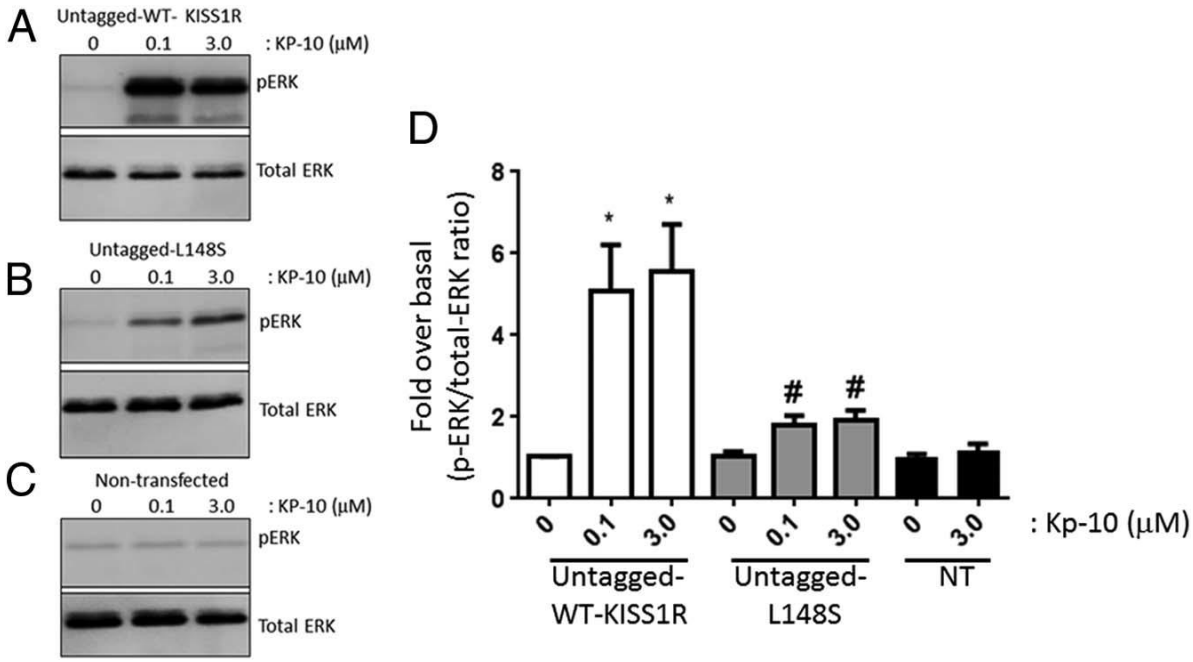

Figure 2.5: L148S triggers ERK1/2 phosphorylation.

Representative autoradiographs from the Babwah laboratory (A-C) and densitometric analysis from the Kaiser laboratory (D) showing the expression of total and phosphorylated ERK1/2 in untagged-L148S, non-transfected (NT) and WT untagged-KISS1R overexpressing HEK 293 cells following 10-min KP-10 treatment (0, 0.1 and 3.0 µM). Western blot analyses were done using monoclonal anti-ERK1/2 and anti-phospho ERK1/2 antibodies. The data represent the mean of 3 independent experiments. *: $P < 0.05$, pERK/total ERK ratio compared with 0 µM in WT group; #: $P < 0.05$, pERK/total ERK ratio compared with 0 µM in L148S group. Error bars represent SEM.

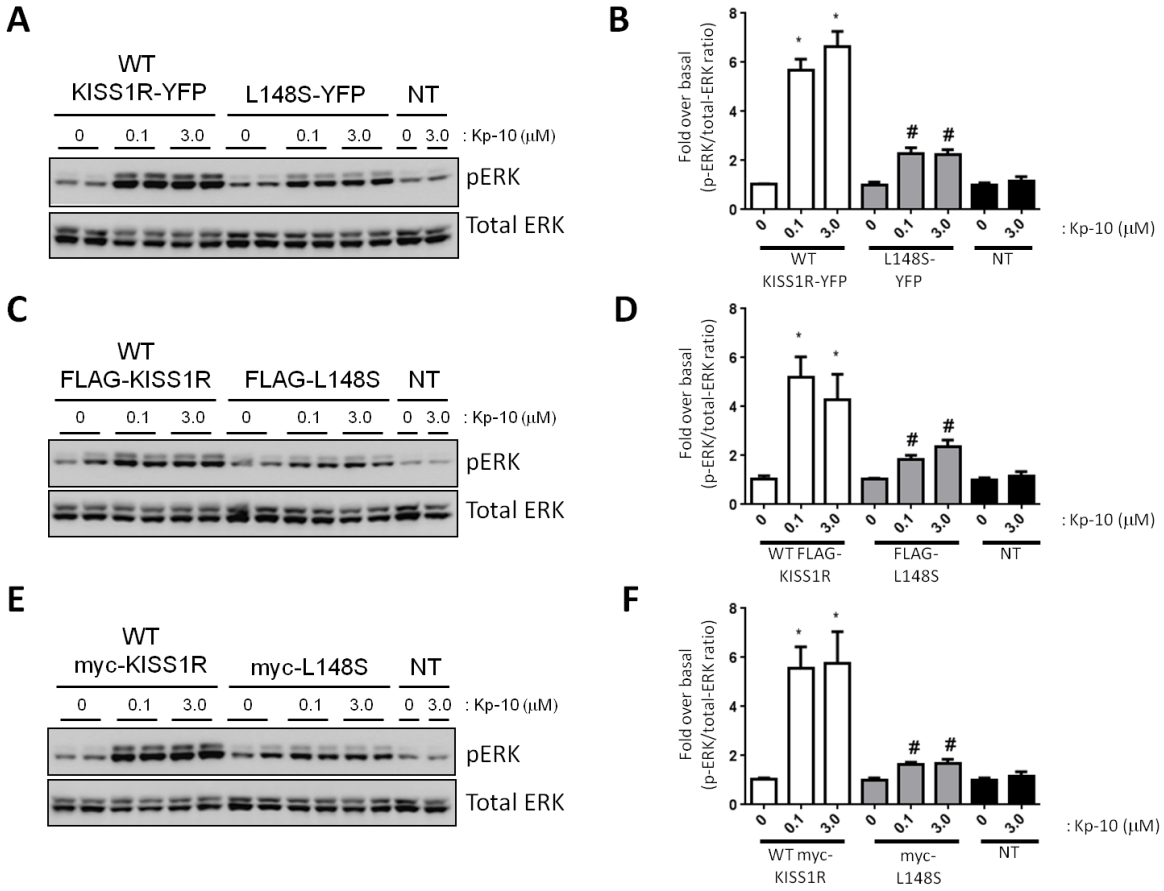

Figure 2.6: L148S triggers ERK1/2 phosphorylation regardless of epitope tag

Representative autoradiographs (A, C and E) and densitometric analysis (B,D and F) from the Kaiser laboratory showing the expression of total and phosphorylated ERK1/2 in WT KISS1R (YFP-, FLAG- and myc-tagged), L148S (YFP-, FLAG- and myc-tagged) and non-transfected (NT) overexpressing HEK 293 cells following 10-min KP-10 treatment (0, 0.1 and 3.0 μM). Western blot analyses were done using monoclonal anti-ERK1/2 and anti-phospho ERK1/2 antibodies. The data represent the mean of 3 independent experiments. *: $P < 0.05$, pERK/total ERK ratio compared with 0 μM in WT group; #: $P < 0.05$, pERK/total ERK ratio compared with 0 μM in L148S group. Error bars represent SEM.

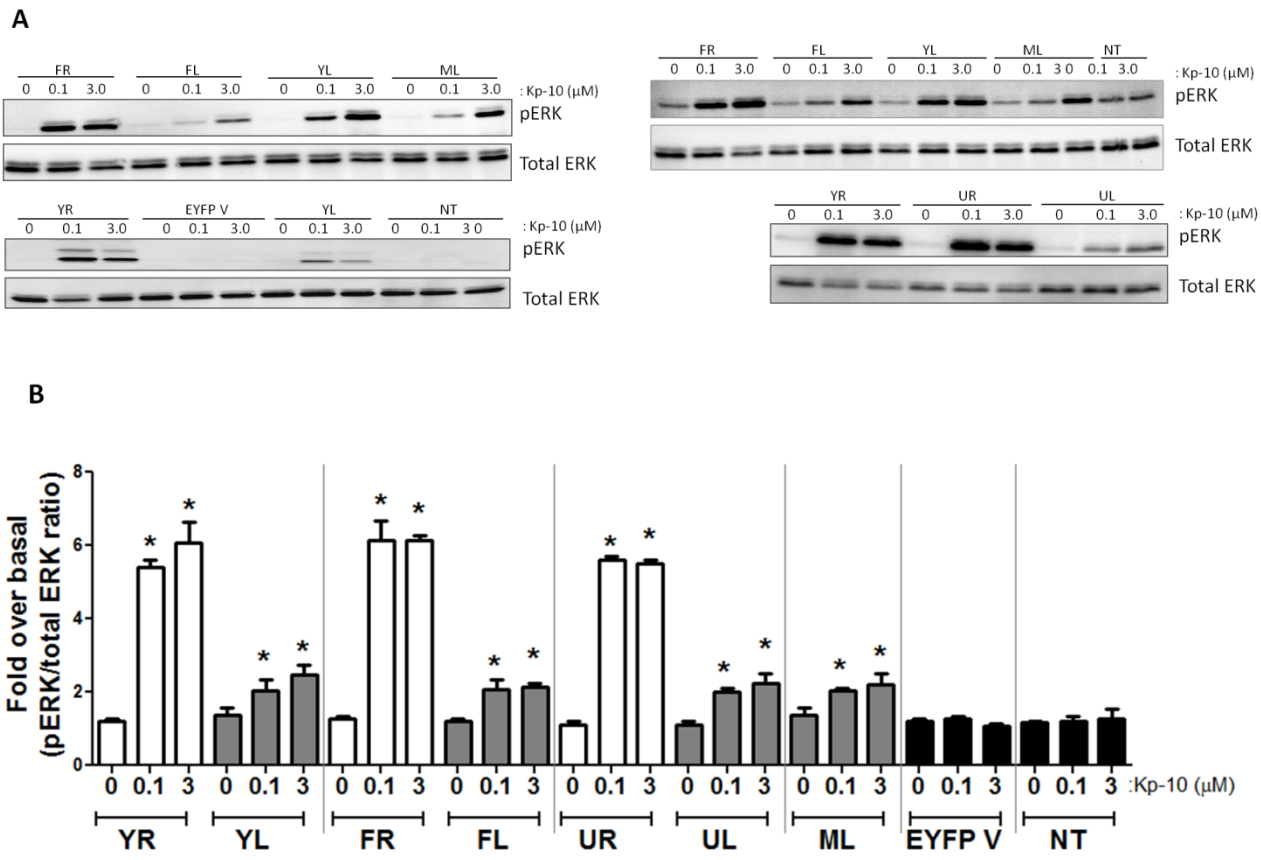

Figure 2.7: L148S triggers ERK1/2 phosphorylation

Representative autoradiographs (A) and densitometric analysis (B) from the Babwah laboratory confirming results in Figs. 2.6 and 2.7 done by the Kaiser Laboratory. YR: KISS1R-YFP, YL: YFP-L148S, FR: FLAG-KISS1R, FL: FLAG-L148S, UR: untagged KISS1R, UL: untagged L148S, ML: myc-L148S, EYFP V: vector control for YFP constructs, NT: non-transfected HEK 293 cells. Cells were treated with KP-10 (0, 0.1 and 3.0μM) for 10 min. Western blot analyses were done using monoclonal anti-ERK1/2 and anti-phospho ERK1/2 antibodies. The data represent the mean of at least 3 independent experiments. *P < 0.05, pERK/total ERK ratio compared with 0 μM of the same transfection in that group; #P < 0.05, pERK/total ERK ratio compared with 0 μM in L148S group. Error bars represent SEM.

2.3.2.2 L148S triggers significant ERK1/2 phosphorylation via the β-arrestin pathway.

Since the $G\alpha_{q/11}$-uncoupled mutant, L148S physically associates with β-arrestin-1 and -2 (Pampillo et al. 2009) we hypothesized that L148S-dependent ERK1/2 phosphorylation (Figs. 2.5, 2.6 and 2.7) is β-arrestin-dependent. This was tested by assessing L148S-dependent ERK1/2 phosphorylation following KP-10 treatment in the presence and absence of β-arrestin-1 and -2. As seen, when L148S was expressed in WT MEFs, following KP-10 treatment, it triggered significant ERK1/2 phosphorylation ($P < 0.05$) (Fig. 2.8). However, when L148S was expressed in MEFs lacking both β-arrestin-1 and -2, the ability to trigger Kp-dependent ERK1/2 phosphorylation was almost completely abolished (Fig. 2.8), thus supporting our hypothesis that L148S-triggered ERK1/2 phosphorylation is β-arrestin-dependent.

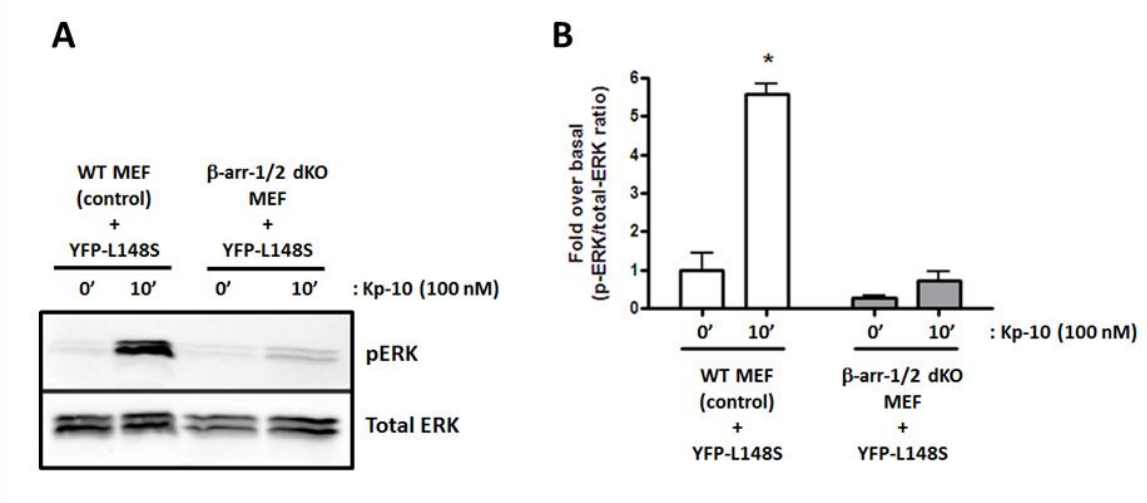

Figure 2.8: L148S triggers ERK1/2 in a β-arrestin-dependent manner.

Representative autoradiograph (A) and densitometric analysis (B) showing the expression of total and phosphorylated ERK1/2 in YFP-L148S overexpressing MEFs. β-arrestin-1/2 dKO MEFs and corresponding WT control MEFs were treated for 10 min with KP-10 at 100 nM. Western blot analysis was done using monoclonal anti-ERK1/2 and anti-phospho ERK1/2 antibodies. The data represent the mean of three independent experiments. *P<0.05, pERK/totak ERK ratio compared with 0μM in WT MEFs. Error bars represent SEM.

2.3.2.3 L148S is strongly uncoupled from $G\alpha_{q/11}$ signaling at Kp-10 concentrations that trigger robust signaling via WT KISS1R.

L148S is impaired in G-protein activation by inhibiting the dissociation of Gα and Gβγ-subunits and, as a result, is uncoupled from $InsP_3$ formation (Seminara et al. 2003 and Wacker et al. 2008). In the present study we also show that, while WT KISS1R triggered $InsP_3$ formation over a wide range of KP-10 concentrations (1.0×10^{-9} to 3×10^{-6} M of KP-10) with an EC_{50} of 1.5×10^{-9} M, L148S was significantly diminished ($P < 0.05$) in its capacity to do so, displaying an EC_{50} of 1.7×10^{-8} M that was not significantly different ($P > 0.05$) from the $InsP_3$-generating capacity of non-transfected cells (Fig. 2.9). Although these findings confirm previous reports that L148S is strongly uncoupled from $G\alpha_{q/11}$ signaling, (Seminara et al. 2003 and Wacker et al. 2008) it was important to confirm these findings in parallel with the evidence that ERK1/2 phosphorylation was still occurring (Figs. 2.5, 2.6 and 2.7). Thus, the small yet significant L148S-dependent ERK1/2 phosphorylation that we observed was unlikely due to differences such as in expression constructs, experimental techniques and cell lines that may exist between the current and previous studies.

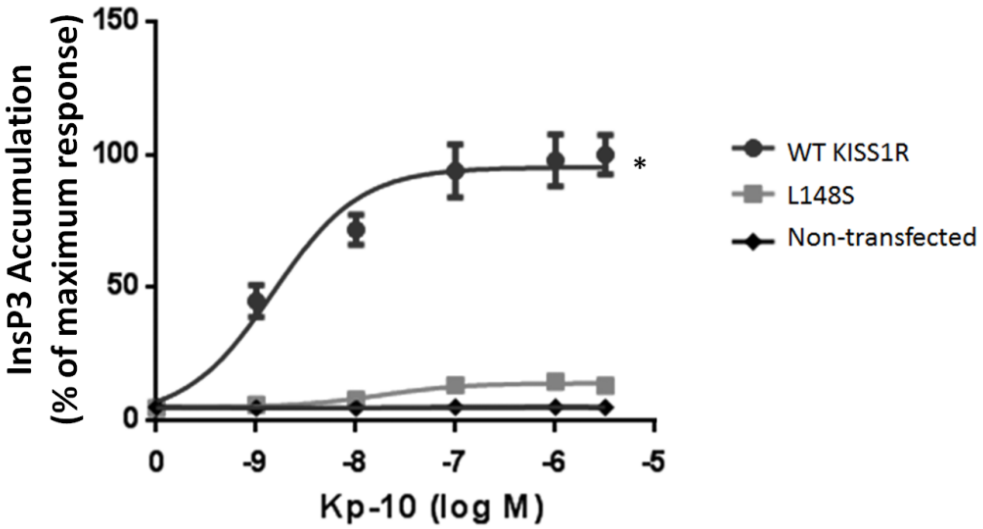

Figure 2.9: L148S is strongly uncoupled from $G\alpha_{q/11}$ signaling

COS7 cells were transiently transfected with WT KISS1R or L148S. Kp-10 stimulated $InsP_3$ accumulation was measured by conversion of pre-labeled [2^3H] myo-inositol to [^{3}H]InsP. Data are percentage of maximum response. *P < 0.01. The result is pooled data from three independent experiments. All assays were performed in triplicate. Error bars represent SEM.

2.3.2.4 WT KISS1R and L148S differentially regulate PIP_2 hydrolysis.

While L148S-expressing cells were not significantly different (P > 0.05) from non-transfected cells in their $InsP_3$-generating capacity, to further strengthen the finding that L148S is functionally uncoupled from the $G\alpha_{q/11}$ signaling pathway, we monitored the spatial distribution of GFP-PH-PLC-δ1following kisspeptin treatment in cells co-expressing either WT KISS1R or L148S. The spatial localization of GFP-PH-PLC-δ1in the cell reflects the activation state of $G\alpha_{q/11}$. In the unstimulated cell, GFP-PH-PLC-δ1 is localized to the plasma membrane but upon stimulation, GFP-PH-PLC-δ1 is released

from the plasma membrane as a consequence of both PIP_2 hydrolysis and the high affinity of the PLC-δ1 PH domain for $InsP_3$ (Hirose et al. 1999). In cells expressing GFP-PH-PLC-δ1, but lacking KISS1R, GFP-PH-PLC-δ1 was localized at the plasma membrane, even after Kp-10 treatment, due to the interaction of the PLC-δ1 PH domain with membrane PIP_2 (Fig. 2.10A-C). However, in cells co-expressing GFP-PH-PLC- δ1 and FLAG-KISS1R (WT), in response to KP-10 treatment, GFP-PH-PLC- δ1 was released from the plasma membrane and accumulated in the cytosol of the cell (Fig. 2.10D-G).

Our previously published study (Babwah et al. 2012) demonstrated that 100 nM KP-10 was sufficient to trigger a robust redistribution of GFP-PH-PLC-δ1 in cells co-expressing WT KISS1R. However, the present study was conducted with 3 μM KP-10 to determine whether a high concentration of KP-10, which triggers a robust L148S-distal signaling response (that is, ERK activation) (Figs. 2.5, 2.6 and 2.7), would stimulate a $G\alpha_{q/11}$-coupled L148S-proximal signaling event (that is, PIP_2 hydrolysis). If it did not, we would conclude that L148S is strongly uncoupled from the $G\alpha_{q/11}$ signaling pathway and that L148S-dependent ERK activation is primarily the consequence of $G\alpha_{q/11}$-independent signaling. As seen, when either FLAG-L148S (Fig. 2.10H-J) or myc-L148S (Fig. 2.10K-M) were co-expressed with GFP-PH-PLC-δ1, 3 μM KP-10 completely failed to stimulate the cellular redistribution of GFP-PH-PLC-δ1 (Fig. 2.10H-M) and was indistinguishable from Kp-treated cells expressing GFP-PH-PLC-δ1 alone (Fig. 2.10A-C). Cells co-expressing GFP-PH-PLC- δ1 with either FLAG-L148S (Fig. 2.10H-J) or myc-L148S (Fig. 2.10K-M) were imaged for up to about 17 minutes following KP-10 treatment. Even if there was weak L148S-dependent $InsP_3$ formation over this period, transient cellular accumulation of $InsP_3$ was still insufficient to trigger detectable levels of PIP2 hydrolysis as determined by a cellular redistribution of GFP-PH-PLC-δ1. Based on the GFP-PH-PLC-δ1cellular redistribution data, it appears that at up to 3 μM KP-10, L148S is strongly uncoupled from the $G\alpha_{q/11}$ signaling pathway.

Figure 2.10:

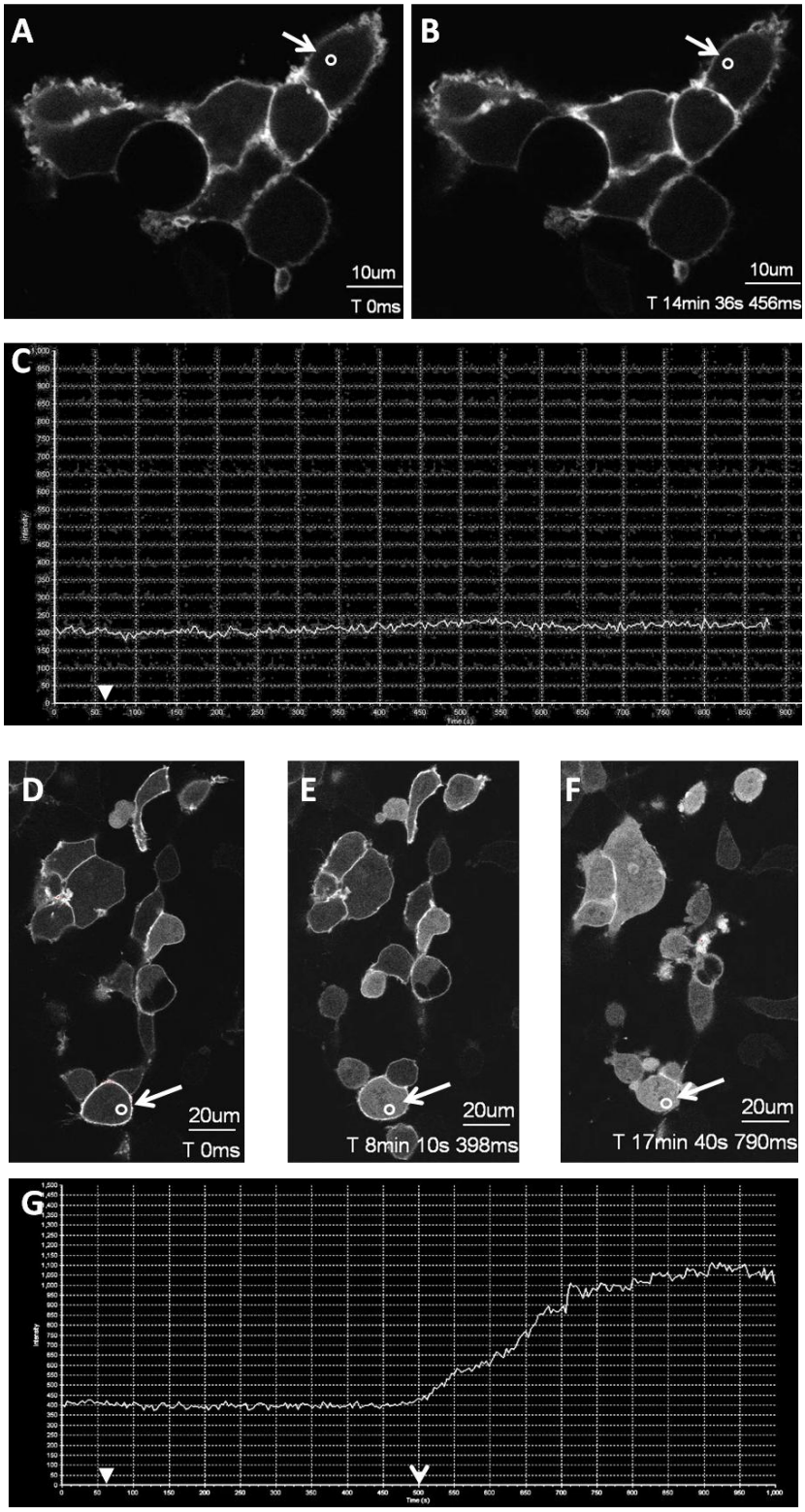
A
10um
T 0ms
B
10um
T 14min 36s 456ms
C
D
20um
T 0ms
E
20um
T 8min 10s 398ms
F
20um
T 17min 40s 790ms
G

Figure 2.10 continued:

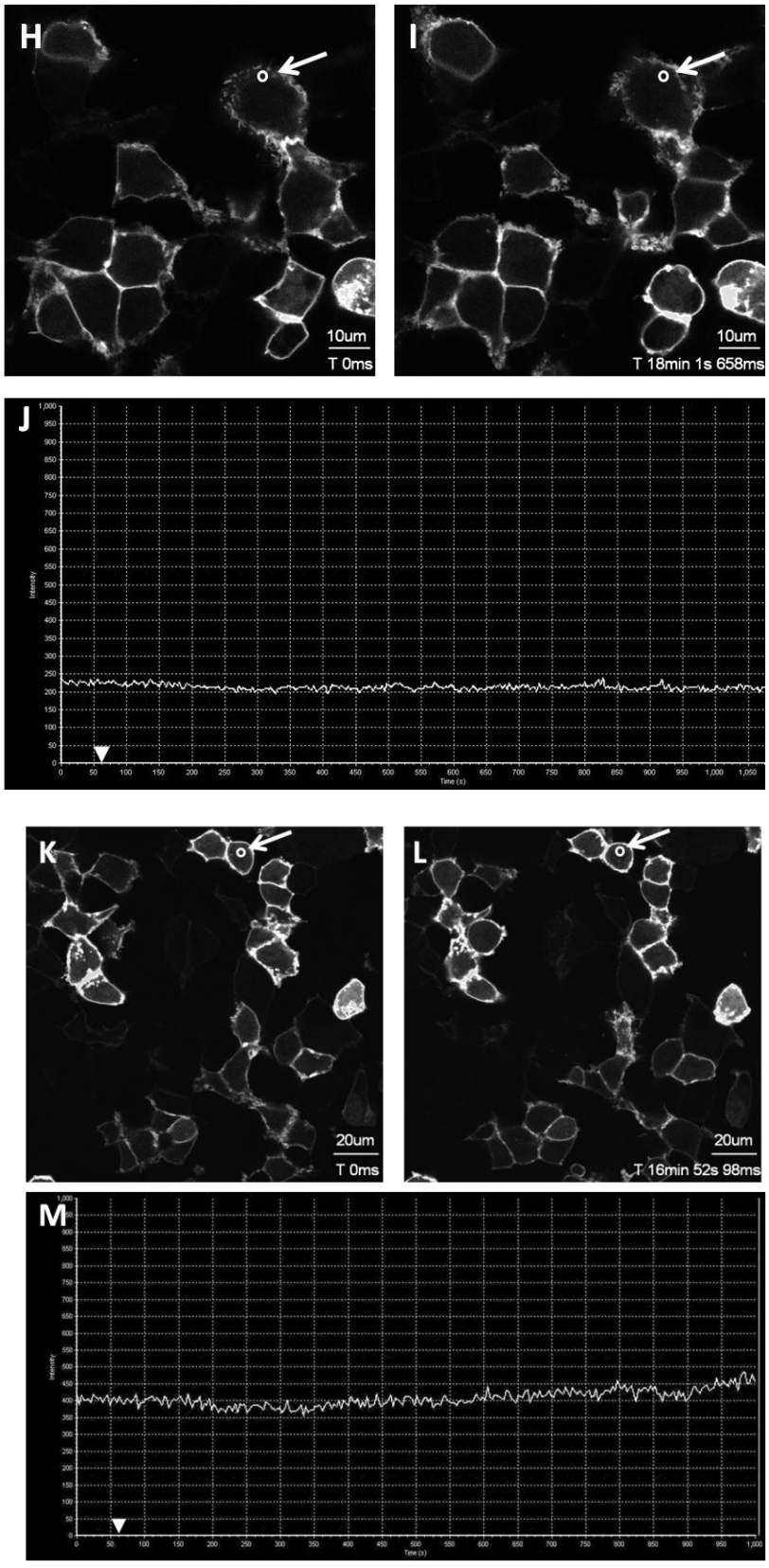

Figure 2.10: L148S is strongly uncoupled from $G\alpha_{q/11}$ signaling.

(A-M) representative images selected from a time series of laser scanning confocal microscopic images showing the plasma membrane and cytosolic localization of GFP-PH-PLC-δ1 in non-transfected HEK 293 cells **(A-C)** and in HEK 293 cells expressing WT KISS1R or L148S **(D-G**: FLAG-WT-KISS1R-expressing cells; **H-J**: FLAG-L148S-expressing cells; **(K-M):** myc-L148S-expressing cells) in the absence of agonist (0 s) and in response to 3 μM KP-10 treatment (added gently and in a drop-wise manner to cells cultured on a confocal dish) approximately one min later (see solid white arrowheads on X-axis of graphs C, G, J and M). Cytosolic regions of interest in cells (shown by small white circle in panels A, B, D-F, H, I, K, L) were analyzed quantitatively by determining the changes in GFP fluorescence over time. These changes are presented graphically (C, G, J and M). These studies were conducted five independent times and representative images from one experiment are shown. **(A-C):** Approximately 14 min after KP-10-treatment, translocation of GFP-PH-PLC-δ1 from the plasma membrane to the cytosol was visually undetectable. This confirms that translocation is dependent on KISS1/KISS1R signaling. **(D-G):** At approximately 440 sec (7.5 min) after adding KP-10 (open arrow-head) GFP-PH-PLC-δ1 began translocating from the plasma membrane to the cytosol indicating that the KISS1R-coupled $G\alpha_{q/11}$ pathway was activated. **(H-J):** Translocation of GFP-PH-PLC-δ1 from the plasma membrane to the cytosol was visually undetectable following 15 min of KP-10-treatment of cells expressing FLAG-L148S. **(K-M):** Translocation of GFP-PH-PLC-δ1 from the plasma membrane to the cytosol was visually undetectable following 16 min of KP-10-treatment of cells expressing myc-L148S.

2.3.2.5 WT KISS1R and L148S differentially regulate STAT3 phosphorylation.

In agreement with our previous reports (Pampillo et al. 2009 and Szereszewski et al. 2010), we demonstrated here that L148S can signal to ERK1/2 through $G\alpha_{q/11}$ – independent mechanisms, likely involving β-arrestins (Figs 2.5 – 2.8). Next we wanted to further analyze differences between the $G\alpha_{q/11}$ –dependent and independent pathways in terms of regulation of intracellular signaling molecules other than ERK1/2. Using a Proteome Profiler system we were able to assess kisspeptin-triggered site-specific phosphorylation of 45 human kinases and compare differences in phosphorylation between untagged WT KISS1R and L148S (Fig. 2.11A). Consistent with previous results in Figs. 2.5-2.7, after 0.1 μM KP-10 stimulation, KISS1R expressing cells had a significant increase in ERK1/2 phosphorylation relative to unstimulated KISS1R expressing cells (Fig. 2.11B) ($P<0.05$). L148S also triggered significant Kp-stimulated ERK1/2 phosphorylation relative to untreated cells (Fig. 2.11B) but this response was diminished compared to KISS1R confirming results seen above in Figs. 2.5, 2.6 and 2.7 ($P<0.05$). While WT KISS1R was able to stimulate significant STAT3 (S727) phosphorylation ($P<0.05$) following 0.1 μM KP-10 stimulation, L148S could not (Fig. 2.11C). This suggests that while ERK1/2 lies downstream of both $G\alpha_{q/11}$ dependent and independent KISS1R signaling, STAT3 may only be part of the $G\alpha_{q/11}$ pathway. Western blot analysis was done to verify the phospho-kinase array results (Fig. 2.12).

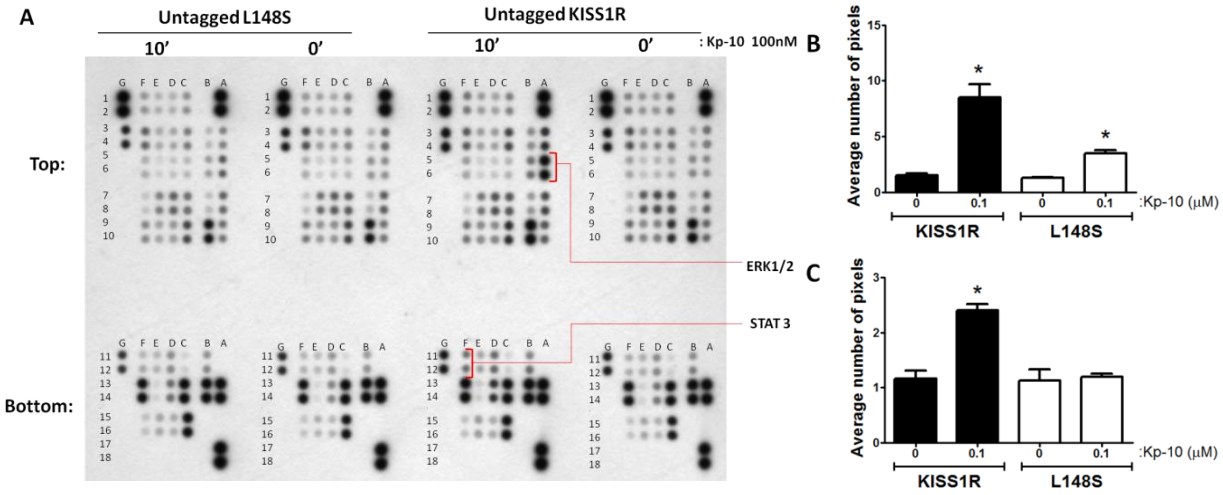

Figure 2.11: Human Phospho-Kinase Array identified signaling molecules that are solely part of the G$\alpha_{q/11}$-dependent pathway.

(A) Representative autoradiograph of the Human Phospho-Kinase Array. (B and C). Densitometric analysis of mean pixel intensity for spots detecting phospho-ERK1/2 and phospho-STAT3. (B) Following Kp-10 treatment, KISS1R expressing HEK 293 cells showed an approximate 8-fold significant increase in ERK1/2 phosphorylation (*P < 0.05) while cells expressing L148S demonstrated a diminished yet significant 2-fold approximate increase in ERK1/2 phosphorylation (*P < 0.05). (C) Following Kp-10 treatment, KISS1R expressing HEK 293 cells showed an approximate 1-fold significant increase in STAT3 phosphorylation (*P < 0.05) while cells expressing L148S demonstrated no significant difference in STAT3 phosphorylation. Densitometric analysis represents the mean of 3 independent experiments where mean pixel intensity is normalized to reference controls on array. Error bars represent SEM. Other molecules identified include p38, CREB and RSK1/2/3 (See Appendix 2).

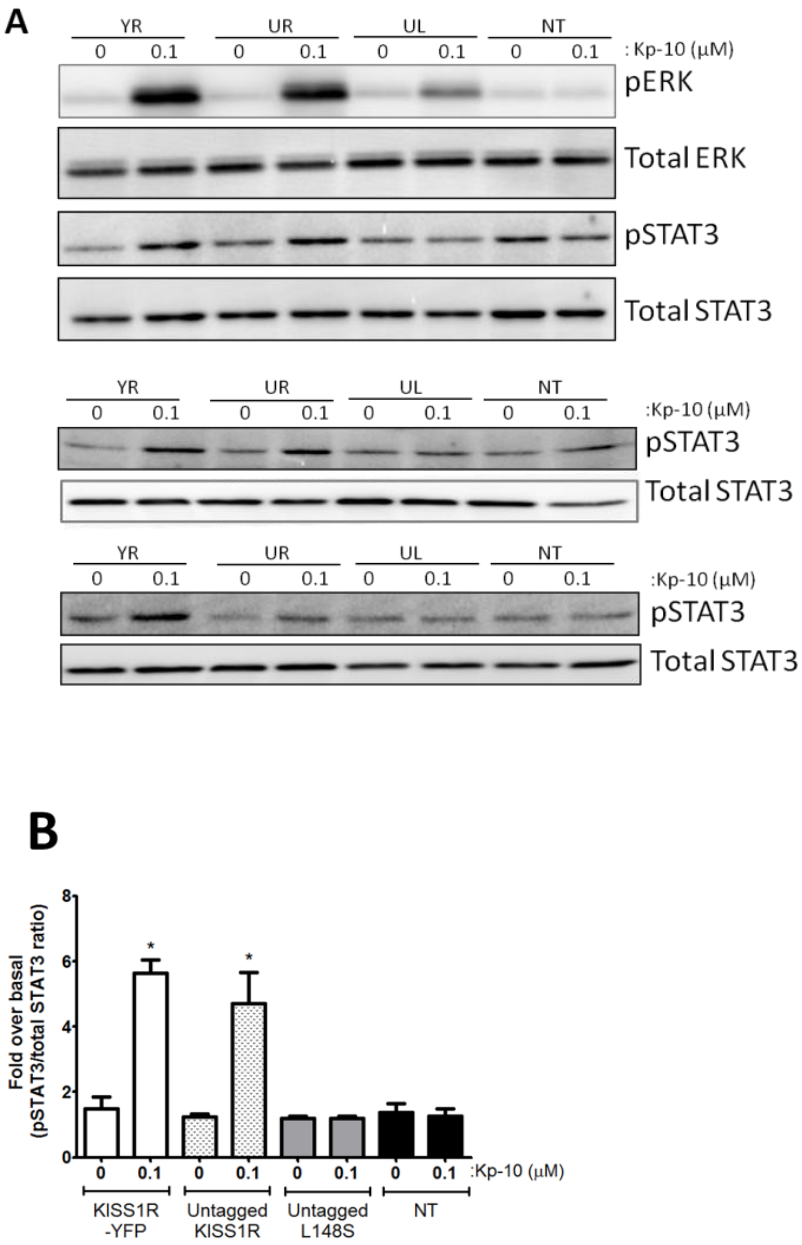

Figure 2.12: L148S cannot trigger STAT3 phosphorylation.

Representative autoradiographs (A) and densitometric analysis (B) showing expression of total and phosphorylated ERK1/2 and STAT3 in YR: KISS1R-YFP, YL: L148S-YFP, UR: untagged KISS1R, UL: untagged L148S, NT: non-transfected HEK 293 cells. Cells were treated with Kp-10 (0 and 0.1 μM) for 10 minutes. While WT KISS1R (YR and UR) are able to stimulate STAT3 phosphorylation, L148S (UL) is not *P<0.05.

2.3.3 β-arrestin-1 and -2 regulate KISS1R homologous desensitization.

Since we now have established that KISS1R signals through $G\alpha_{q/11}$-independent mechanisms, one of which is β-arrestin, we wanted to investigate if β-arrestin also was involved in the attenuation of KISS1R signaling. Traditionally, β-arrestins mediate GPCR signal termination by homologous desensitization (Conner et al. 1997, Bohn et al. 1999, Kohout et al. 2001, Ferguson 2001, Luttrell et al. 2010). We have shown that GRK-2 was involved in KISS1R desensitization and indirectly suggested that β-arrestins may also be involved (Pampillo et al. 2009 and Szereszewski et al. 2010). Here we investigate more directly the role of β-arrestin in KISS1R homologous desensitization. We used three cell models and three experimental approaches to validate our findings in order to show that the results were conserved and not cell or technique-specific. Since inositol phosphate ($InsP_3$) formation is an early and direct outcome in KISS1/KISS1R signaling through the $G\alpha_{q/11}$ pathway, we used it here as an inverse indicator for the termination of this cascade by homologous desensitization. By measuring $InsP_3$ formation over a range of kisspeptin concentrations for a fixed time period as described previously (Pampillo et al. 2009) we were able to investigate the effect of β-arrestin expression on KISS1R desensitization (Fig. 2.13). Using an immortalized mouse GnRH neuronal cell line, GT1-7, we used shRNA to create lines which stably downregulated β-arrestin-1 and -2 and verified the decreased expression by western blot (Fig. 2.13A and B). If β-arrestin regulated KISS1R homologous desensitization we would expect that the shRNA knock-down lines would undergo less signal termination demonstrated by greater levels of $InsP_3$ accumulation. This is exactly what we see in the GT1-7 (Fig. 2.13A) where both β-arrestin-1 and -2 KD lines show more $InsP_3$ accumulation at all Kp-10 concentrations compared to the line expressing KISS1R. At $3x10^{-5}$ M KP-10, β-arrestin-2 KD produced 3-fold more $InsP_3$ than the receptor only line ($P<0.05$). This result is further recapitulated in the MEF cell lines which were derived from genetically modified mice that lacked β-arrestin-1, -2 or both isoforms (Fig. 2.13D) (Kohout et al 2001). At $3x10^{-5}$ M KP-10, all of these MEF KO lines had significantly more $InsP_3$ accumulation than their control lines. While the single β-arrestin knockout MEFs had 3-4 fold more $InsP_3$ compared to parental lines, the double β-arrestin-1-2 knockout MEFs had 20-fold more $InsP_3$

accumulation ($P<0.05$) further demonstrating the importance of both β-arrestins for KISS1R desensitization. Finally we used a reciprocal approach in the primate cell line, COS-7. Due to the relatively low levels of endogenous β-arrestins in COS-7 cells (Menard et al. 1997) we were able to use this line to over-express β-arrestin and as we expected the converse held true. Overexpression of β-arrestin triggered an increase in KISS1R desensitization as demonstrated by the downward and rightward shift in the dose response curves (Fig. 2.13C). Both the β-arrestin-1 and the β-arrestin-2 overexpressed cells show significantly less $InsP_3$ accumulation (3 and 2-fold respectively) than the receptor only-expressing line at $3x10^{-5}$ M KP-10 ($P < 0.05$). Taken together, these results prove that the β-arrestin regulate KISS1R homologous desensitization.

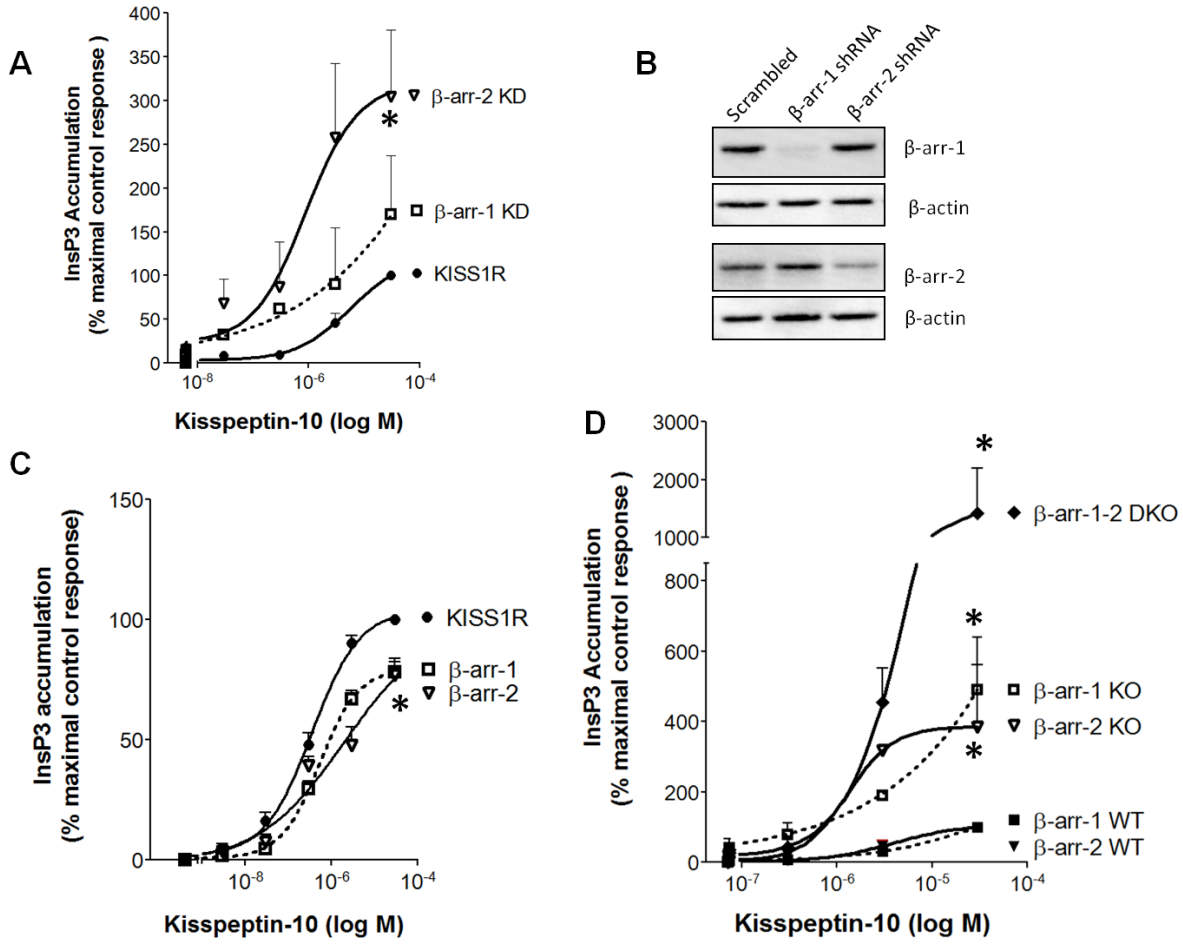

Figure 2.13: β-arrestin-1 and -2-dependent homologous desensitization of KISS1R is conserved in various cell types.

(A). GT1-7 cells stably transfected with 10 µg FLAG-KISS1R only or with shRNA against either β-arrestin-1 (β-arr-1 KD) or β-arrestin -2 (β-arr-2 KD). Over a range of kisspeptin concentrations, the lines with reduced expression of either β-arrestin-1 or -2 produced a greater quantity of $InsP_3$ at each KP-10 dose. Data points represent the mean for three independent experiments. $InsP_3$ accumulation in the β-arrestin-2 KD line is significantly more than the receptor only line at $3x10^{-5}$ M KP-10. *P < 0.005. (B). Representative western blot showing 86% and 72% diminished protein expression of β-arrestin-1 and -2 respectively, in the GT1-7 shRNA stables relative to scrambled shRNA control. A similar level of down-regulation was detected at the level of mRNA expression

(data not shown). (C). COS-7 cells transiently transfected with 10 µg FLAG-KISS1R alone or with 5 µg EYFP-β-arrestin-1 or -2. COS-7 cells with increased expression of either β-arrestin-1 or -2 produced a lower quantity of $InsP_3$ over a range of kisspeptin concentrations. At $3x10^{-5}$ M KP-10, both the β-arrestin-1 and the β-arrestin-2 overexpressed cells show significantly less $InsP_3$ than the KISS1R only expressing cells *P<0.05. (D). MEFs genetically lacking β-arrestin-1 or -2 or both isoforms were transiently transfected with 10 µg KISS1R-EYFP. All MEF cell lines expressing diminished or no β-arrestin produced more $InsP_3$ and at $3x10^{-5}$ M was significantly greater compared to the WT parental lines. *P<0.05. Data points for all curves represent the mean for three independent experiments. Error bars represent SEM.

2.3.4 KISS1R signals via $G\alpha_{q/11}$ -independent mechanisms to regulate fertility

At this point we just demonstrated that both KISS1R-triggered LH secretion and L148S-triggered ERK1/2 phosphorylation can occur in a β-arrestin-dependent manner. These results therefore suggest an important role for β-arrestin in regulating GnRH secretion and fertility and we would expect mice lacking β-arrestin to display infertility or sub-fertility. While we were able to detect diminished kisspeptin-stimulated LH release, overall fertility as described by brood size, was unaffected in the single β-arrestin knockout mice due to functional isoform compensation between the β-arrestin proteins. Since β-arrestin double knockout mice are perinatal lethal this precluded further investigation of the physiological impact of β-arrestins on fertility.

Nevertheless, the *in vivo* and *in vitro* studies detailed above suggest that $G\alpha_{q/11}$ – independent pathways play a role in regulating GnRH release and one of these pathways may involve β-arrestin. We therefore took slightly different approach by investigating the importance of $G\alpha_{q/11}$ –independent pathways in the GnRH neuron affecting GnRH secretion and fertility. To accomplish this we created the GnRH-neuron specific $G\alpha_{q/11}$ double knockout mouse model. We hypothesized that $G\alpha_{q/11}$-independent mechanisms in the GnRH neuron would be able to trigger sufficient GnRH secretion to attain and

maintain puberty and fertility in mice. Our results revealed that while puberty was delayed it still occurred and males were sub-fertile indicating that the endogenous $G\alpha_{q/11}$-independent pathways sustained partial function of the GnRH neuron.

2.3.4.1 Generation of GnRH-neuron specific *Gnaq;Gna11* double deletion mice.

A conditional deletion of $G\alpha_{q/11}$ in GnRH neurons was generated by breeding *Gnaq*$^{fl/fl}$*;Gna11*$^{-/-}$ mice (Offermans et al. 1998, Wettschureck et al. 2001 and Kero et al. 2007) with *Gnrh-Cre* transgenic mice (Yoon et al. 2005). The *Gnrh-Cre* line used in these studies allows for Cre expression in GnRH neurons only and has been extensively characterized and used for the modification of several genes in this cell type (Yoon et al. 2005, Skynner et al. 1999 and Wintermantel et al. 2006, Wolfe et al. 2008, Leshan et al. 2009). The *Gnaq*$^{fl/fl}$*;Gna11*$^{-/-}$ parental line has also been extensively characterized and used with many conditional Cre expressing lines to understand the roles of $G\alpha_{q/11}$ signaling in various tissues and organs in the body (Kero et al. 2007, Wettschureck et al. 2001, 2005, 2006, 2007, Dagmara et al. 2005). Upon expression and action of the Cre recombinase enzyme, exon 6 of the *Gnaq* gene is excised resulting in loss of function of $G\alpha_q$ in GnRH neurons the background of a full body $G\alpha_{11}$ deletion (Offermans et al. 1998, Wettschureck et al. 2001, 2005, Kero et al. 2007 and Breen et al. 2013). Experimental mice are designated (dKO) or *(Gnaq*$^{d/d}$*)* and the control littermates are designated *(Gnaq*$^{fl/fl}$*)*.

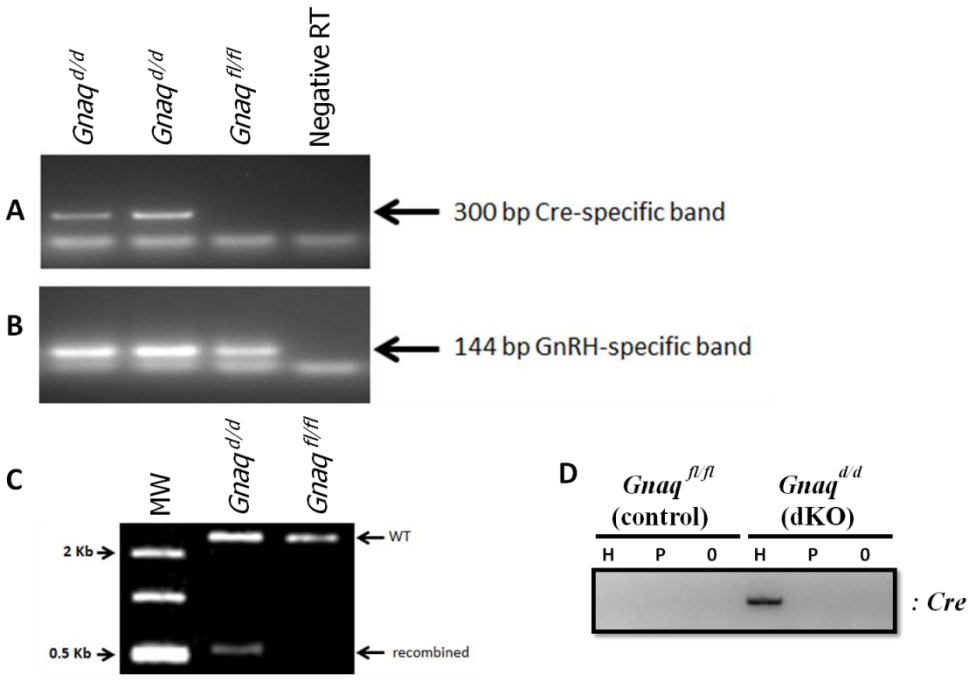

Figure 2.14: Confirmation of the successful creation of GnRH-neuron specific $G\alpha_{q/11}$ double knockout mice.

For all these experiments, 8 week old littermates were used. A. RT-PCR analysis of hypothalamic tissue confirms the expression of Cre recombinase in the hypothalamus of the *Gnaq*$^{d/d}$ mice but not in the *Gnaq*$^{fl/fl}$ littermate or negative RT-PCR control. B. RT-PCR analysis of hypothalamic tissue confirms the expression of GnRH all samples as expected and not the negative RT-PCR control. C**.** PCR analysis of genomic DNA from hypothalamic tissue produces a 2.3 kb product in mice bearing a floxed allele (*Gnaq*$^{fl/fl}$) and a 0.5 kb recombination product (*Gnaq*$^{\triangle}$) (confirming Cre-dependent excision of the sequence between the LoxP sites) in mice bearing the floxed allele and also expressing Cre Recombinase. Hypothalamic tissue contains a mix of GnRH neurons and non-GnRH cells so both products (2.3 kb and 0.5 kb) are detected in the hypothalamus of the *Gnaq*$^{d/d}$ mouse while only the 2.3 kb product is detected in the *Gnaq*$^{fl/fl}$ littermate. D. RT-PCR analysis showing *Cre* expression is only detected in the hypothalamus (H) of the (*Gnaq*$^{d/d}$) mouse and not in the other tiers of the HPG axis; Pituitary (P) and Ovary (O) nor in the *Gnaq*$^{fl/fl}$ littermate.

2.3.4.2 Cre Recombinase activity in *Gnrh-Cre* mice used to generate *Gnaq*$^{d/d}$ mice.

The well characterized *Gnrh-Cre* line used for this study essentially targets all (97%) GnRH neurons (Srinivas et al. 2001 and Yoon et al. 2005, Kirilov et al 2013). For our purposes it was important that recombination occurred during early development, before pubertal onset. Therefore to determine when recombination of *Gnaq* occurred we crossed the *Gnrh-Cre* line to a *ROSA26::LoxP-Stop-LoxP-GFP* reporter line (Soriano 1999) and examined brain slices from offspring at E18, 1, 2 and 3 weeks of age. At each age (E18, 1, 2 and 3 weeks) three independent analyses were done where neuronal soma were scored for both ROSA26::GFP and GnRH immunostained signals in ten consecutive tissue coronal brain sections spanning the rostral-caudal hypothalamic axis. We found that as early as E18 (Fig. 2.15), Cre recombination (as determined by GFP expression) occurred in at least 97% of all GnRH-positive neurons. The possibility remains that the remaining 3% of GnRH neurons did not possess a recombination event or that GFP fluorescence was too low to be efficiently detected. We also detected GFP-expressing cells in the intermediate division of the lateral septum (LSi) which has been already describes as a site where GnRH is transiently expressed in early development in the mouse (Skynner et al.1999). From this result we conclude that $G\alpha_{q/11}$ was effectively deleted from essentially all GnRH neurons and possibly from a very small population of LSi neurons in *Gnaq*$^{fl/fl}$*; Gna11*$^{-/-}$ *; Gnrh-Cre*$^{+}$ mice.

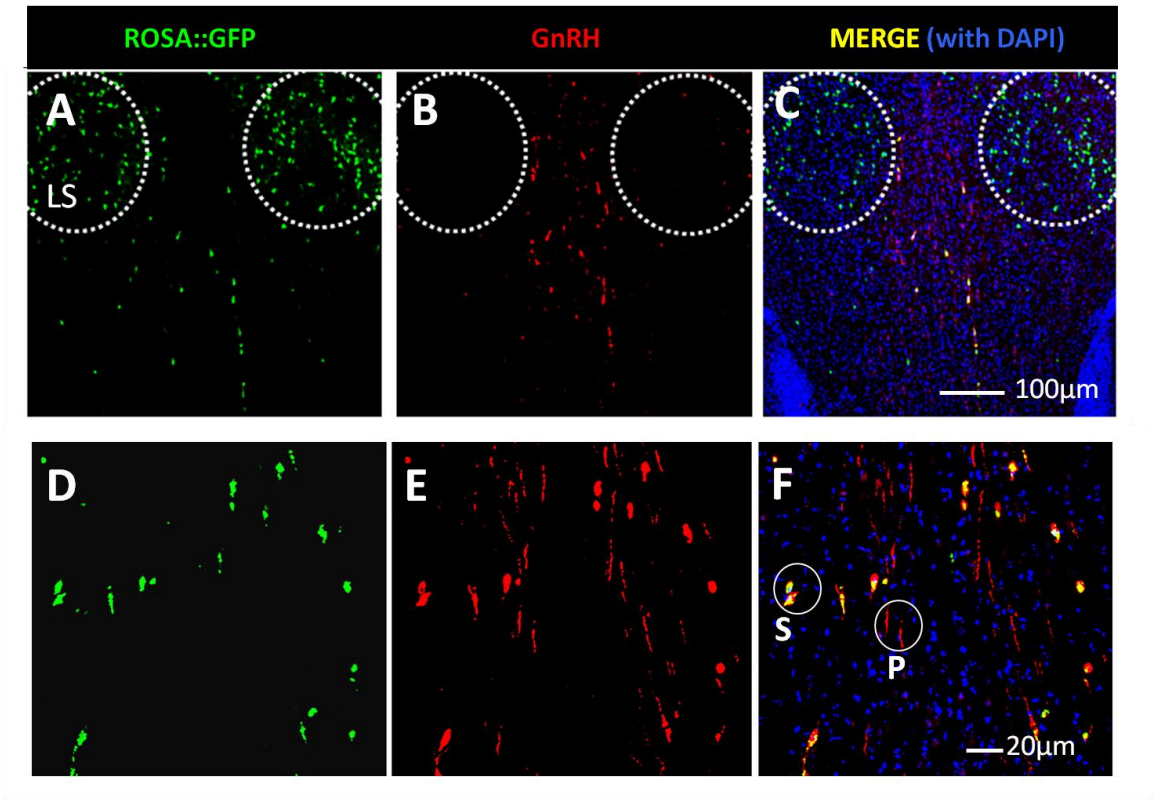

Figure 2.15: The *Gnrh-Cre* line shows high Cre recombinase activity where essentially all Gnrh neurons co-express ROSA26::GFP.

A-F. Representative image of a 30µm thick coronal brain section at the level of the OVLT in E18 mice generated by crossing a Gnrh-Cre mouse to a ROSA26::loxP-Stop-loxP-GFP reporter mouse and subsequently immunostained for GnRH and counterstained for nuclei with DAPI. **A-C.** Low power image of a coronal brain slice showing show Cre recombinase activity in the lateral septum (LS) and peri-ventricular zone of the rostral hypothalamus. Cre activity in the lateral septum (LS) is well described in this line and occurs during fetal development (Skynner et al. 1999). It is believed that in early development these cells express GnRH but stop doing so at a later date (x20 magnification). **D-F.** High power image (x40 magnification) of the periventricular zone of the same coronal slice. The GFP expression seemed to be restricted to the soma (S) while GnRH was detected in both the soma and processes (P) of the GnRH neurons. (LS: Lateral Septum, S: Soma; P: processes).

2.3.4.3 Loss of $G\alpha_q$ and $G\alpha_{11}$ has no effect on GnRH immunoreactive neuronal population size and distribution in the hypothalamus.

In order to use the GnRH-neuron-specific $G\alpha_{q/11}$ dKO mouse to investigate the impact of $G\alpha_{q/11}$ –independent signaling on fertility, we first needed to demonstrate that the loss of both *Gnaq* and *Gna11* in the GnRH neuron did not disrupt GnRH neuronal migration and/or survival during early mouse development. The number and location of GnRH neurons in coronal brain slices from 8 week old $Gnaq^{d/d}$ mice was compared to littermate controls ($Gnaq^{fl/fl}$) (collaborative study with Dr. Tobet, Colorado State University). Representative images from coronal sections at the level of the OVLT are shown (Fig. 2.16A and B). The mean number of GnRH immunoreactive neurons in each section was plotted as a histogram (n = 6) for both genotypes (Fig. 2.16D). As expected, the highest number of GnRH immunoreactive neurons was generally found at or near the OVLT with fewer cells in sections rostral and caudal to the OVLT (Gill et al. 2008). Genotype ($Gnaq^{d/d}$ vs. $Gnaq^{fl/fl}$) had no significant effect on the total GnRH immunoreactive cell counts ($P > 0.1$) (Fig. 2.16C). Overall, these findings suggest that the GnRH neuronal population was fundamentally intact and that the $Gnaq^{d/d}$ mice could be used for analyzing the importance of $G\alpha_{q/11}$ independent signaling on GnRH secretion.

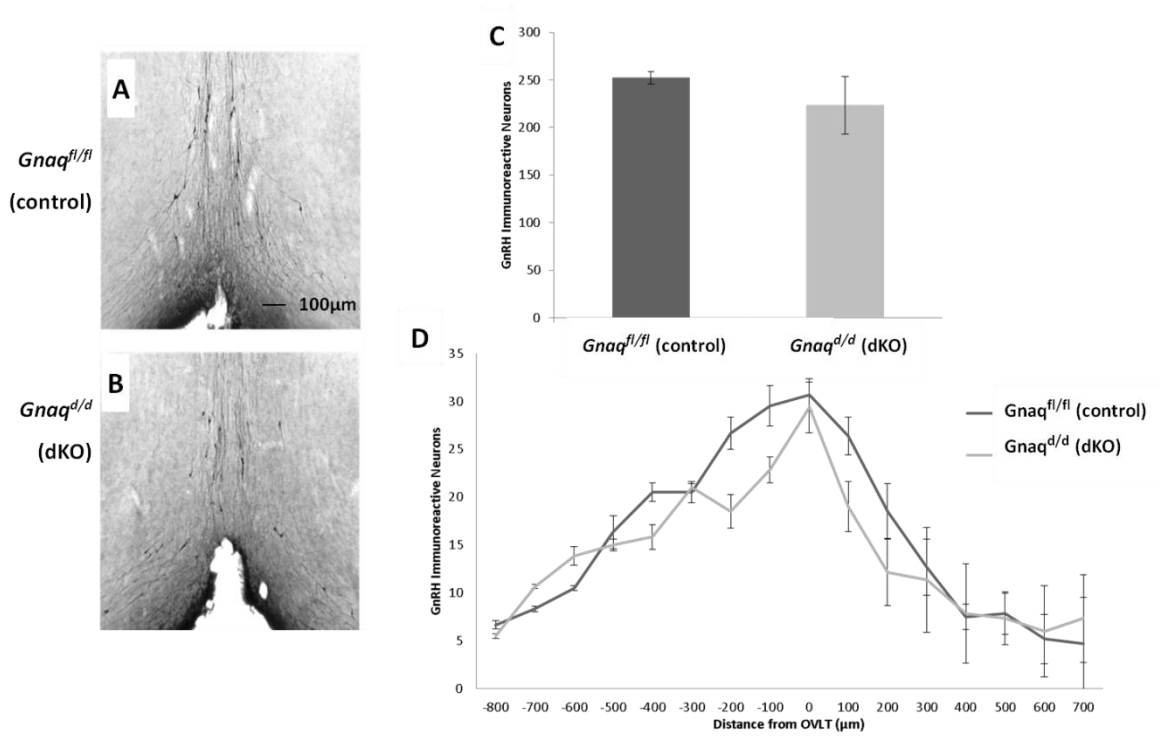

Figure 2.16: Loss of *Gnaq* and *Gna11* has no effect on the GnRH immunoreactive neuronal population size and distribution in the hypothalamus.

(A and B): Photomicrographs of coronal brain sections at the level of the OVLT from 8 week old *Gnaq*$^{d/d}$ *and Gnaq*$^{fl/fl}$ mice. GnRH neurons were revealed in the brain slices by IHC using the EL14 antibody and the OVLT was used as a neuroanatomical reference point for rostral to caudal alignment of coronal sections. (C): Total number of GnRH immunoreactive neurons identified with the EL14. Error bars represent SEM (n=6 for both genotypes). (D): Histogram displaying the rostral-caudal distribution of GnRH immunoreactive neurons from WT and KO mice aligned at the OVLT. Plot represents average GnRH immunoreactive neurons per 50 µm coronal section. GnRH immunoreactive neurons were identified with the EL14 antibody.

2.3.4.4 Pubertal onset is delayed but not impeded in *Gnaq*$^{d/d}$ mice.

External anatomic abnormalities were not detected in *Gnaq*$^{d/d}$ mice when compared to their littermate controls *Gnaq*$^{fl/fl}$. Weight gain over time was comparable between the genotypes for each gender Females; *Gnaq*$^{fl/fl}$ vs. *Gnaq*$^{d/d}$ = 20.7± 0.9g; n = 13 and 22.9± 0.7g; n = 14(P > 0.1). Males; *Gnaq*$^{fl/fl}$ vs. *Gnaq*$^{d/d}$ = 26.2 ± 0.6g; n = 22 and 27.1± 0.9g; n = 23 (P > 0.1) (Fig. 2.16). We used multiple established indices to assess the reproductive phenotype if the *Gnaq*$^{d/d}$ mice. Pubertal onset in females was assessed by vaginal opening (VO) and in males by preputial separation (PPS) and anogenital distance (AG) (Fig. 2.18). (Lapatto et al. 2007, Parakarainen et al. 2005, Novaira 2014, Herbison et al. 2008 and Chan et al. 2009 Caligioni 2009).Over the age range assessed, anogenital distance (mm) in males was not significantly different between genotypes except at 16 day old where control *Gnaq*$^{fl/fl}$ measured approximately 1.2 mm longer than *Gnaq*$^{d/d}$ littermates (Fig. 2.18A) (P < 0.05). PPS was significantly delayed by about 4 days in *Gnaq*$^{d/d}$ males compared to their *Gnaq*$^{fl/fl}$ littermates (Fig. 2.18 B) (P<0.0001). VO was also significantly delayed by about 4 days in *Gnaq*$^{d/d}$ females compared to *Gnaq*$^{fl/fl}$ littermates (Fig. 2.18 C) (P<0.05). Delays in PPS and VO are not due to differences in body weight since there was no significant difference between the genotypes (Fig. 2.17).

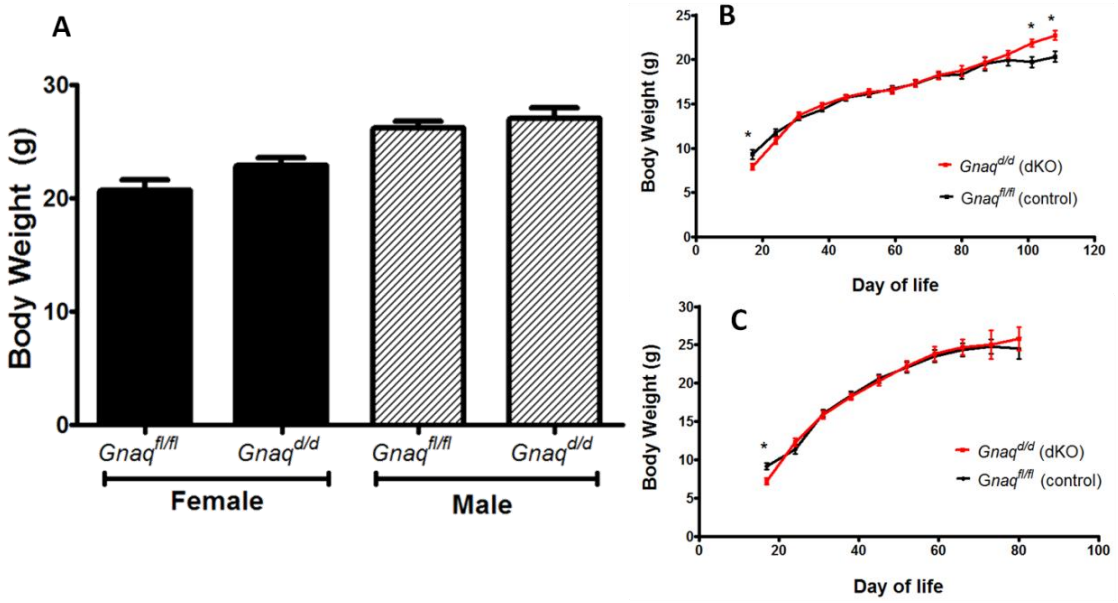

Figure 2.17: Loss of Gnrh neuron-specific *Gnaq* and *Gna11* has no effect on the weight gain of mice.

(A). Average body weight of 8 week old mice show that while males tended to be heavier than females there was no significant difference in body weight between genotypes within the same gender. Females; $Gnaq^{fl/fl}$ vs. $Gnaq^{d/d}$ = 20.7± 0.9 g; n = 13 and 22.9± 0.7 g; n = 14(P>0.1). Males; $Gnaq^{fl/fl}$ vs. $Gnaq^{d/d}$ = 26.2 ± 0.6 g; n = 22 and 27.1± 0.9 g; n = 23 (P>0.1). Error bars represent SEM. (B). Average female weight over age. At 16 days old, $Gnaq^{fl/fl}$ were significantly heavier than $Gnaq^{d/d}$ littermates (9.3 ± 0.5 g; n = 17 vs. 7.9 ± 0.4 g; n =18 (P<0.05). At 100 and 108 days this result was reversed where $Gnaq^{d/d}$ were significantly heavier than $Gnaq^{fl/fl}$ littermates. (19.7 ± 0.6 g; n = 7 vs. 21.9 ± 0.4 g; n = 6 (P < 0.05)) and (20.3 ± 0.6 g; n = 7 vs. 22.7 ± 0.5 g; n = 6 (*P < 0.05)). Error bars represent SEM. (C). Average male weight over age was not significantly different except at 16 days old $Gnaq^{fl/fl}$ were significantly heavier than $Gnaq^{d/d}$ littermates (9.2 ± 0.4 g; n = 15 vs. 7.2 ± 0.5 g; n = 11 (*P < 0.05)). Error bars represent SEM.

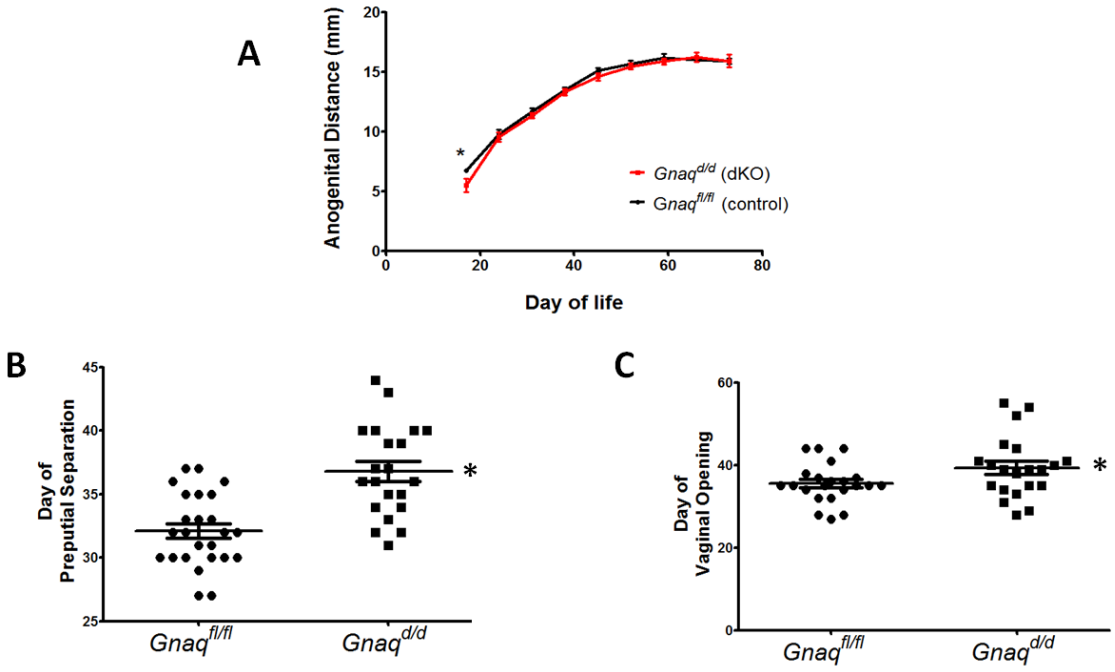

Figure 2.18: Loss of *Gnaq* and *Gna11* in the GnRH neuron delays but does not prevent pubertal onset.

(A). Anogenital distance (mm) in males over age was not significantly different between genotypes except for 16 days old where $Gnaq^{fl/fl}$ > $Gnaq^{d/d}$; 6.7 ± 0.2 mm; n = 8 vs. 5.5 ± 0.6 mm; n = 6 ($P < 0.05$). (B). Graphical representation of the time course of the day of preputial separation in males showing that $Gnaq^{d/d}$ took significantly longer than $Gnaq^{fl/fl}$ littermates; 36.8 ± 0.8 days; n = 21 vs. 32.1 ± 0.6 days; n = 25 (*$P < 0.0001$). (C). Graphical representation of the time course of the day of vaginal opening in females showing that $Gnaq^{d/d}$ took significantly longer than $Gnaq^{fl/fl}$ littermates; 39.4 ± 1.6 days; n = 21 vs. 35.6 ± 1.0 days; n = 22 ($P < 0.05$) Error bars represent SEM.

2.3.4.5 Estrous cycling is abnormal in *Gnaq*$^{d/d}$ mice but sporadic estrus phases are still observed.

To further characterize female reproductive development we used a combination of daily vulva examination and vaginal cytological assessment following vaginal lavage to evaluate estrous cyclicity over a period of 17 days (Novaira et al. 2014). Each female was tested at two ages, 8-11 weeks and 13-16 weeks of age. Control *Gnaq*$^{fl/fl}$ mice had an average cycle length of 5.23 ± 0.21 days (n = 6) (Fig. 2.19A and B). *Gnaq*$^{d/d}$ mice did not display a normal estrous cycling pattern so cycle length could not be calculated (n=6). In order to make a comparison, the total number of times the mice were found in estrus over the assessment period was analyzed (Chan et al. 2009). We found that at both age ranges examined *Gnaq*$^{d/d}$ mice displayed significantly fewer estrous stages than control *Gnaq*$^{fl/fl}$ litermates; 8-11 weeks; 1.3 ± 0.4; n = 6 vs 3.5 ± 0.2; n = 6 ($P<0.05$). 11-13 weeks; 3.7 ± 0.4; n = 6 vs. 1.5 ± 0.4; n = 6 ($P < 0.05$) (Fig. 2.19C).

Figure 2.19:

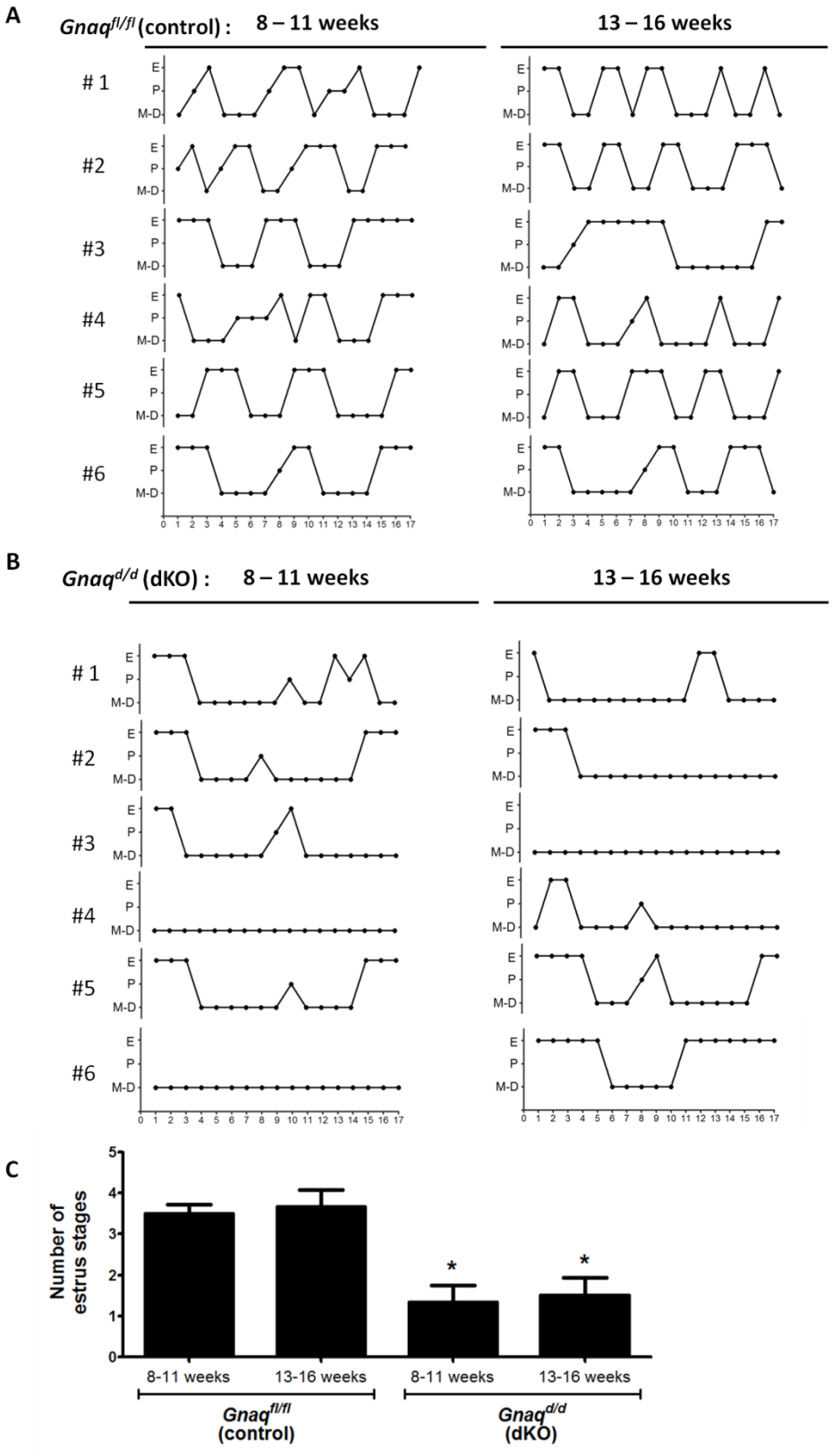
A
Gnaq fl/fl (control) :
8 – 11 weeks
13 – 16 weeks
1
#2
#3
#4
#5
#6
E
P
M-D
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
Gnaq d/d (dKO) :
8 – 11 weeks
13 – 16 weeks
C
Number of estrus stages
0
1
2
3
4
5
*
*
8-11 weeks
13-16 weeks
8-11 weeks
13-16 weeks
Gnaq fl/fl (control)
Gnaq d/d (dKO)

Figure 2.19: Loss of *Gnaq* and *Gna11* in the GnRH neuron causes abnormal estrous cyclicity but does not prevent sporadic estrus from occuring.

(A-B). Graphic representation of estrous cycle as determined by vulva observation and vaginal cytology followed for 17 days at 8-11weeks of age then again at 13-16weeks (n = 6). (A). Control $Gnaq^{fl/fl}$ females with average cycle length of 5.2 ± 0.2 days (n = 6). (B). $Gnaq^{d/d}$ females did not display normal cyclicity so cycle length could not be calculated. (C). Total number of estrus stages over assessment period. $Gnaq^{d/d}$ mice displayed significantly fewer estrous stages than control $Gnaq^{fl/fl}$ litermates at both ages. 8-11 weeks; 1.3 ± 0.4, n = 6 vs 3.5 ± 0.2, n = 6 (*P<0.05). 11-13 weeks; 3.7 ± 0.4; n = 6 vs. 1.5 ± 0.4; n = 6 (P < 0.05). Error bars represent SEM.

2.3.4.6 Reproductive tract abnormalities and impaired fertility in $Gnaq^{d/d}$ mice

Post-pubertal (8 weeks old) untested and previously unmated $Gnaq^{d/d}$ male and female mice were paired to fertile proven dams and studs respectively for 18-21 days. 9 of 15 male $Gnaq^{d/d}$ mice (60%) were able to impregnate females; however 100% of $Gnaq^{d/d}$ females (15 in total) failed to become pregnant (Fig. 2.20C). When $Gnaq^{d/d}$ males and females were paired to each other under the exact same conditions, as expected, no successful mating occurred demonstrating that the double deletion of *Gnaq* and *Gna11* the GnRH neuron compromises reproductive capacity. As seen in Fig. 2.20A and B, female reproductive tracts are significantly smaller (37%) in $Gnaq^{d/d}$ mice compared to $Gnaq^{fl/fl}$ controls (36.7 ± 5.4 mg; n = 14 vs. 99.3 ± 9.3 mg; n =13 (P < 0.0001). This result is not influenced by body weight since there is no significant difference between genotypes (Fig. 2.17). Relative to littermates, histological analysis of ovaries and uteri of $Gnaq^{d/d}$ females show clear evidence of abnormalities (Fig. 2.21E and F). $Gnaq^{d/d}$ ovaries (Fig. 2.21E) shows an absence of corporea lutea (CL) indicating that oogenesis was disrupted since primary, preantral and antral follicles are present. So the infrequent transition to estrus stages observed with $Gnaq^{d/d}$ females previously (Fig. 2.19) is not associated with ovulation. Male testicular weight was also reduced in $Gnaq^{d/d}$ mice

compared to *Gnaq*$^{fl/fl}$ controls showing a 61% reduction (Fig. 2.20A and B), 131.8 ± 8.6 mg; n = 23 vs. 216.8 ± 13.7 mg; n = 22 (P<0.0001). Histological analysis of the testis show that *Gnaq*$^{d/d}$ mice had decreased amounts of luminal sperm compared to *Gnaq*$^{fl/fl}$ controls (Fig. 2.21D).

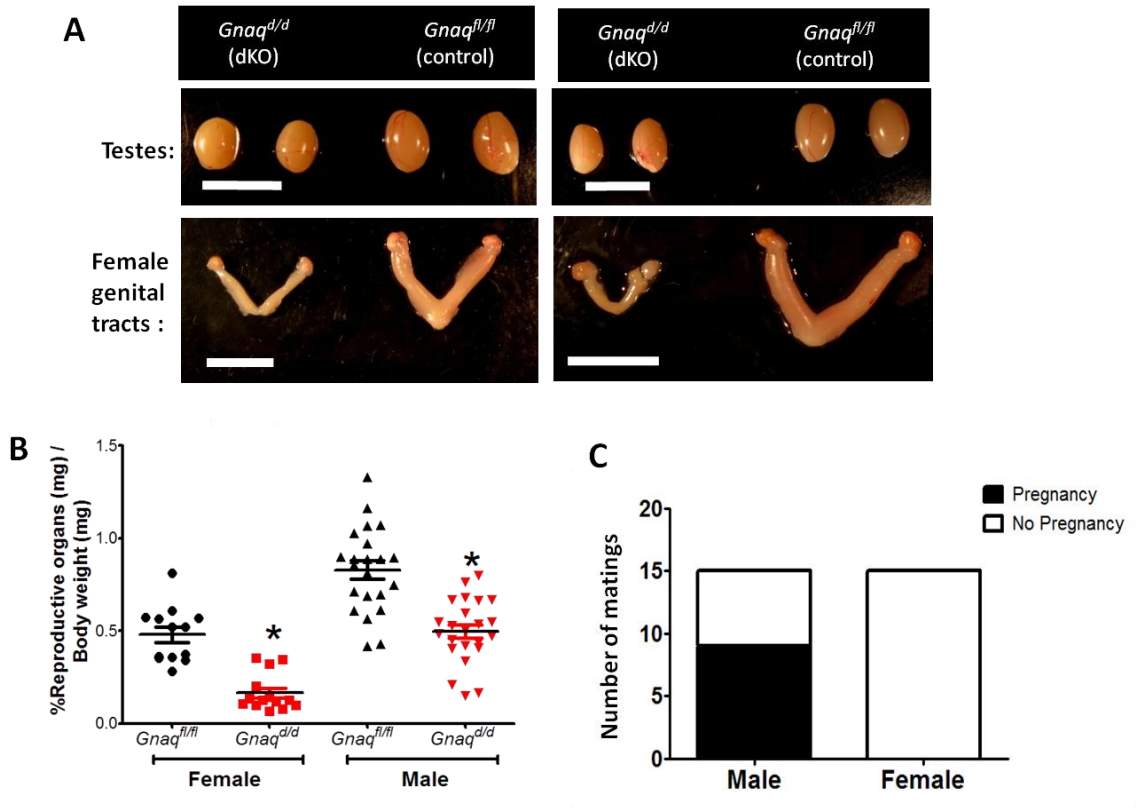

Figure 2.20: Loss of *Gnaq* and *Gna11* in the GnRH neuron causes reproductive tract abnormalities and impaired fertility.

(A-B). Gross gonadal anatomy and weight show that *Gnaq*$^{d/d}$ female reproductive tracts are 37% smaller than controls (36.7 mg ± 5.4 n = 14 vs. 99.3 mg ± 9.3 n =13 (P < 0.0001). Male testicular weight was also significantly reduced in *Gnaq*$^{d/d}$ mice (61%), 131.8 mg ± 8.6 n =23 vs. 216.8 mg ± 13.7 n =22 (*P<0.0001). Average represents SEM. Reproductive tracts and gonads were taken from unmated and untreated 8-16 week old mice. Females were in metestrus. Scale bars = 10 mm. (C). Fertility assessment of *Gnaq*$^{d/d}$ mice showed that while 60% *Gnaq*$^{d/d}$ males (9/15) engaged in successful matings, 100% of females were infertile (15/15).

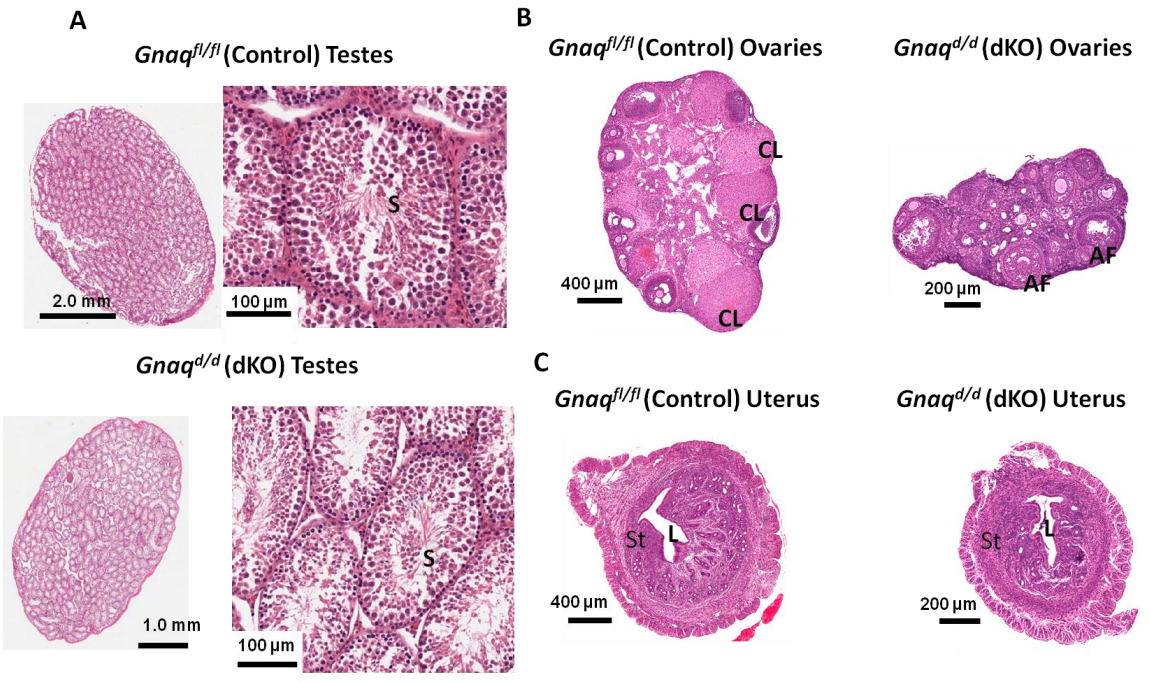

Figure 2.21: Gonadal histology of *Gnaq*$^{d/d}$ mice demonstrates abnormal phenotype.

(A). Representative sections of testes shows reduced luminal spermatozoa (S) in the seminiferous tubules of *Gnaq*$^{d/d}$ males compared to *Gnaq*$^{fl/fl}$ controls which show lumens filled with sperm. Spermatogenisis was variable in *Gnaq*$^{d/d}$ males and those with more sperm correlated to their fertility while those with little or none were sterile (not shown). (B). Representative section of *Gnaq*$^{d/d}$ ovary shows an absence of corporea lutea (CL) indicating that oogenesis was disrupted since primary, preantral and antral follicular (AF) stages are present. *Gnaq*$^{fl/fl}$ control females show follicles at all stages of development including many CL. (C). Representative section of *Gnaq*$^{d/d}$ uterus were smaller and showed fewer glandular structures than *Gnaq*$^{fl/fl}$ control females (St) Stroma; (L) Uterine lumen.

2.3.5 KISS1R signals through $G\alpha_{q/11}$ -independent mechanisms to regulate LH secretion

In vitro studies in the Babwah laboratory showed that both WT KISS1R and L148S trigger ERK1/2 phosphorylation independently of $G\alpha_{q/11}$ (Figs. 2.5-2.8) (Pampillo et al. 2009, Szereszewski et al 2010). We also demonstrated that KISS1R signals via β-arrestin to regulate GnRH secretion (Fig. 2.4). Then we showed that the endogenous function of the GnRH neuron was still somewhat intact in the *Gnaq*$^{d/d}$ mice since they displayed delayed pubertal onset and were still partially fertile (Figs. 2.17-2.21). We now hypothesized that like the endogenous function, Kiss1/Kiss1r would be able to signal through these $G\alpha_{q/11}$-independent pathways to trigger Kp-dependent LH secretion thereby providing a potential explanation for the sustained fertility seen in the *Gnaq*$^{d/d}$ mice. Our results revealed that although significantly less than control littermates, Kp-dependent LH secretion was still significantly more than basal levels in GnRH-neuron specific $G\alpha_{q/11}$ double knockout mice. Taken together these findings confirm a novel role for $G\alpha_{q/11}$-independent signaling in the GnRH neuron in mediating endogenous as well as Kp-dependent GnRH release and thereby fertility in mice.

2.3.5.1 Kisspeptin-triggered gonadotropin and testosterone secretion is significantly reduced in *Gnaq*$^{d/d}$ mice.

To test this hypothesis, all females used for hormonal secretion assays were ovariectomized prior to KP-54 stimulation in order to preclude the effects of the estrus cycle on GnRH secretion. Exogenous KP-54 was administered by i.p. injection at a dose of 100nmol/Kg and blood was collected for LH and FSH analysis 1 h later. Serum gonadotropins were used as well established surrogate markers for GnRH secretion (Seminara et al 2003, Chan et al 2009, Gottsch et al 2004 and Ramaswamy et al 2007). As expected, the level of kisspeptin-stimulated gonadotropin secretion by the *Gnaq*$^{d/d}$ females was significantly less than *Gnaq*$^{fl/fl}$ females ($P < 0.005$) however it was still significantly greater than vehicle treatment ($P < 0.005$) (Fig. 2.22A and B). Both control (*Gnaq*$^{fl/fl}$) and *Gnaq*$^{d/d}$ (dKO) females showed significant increases in LH and FSH secretion after kisspeptin stimulation when compared to vehicle treatment (Fig. 2.22A

and B). These results indicate that there is $G\alpha_{q/11}$-independent kisspeptin-stimulated GnRH secretion in the female mouse.

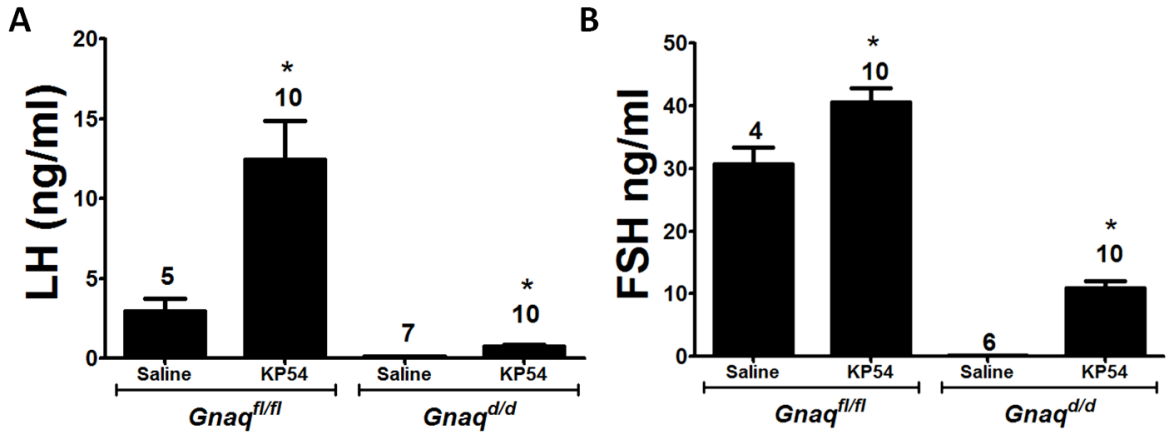

Figure 2.22: Loss of *Gnaq* and *Gna11* in the GnRH neuron diminishes but does not completely abolish KP-54 triggered LH and FSH secretion in the female mouse.

In this study, 9 week old ovariectomized virgin female mice were used. A. LH secretion for KP-54 treated control $Gnaq^{fl/fl}$ females was significantly greater than vehicle treatment (12.4 ± 2.4 ng/ml LH; n =10 vs. 2.9 ± 0.8 ng/ml LH; n =5, P< 0.001) and similarly LH secretion for KP-54 treated $Gnaq^{d/d}$ females was significantly greater than vehicle treatment (0.8 ± 0.1 ng/ml LH; n =10 vs. 0.1 ± 0.02 ng/ml LH; n =7, P< 0.05). B. FSH secretion for KP-54 treated control $Gnaq^{fl/fl}$ females was significantly greater than vehicle treatment (40.5 ± 2.4 ng/ml FSH; n =10 vs. 30.7 ± 2.7 ng/ml FSH; n =4, P< 0.005) and similarly LH secretion for KP-54 treated $Gnaq^{d/d}$ (dKO) females was significantly greater than vehicle treatment (10.9 ± 1.2 ng/ml FSH; n = 10 vs. 0.18 ± 0.005 ng/ml FSH; n = 6 , P<0.001). Error bars represent SEM.

To further test if the hypothesis that in the *Gnaq*$^{d/d}$ mice, kisspeptin would trigger GnRH secretion independently of G$\alpha_{q/11}$ held true in males as it did in females, 7 week old *Gnaq*$^{d/d}$ and littermate control mice were used. Unlike females which were ovarectomized, intact males were used in this study since they do not experience dramatic fluctuations in GnRH secretion. Serum was assayed for LH, FSH and testosterone under basal and stimulated conditions. As observed with the *Gnaq*$^{d/d}$ females, in *Gnaq*$^{d/d}$ males, kisspeptin triggered significant increase in LH and FSH compared to vehicle. This indicates that Kiss1r can cause GnRH secretion via a previously unreported G$\alpha_{q/11}$-independent pathway in the male mouse (Fig. 2.23A, B and C). Serum testosterone further supports the existence of G$\alpha_{q/11}$-independent gonadotropin secretion downstream of Kiss1/Kiss1r signaling (Fig. 2.23C).

Figure 2.23:

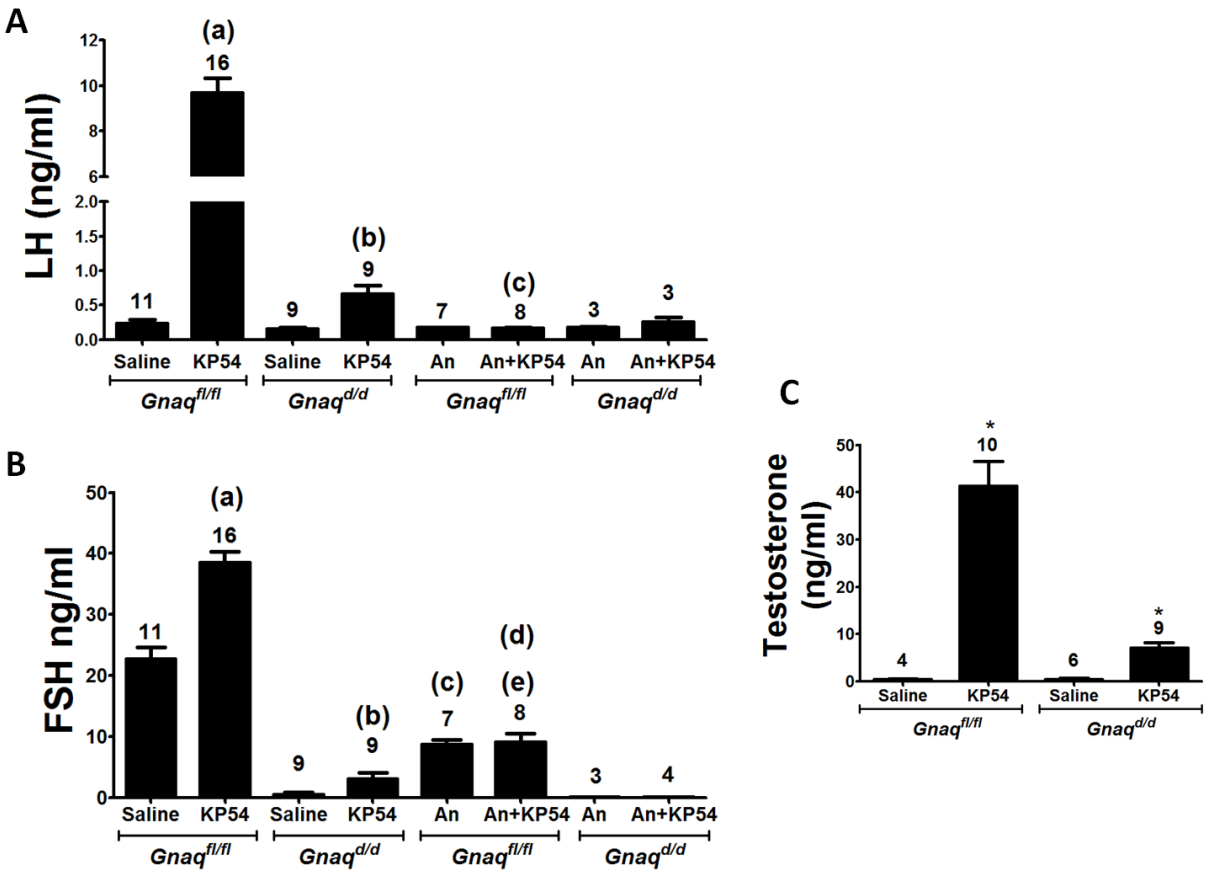

Figure 2.23: Loss of *Gnaq* and *Gna11* in the GnRH neuron diminishes but does not completely abolish KP-54 triggered gonadotropin and testosterone secretion in the male mouse.

In this study, 9 week old intact, virgin male mice were used and were treated with saline (vehicle) or KP-54 for 1 hour before blood collection or were pre-treated with Antide (An) (GnRH antagonist) prior to saline/KP-54 administration and blood collection.

Figure 2.23A: LH secretion in response to treatment.

- In $Gnaq^{fl/fl}$ mice KP-54 triggered significantly more secretion than saline treated $Gnaq^{fl/fl}$ mice (9.7 ± 0.6 ng/ml LH; n =16 vs. 0.24 ± 0.05 ng/ml LH; n =11) **(a)** P< 0.0001.
- In $Gnaq^{d/d}$ mice KP-54 triggered significantly more secretion than in saline treated $Gnaq^{d/d}$ mice (0.67 ± 0.12 ng/ml LH; n = 9 vs. 0.15 ± 0.02 ng/ml LH; n = 9) **(b)** P< 0.05. Also, no significant difference in LH secretion was observed between $Gnaq^{d/d}$ mice treated with Antide+KP-54 or saline.
- In $Gnaq^{fl/fl}$ mice KP-54 triggered significantly more secretion than Antide+KP-54 treated $Gnaq^{fl/fl}$ mice 9.7 ± 0.6 ng/ml LH; n =16 vs. 0.17 ± 0.008 ng/ml LH; n = 8) **(c)** P< 0.05. Also, no significant difference in LH secretion was observed between $Gnaq^{fl/fl}$ mice treated with Antide+KP-54 or saline.

Figure 2.23B: FSH secretion in response to treatment.

- In $Gnaq^{fl/fl}$ mice KP-54 triggered significantly more secretion than saline treated $Gnaq^{fl/fl}$ mice (38.5 ± 1.8 ng/ml FSH; n =16 vs. 22.8 ± 1.9 ng/ml FSH; n =11) **(a)** P< 0.05.
- In $Gnaq^{d/d}$ mice KP-54 triggered significantly more secretion than in saline treated $Gnaq^{d/d}$ mice (3.1 ± 1.0 ng/ml FSH; n = 9 vs. 0.6 ± 0.3 ng/ml FSH; n = 9) **(b)** P< 0.05.
- In $Gnaq^{fl/fl}$ mice Antide treatment significantly reduces FSH secretion compared to saline treated $Gnaq^{fl/fl}$ mice (8.7 ± 0.8 ng/ml FSH; n = 7 vs 22.8 ± 1.9 ng/ml FSH; n = 11) **(c)** P< 0.05.
- In $Gnaq^{fl/fl}$ mice Antide +Kp-54 significantly reduces FSH secretion compared to saline and KP-54 treatment in $Gnaq^{fl/fl}$ mice (9.2 ± 1.4 ng/ml FSH; n = 8 vs. 22.8 ± 1.9

ng/ml LH; n = 11) and (9.2 ± 1.4 ng/ml FSH; n = 8 vs. 38.5 ± 1.8 ng/ml FSH; n = 16). **(d)** and **(e)** P< 0.05

Figure 2.23C: Testosterone secretion in response to treatment.

- In *Gnaq*$^{fl/fl}$ mice KP-54 triggered significantly more secretion than saline treated *Gnaq*$^{fl/fl}$ mice (41.3 ± 5.2 ng/ml T; n = 10 vs. 0.4 ± 0.2 ng/ml T; n = 4) * P< 0.05.
- In *Gnaq*$^{d/d}$ mice KP-54 triggered significantly more secretion than saline treated *Gnaq*$^{d/d}$ mice (7.1 ± 1.1 ng/ml T; n = 9 vs. 0.5 ± 0.3 ng/ml T; n = 6) *P< 0.05

Some studies have suggested that the variable phenotype seen in patients with IHH and also in mice lacking either *Kiss1* or *Kiss1r* is due to direct KISS1/KISS1R action on pituitary gonadotropes causing LH secretion (Gottsch et al. 2004, Seminara et al. 2003, Chan et al. 2009, d'Anglemont de Tassigny et al. 2007, and Tenebaum-Rakover et al. 2007) . If this was indeed the case, it could provide an alternate explanation for the sub-fertile phenotype seen with our *Gnaq*$^{d/d}$ mice. Moreover, since kisspeptin was administered systemically, the diminished yet significant increase in LH, FSH and testosterone secretion may also be explained by direct action of Kiss1 on the pituitary. While more recent studies using GnRH neuronal specific knockouts of KISS1R have demonstrated that the GnRH neuron is in fact the singular site for Kiss1/Kiss1r regulation of the HPG axis, it was still important for us to show that in our *Gnaq*$^{d/d}$ the kisspeptin stimulated hormonal responses were due to GnRH action on the pituitary (Kirilov et al 2013, Novaira et al 2014 and Mesanger et al 2005). To do this we pre-treated mice with a GnRH specific antagonist, antide, prior to kisspeptin administration (Chan et al 2009 and Gottsch et al 2004) and we found that antide effectively ablated kisspeptin-dependent LH, FSH and testosterone secretion in both *Gnaq*$^{d/d}$ and littermate controls (Fig 2.23). Therefore kisspeptin-triggered hormone secretion is the result of its action on the GnRH neuron and not at the pituitary level thus supporting our hypothesis that KISS1R signals independently of $G\alpha_{q/11}$ in the GnRH neuron to mediate GnRH secretion.

2.3.5.2 Hypothalamic and pituitary gene expression in *Gnaq*$^{d/d}$ mice

Because genetic deletion of genes may sometimes lead to the development of unexpected molecular compensatory mechanisms we compared the expression of major central genes involved in the regulation of reproduction in *Gnaq*$^{d/d}$ mice relative to WT animals from the same parental strain (C57BL/6J). We used WT instead of littermate controls since these are *Gnaq*$^{fl/fl}$ mice with *Gna11* deletion from entire genome. While *Gnaq*$^{fl/fl}$ has no overt phenotype (*Gnaq* compensates fully for loss of *Gna11*) it may have some subtle unknown effect at the level of gene expression (Offermans et al 1997, Wettschureck et al 2001). We choose to investigate the following genes to ensure that differences in hormonal secretion (Figs. 2.22 and 2.23) were not due to differences in gene transcription at the central level of the HPG axis; *Kiss1*, *Kiss1r*, *Gnrh-1*, *Gnrh-r1*, *Lhb*, *Fshb*.

Our results show that *Kiss1r* is expressed in hypothalamus and pituitary at equivalent levels in WT and *Gnaq*$^{d/d}$ mice (Fig. 2.24). Kiss1r pituitary expression is expected and has been described in previous studies, however it has been shown to not play a major role in gonadotropin secretion (Richard et al 2009, Kirilov et al. 2013 and Novaira et al. 2014). This result also indicates that loss of $G\alpha_{q/11}$ in the GnRH neuron did not lead to compensatory up-regulation of Kiss1R in the hypothalamus or pituitary (Gottsch et al 2004, Chan et al. 2009 and Richard et al 2009).

As expected, *Kiss1* hypothalamic expression is significantly higher in *Gnaq*$^{d/d}$ than WT mice due to reduced negative feedback of gonadal steroids (Fig. 2.24). Negative steroid feedback is shown to act at the level of the hypothalamus, not pituitary, specifically on kisspeptin neurons in the arcuate nucleus (ARC) (Shahab et al. 2005, Smith et al. 2005, Bateman and Patisaul et al. 2008 and Frazao et al. 2013). Since we showed that *Gnaq*$^{d/d}$ mice have lower levels of circulating gonadal steroids (Fig. 2.23C) and this would mean less negative feedback to reduce *Kiss1* expression in those ARC kisspeptin neurons.

Hypothalamic *Gnrh-1* mRNA expression is significantly greater in *Gnaq*$^{d/d}$ compared to WT mice indicating that $G\alpha_{q/11}$ signaling regulates GnRH synthesis as well as secretion in the GnRH neuron (Fig. 2.24). This is not surprising since *Kiss-1* expression is also

significantly increased in *Gnaq*$^{d/d}$ mice and studies done in immortalized mouse GnRH neuronal cell lines (GT1-7 and GN-11) have demonstrated that kisspeptin positively regulates GnRH synthesis and secretion (Novaira et al. 2009, Mellon et al. 1990, Mayer et al. 2009).

GnRH-R1 is expressed on anterior pituitary gonadotropes which are stimulated by hypothalamic GnRH to secrete LHβ and FSHβ into systemic circulation. As expected, *Gnrh-r1* is strongly expressed in the pituitary and is significantly higher in WT than *Gnaq*$^{d/d}$ mice. GnRH stimulation is required for *Gnrhr-1*gene transcription so in the *Gnaq*$^{d/d}$ mice where there is impaired GnRH secretion as demonstrated by decreased gonadotropin levels (Figs. 2.22 and 2.23), there will therefore also be reduced *Gnrhr-1* mRNA expression in pituitary gonadotropes (Kaiser et al. 1994 and Bedecarrats and Kaiser 2007). *Lhb* (gene encoding for LHβ) and *Fshb* (gene encoding for FSHβ) are expressed significantly higher in WT pituitary than *Gnaq*$^{d/d}$. This further supports the finding that GnRH secretion is impaired in *Gnaq*$^{d/d}$ mice since GnRH is required for gonadotropin gene expression (Kaiser et al 1997 and Noel et al 2011).

Overall, based on relative gene expression, there is no evidence to support compensatory up-regulation of gene expression due to $G\alpha_{q/11}$ deletion in hypothalamic GnRH neurons. In *Gnaq*$^{d/d}$ mice there is increased *Kiss-1* expression (due to lack of negative steroid feedback) this stimulates increased *GnRH-1* transcription since kisspeptin regulates both GnRH transcription and secretion (Fig. 2.24) (Novaira et al. 2009). However in the absence of $G\alpha_{q/11}$, GnRH secretion is compromised, demonstrated by the significantly lower levels of gonadotropin gene expression and basal secretion in *Gnaq*$^{d/d}$ mice (Figs. 2.22, 2.23 and 2.24). Nonetheless, kisspeptin treatment in these mice is still able to stimulate significant gonadotropin and sex steroid release via a $G\alpha_{q/11}$ independent pathway (Figs. 2.22 and 2.23).

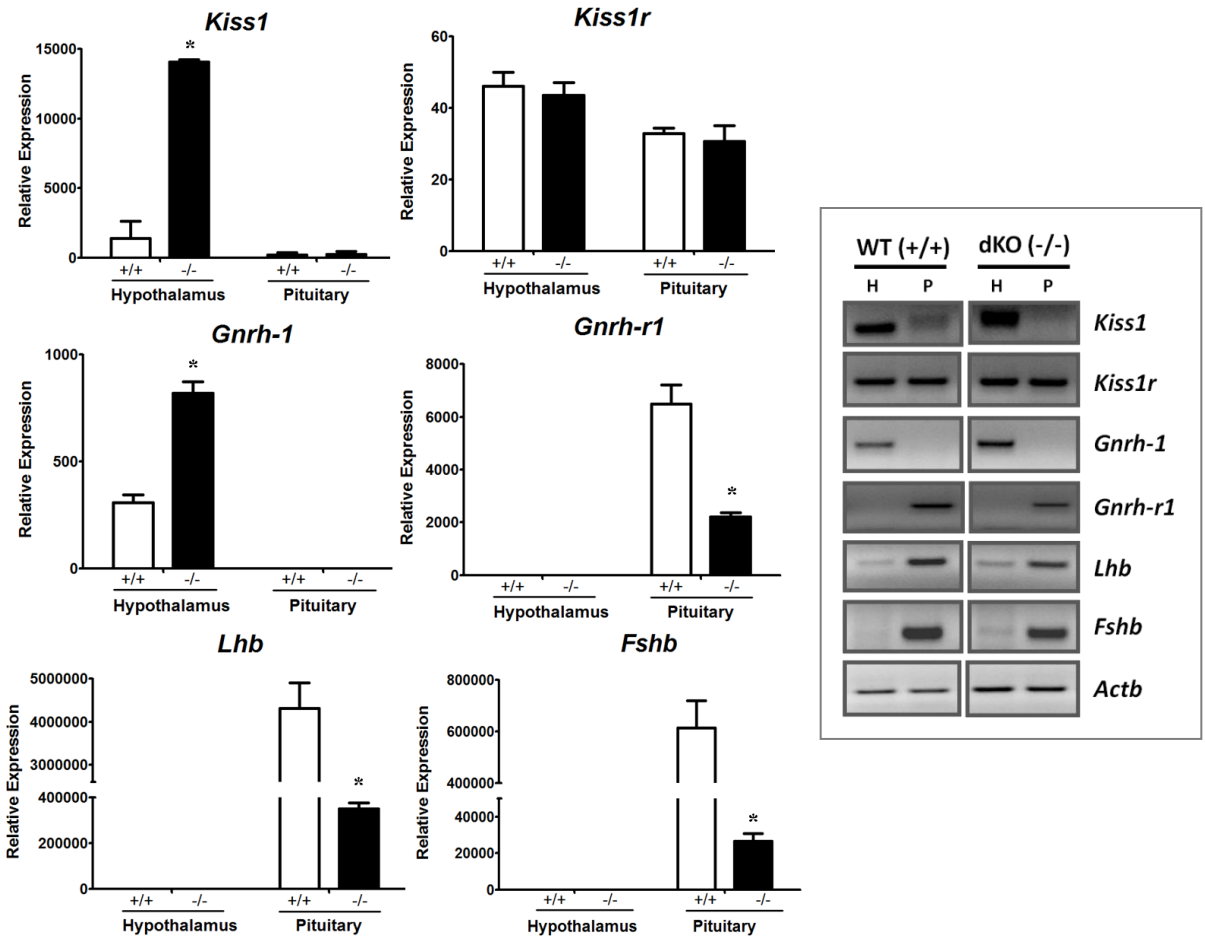

Figure 2.24: Loss of *Gnaq* and *Gna11* in the GnRH neuron does not affect expression of genes regulating reproduction in the mouse hypothalamus and pituitary.

In this study 7 week old male *Gnaq*$^{d/d}$ (-/-) and WT (+/+) mice were used. Hypothalami and pituitaries were used for quantitative RT-PCR (qPCR) measurement of relative gene expression. There is significantly more hypothalamic *Kiss1* and *GnRH-1* in *Gnaq*$^{d/d}$ mice and significantly less expression of *Gnrh-r*, *Lhb* and *Fshb* in *Gnaq*$^{d/d}$ pituitary. No significant difference in *Kiss1r* expression is observed. All gene expression normalized to housekeeping gene *Actb* (encodes β-actin). Inset shows representative 1% agarose gel with qPCR products. *P<0.01. Each bar is an average of 3 independent analyses. Error bars represent SEM.

2.3.5.3 *Gnaq*$^{d/d}$ mice do not signal through *Gna14* in the absence of *Gnaq* and *Gna11*.

Our *Gnaq*$^{d/d}$ mouse model employed Gnrh-Cre to allow GnRH-neuron specific inactivation of $G\alpha_q$, and $G\alpha_{11}$. From our *in vivo* findings, the *Gnaq*$^{d/d}$ mice are still able to secrete sufficient GnRH in response to kisspeptin to give rise to a small yet significant increase in serum gonadotropins and gonadal steroids (Figs. 2.22 and 2.23). The *Gnaq*$^{d/d}$ mice also exhibited a modest reproductive capacity where males could sometimes impregnate proven dams and females were still able to undergo follicle development (Figs. 2.20 and 2.21). This is not surprising since reproduction is critical for the survival of any species and multiple redundant and back-up systems are probably in place to maintain some level of fertility. We hypothesized that this kisspeptin-triggered GnRH secretion in the *Gnaq*$^{d/d}$ mice was due to a $G\alpha_{q/11}$ –independent pathway downstream of KISS1R. Coupled with our *in vivo* results showing that lack of either β-arrestin isoform (1 or 2) significantly impaired kisspeptin-triggered GnRH secretion (Fig. 2.4) and a KISS1R mutant; L148S signals to ERK1/2 (Figs. 2.5,2.6, 2.7 and 2.8), we propose that β-arrestins are part of the $G\alpha_{q/11}$ –independent pathway downstream of KISS1R that regulates GnRH secretion.

The four members of the $G\alpha_q$ family of proteins are $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$ encoded by genes *Gnaq, Gna11, Gna14* and *Gna15* respectively (Exton, 1996 and Rhee, 2001). $G\alpha_q$, and $G\alpha_{11}$ are ubiquitously expressed and most receptors are able to activate either isoform equally hence the "$G\alpha_{q/11}$" terminology used in the literature (Wange et al., 1991, Wu et al., 1992, Offermanns et al., 1994, 1999 and 2003). Murine $G\alpha_{15}$ (human equivalent $G\alpha_{16}$) is only expressed in a subset of hematopoietic cells and $G\alpha_{14}$ expression is restricted to the lung, kidney and testis (Amatruda et al. 1991, Wilkie et al. 1991, Tanaka et al., 2000, Offermanns, 1999 and 2003). To further support our hypothesis that KISS1R can signal via β-arrestins ($G\alpha_{q/11}$–independent) to trigger GnRH secretion we firstly investigated hypothalamic expression of other member of the $G\alpha_q$ family; $G\alpha_{14}$ and $G\alpha_{15}$.

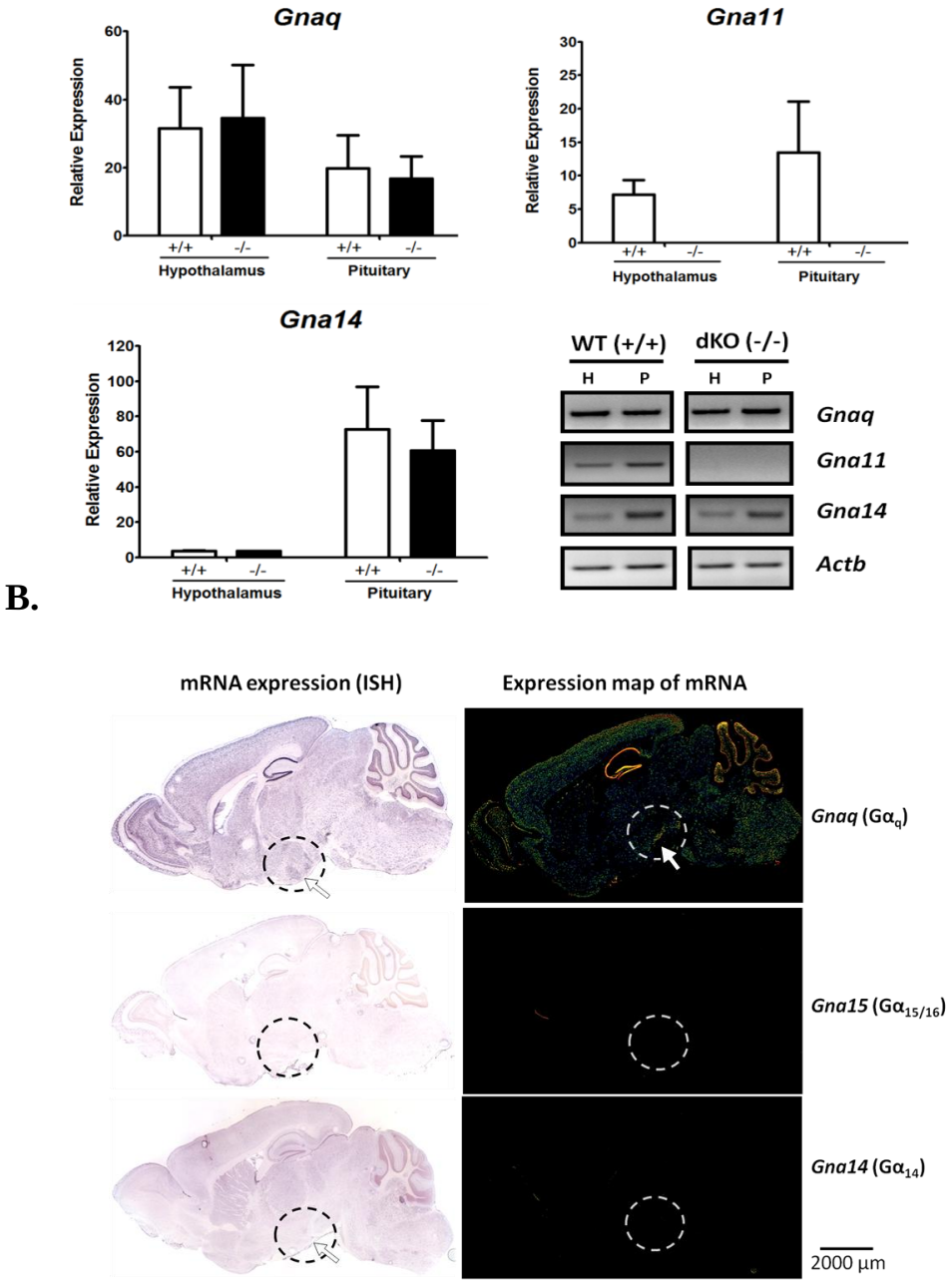

Figure 2.25: Loss of *Gnaq* and *Gna11* in the GnRH neuron does not affect expression of other Gq family of genes, specifically Gna14 and Gna15.

In this study 7 week old male *Gnaq*$^{d/d}$ (-/-) and WT (+/+) mice were used. Hypothalami and pituitaries were used for quantitative RT-PCR (qPCR) measurement of relative gene expression of *Gnaq*, *Gna11* and *Gna14*. All samples were normalized to *Actb* (β-actin).

Inset shows representative 1% agarose gel with qPCR products. No significant difference in G-protein gene expression is observed between *Gnaq*$^{d/d}$ and WT mice. Error bars represent SEM. Each bar is an average of 3 independent analyses. B. Representative images of sagittal brain sections of 8 week old male mice from the Allen Mouse Brain Atlas showing the localization of *Gnaq, Gna14* and *Gna15* by in situ hybridization (left panels) and having background signal filtered out so that only expression patterns are visible (right panels). Circled region represents the approximate localization of the hypothalamus. Scale bar is 2000 μm. (Image credit: Allen Mouse Brain Atlas, 2014)

As expected there is no significant difference in expression between genotypes (*Gnaq*$^{d/d}$ vs. WT). Since $G\alpha_q$ was only deleted in GnRH neurons the hypothalamic *Gnaq*$^{d/d}$ sample still shows strong expression of $G\alpha_q$ since it will be expressed normally in all other hypothalamic cells (Fig. 2.25A). *Gnaq*$^{d/d}$ mice also were created in the background of full body $G\alpha_{11}$ deletion and which is why only the WT hypothalamus and pituitary show *Gna11* expression (Fig. 2.25A). Unlike *Gnaq*, both *Gna14* and *Gna15* are weakly expressed the brain with no detectable expression in the hypothalamus as demonstrated by quantitative real-time PCR (Fig. 2.25A) and in situ hybridization by the Allan Institute for Brain Science (Fig. 2.25B). This is in agreement with other published studies (Wilkie et al., 1991, Amatruda et al., 1991, Tanaka et al., 2000). Functional studies done by Wacker et al., 2008 demonstrated that when overexpressed in a heterologous system of HEK 293 cells, KISS1R displays *Gna15*-concentration dependent PIP_2 hydrolysis. These studies were however performed in HEK 293 cells and our present study focuses on the hypothalamic GnRH neuron where there is no compelling evidence that $G\alpha_{15}$ is expressed in the hypothalamus (Fig. 2.25B) (Wilkie et al., 1991, Amatruda et al., 1991, Tanaka et al., 2000). Therefore, while KISS1R may be able to signal through $G\alpha_{15}$ this is not a mechanism likely to be employed in the GnRH neuron. To date, it has not been determined whether or not KISS1R can signal via $G\alpha_{14}$ and therefore one of our goals was to investigate this, despite the evidence suggesting that GnRH neurons do not express *Gna14* (Fig. 2.25).

We co-transfected HEK 293 cells with WT KISS1R and various concentrations of *Gnaq* and *Gna14* then measured ERK1/2 phosphorylation after 10 minutes of 100nM KP-10 (Fig. 2.26). We see that while KISS1R displays a concentration-dependent ERK1/2 phosphorylation response with increasing amounts of *Gnaq*, this response is not seen with increasing concentrations of *Gna14* indicating that KISS1R does not signal through *Gna14*. Overall, the results in Figs 2.25 and 2.26 strongly indicate that the $G\alpha_{q/11}$–independent cascade downstream from KISS1R is not via *Gna14* or *Gna15*.

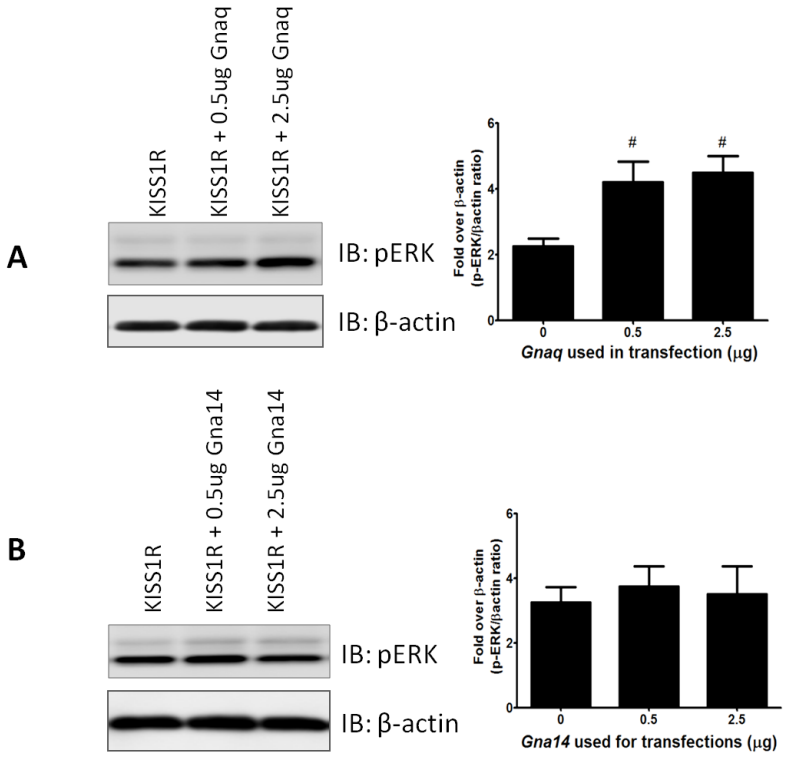

Figure 2.26: KISS1R *does not signal through $G\alpha_{14}$ to trigger ERK1/2 phosphorylation.*

Representative autoradiographs and densitometric analysis showing the expression of phosphorylated ERK1/2 and β-actin as a loading control. A. KISS1R and $G\alpha_q$ co-transfected cells show a significant increase in ERK1/2 phosphorylation with increasing $G\alpha_q$ concentrations. B. KISS1R and $G\alpha_{14}$ co-transfected cells show no significant difference in ERK1/2 phosphorylation with increasing $G\alpha_{14}$ concentrations. Data represents the mean of 3 independent experiments #P<0.05 pERK/ β-actin ratio compared to KISS1R only. Error bars represent SEM.

2.4 Discussion

Since the initial discovery of the role of the KISS1/KISS1R system in regulating the timely onset of puberty, many studies have since confirmed the integral role of this system in reproductive function and fertility. Firstly genetic analysis have revealed even more KISS1R mutations (12 to date) in patients diagnosed with infertility and IHH (Cerrato et al. 2007, Wahab et al. 2011, Nimiri et al. 2011, Pinilla et al. 2012). Secondly, mice genetically modified to lack *Kiss1* or *Kiss1r* expression are infertile and phenocopy human IHH patients (Funes et al. 2003, Seminara et al. 2003, Lapatto et al. 2007, Kirilov et al. 2013, Novaira et al. 2014). Thirdly, central and peripheral administration of kisspeptin potently stimulates GnRH and gonadotropin secretion in rodents, sheep and primates and kisspeptin antagonists ablate this response (d'Anglemont de Tassigny et al. 2007, Gottsch et al. 2004, Navarro et al. 2004. Irwig et al. 2004, Han et al. 2005, Shahab et al. 2005, Keen et al. 2008, Ramaswamy et al. 2007, Roseweir et al. 2009, Guierro et al. 2012, Pineda et al. 2010). Finally, kisspeptin agonists have been used to stimulate the HPG axis in both healthy and unhealthy male and female patients (Jayasena et al. 2010 and Chan et al. 2011). Recently, kisspeptin has even been used to facilitate successful *in vitro* fertilization resulting in full term pregnancy and live birth of a healthy baby (Jayasena et al. 2014).

In contrast to the *in vivo* reports above, a much smaller number of studies have provided substantial mechanistic insight into how KISS1R signals intracellularly to regulate biological functions and how receptor-based mutations affect these signaling events (Pampillo et al. 2009, Szereszewski et al. 2010, Ahow et al. 2014, Nimiri et al. 2011, Chevrier et al. 2013, Teles et al. 2008, Liu et al. 2008, Wacker et al. 2008, Bianco et al. 2011, Babwah et al. 2012, Zajac et al. 2011, Cvetkovic et al. 2013). Based on these studies it is now well established that the KISS1/KISS1R system potently stimulates GnRH secretion in a $G\alpha_{q/11}$-dependent manner. Previous publications have also suggested that KISS1R is capable of signaling to ERK1/2 independently of $G\alpha_{q/11}$, in a β-arrestin-dependent manner (Pampillo et al. 2009, Szereszewski et al. 2010). β-arrestin dependent signaling has now been established for many GPCRs including but not limited to, $AT_{1A}R$, β_2AR, PAR, V2R and NK-1 (Ahn et al. 2004, shenoy et al. 2006, Ren et al. 2005, Getsy-

Palmer et al. 2006, Wei et al. 2003, Tohgo et al. 2002, DeFea et al. 2000, Luttrell et al. 2001). β-arrestin has been shown to mediate insulin secretion and since ERK1/2 is critical step in GnRH secretion it is therefore plausible that KISS1R can signal through β-arrestin to promote GnRH secretion (Sonoda et al. 2008, Ravier et al. 2014, Silvestre et al. 2008, Castellano et al. 2006). We therefore hypothesize that KISS1R can signal through $G\alpha_{q/11}$ and $G\alpha_{q/11}$ –independent mechanisms, the latter most likely involving β-arrestins, to mediate GnRH secretion and overall fertility.

Many *in vivo* studies demonstrated that KISS1R rapidly desensitizes in the continuous presence of the kisspeptin agonist however the underlying mechanism was unknown (Seminara et al. 2006, Plant et al. 2006, Thompson et al. 2006, Ramaswamy et al. 2007, Messager et al. 2005). We demonstrated that GRK2 mediated KISS1R homologous desensitization and suggested that β-arrestin may be involved (Pampillo et al. 2009). Here we provide direct proof that β-arrestins mediate KISS1R homologous desensitization on a molecular level. Physiologically this is an important finding since kisspeptin agonists are proposed as a more effective therapy compared to GnRH agonists currently used for endometriosis, sex-steroid dependent cancers and uterine fibroids. This treatment strategy is based on KISS1R being more rapidly desensitized than GnRHR (Pinilla et al. 2012 and Millar, 2010). On a molecular level, this is also a significant and novel finding since some GPCRs such as GnRHR desensitize in a β-arrestin-independent manner. Also it supports the hypothesis that KISS1R engages in β-arrestin-dependent signaling since desensitization is a critical initial step in this process (Luttrell et al. 2002, Luttrell et al. 2010). In this study we demonstrated that a loss of either β-arrestin-1 or -2 results in about 20-25% reduction in kisspeptin-triggered LH secretion while had no effect on GnRH-triggered LH secretion at the level of the pituitary (Ahow et al. 2014). This reduced kisspeptin-triggered LH secretion had no effect on fertility based on brood size and is consistent with previous reports that describe limited secretion still being sufficient to support fertility (Popa et al. 2013, Mayer et al. 2011, Herbison et al. 2008). It is possible that isoform compensation did occur to some extent where the β-arrestin isoform that was still expressed was able to perform most of the functions of the isoform that was absent. However since we still observed reduced kisspeptin-triggered LH response in the single β-arrestin knockout mice it is plausible that if compensation did occur it was not

complete. Additionally, since both β-arrestin-1 and -2 mediate LH secretion we predict that their combined loss would result in a much larger reduction in LH secretion, reflecting an even greater role for β-arrestin in promoting GnRH secretion. It is also possible that combined loss of both isoforms would result in a sub-fertile phenotype, but unlikely an infertile phenotype since KISS1R signaling through $G\alpha_{q/11}$ would still be intact. However, since β-arrestin-1 and -2 double knockout mice die around birth and floxed Arrb mice are unavailable, we could not test this prediction (Zhang et al. 2010 and Zhang et al. 2011).

Since we could not further this study in β-arrestin double knockout mice we then considered the significance of all $G\alpha_{q/11}$-independent mechanisms on KISS1R signaling and biological response. To investigate this further we tested the hypothesis that naturally occurring $G\alpha_{q/11}$-uncoupled KISS1R mutants might maintain the ability to couple and signal via $G\alpha_{q/11}$-independent mechanisms. In order to be used in this study, a given KISS1R mutant must fulfill the following criteria. It must localize to the plasma membrane, bind kisspeptin and interact with signaling molecules in the G-protein cascade to a similar extent as WT KISS1R. In addition to fulfilling these criteria, L148S is also one of a few KISS1R mutants that have been functionally well-characterised in the literature (Wacker et al. 2008). Furthermore, based on its strong interaction with β-arrestin (Szereszewski et al. 2010) we hypothesize that the β-arrestin pathway represents a major conduit for L148S signaling (Wacker et al. 2008). We demonstrated that the diminished yet significant kisspeptin-triggered ERK1/2 phosphorylation downstream of L148S was $G\alpha_{q/11}$ –independent and confirmed that loss of β-arrestin expression abolished this response (Ahow et al. 2014). The finding that L148S displays $G\alpha_{q/11}$ – independent signaling is important because it reveals that some mutant KISS1Rs which are classified as loss-of-function based on $InsP_3$ formation might in fact be capable of signaling and therefore need to be more extensively functionally characterised.

Differential activation of PLCβ and $InsP_3$ formation is not the only way in which the KISS1R coupled $G\alpha_{q/11}$ –dependent response is functionally distinct from that of the $G\alpha_{q/11}$ –independent pathway. While both pathways trigger ERK1/2 phosphorylation, we identified STAT3 phosphorylation as a $G\alpha_{q/11}$ pathway-specific response since it is

abolished following L148S activation. Signal transducer and activator of transcription 3 (STAT3), also known as acute-phase response factor, is a transcription factor that is activated in the cytosol and translocates to the nucleus. This has important implications for the development of clinical therapies and further studies are necessary to fully elucidate and separate these KISS1R signaling cascades. We previously showed that KISS1R activated ERK1/2 translocates to the nucleus and mediates changes in gene expression in GT1-7 cells (Szereszewski et al. 2010). STAT3 is activated by members of the MAPK pathway and it therefore may be involved in KISS1R-mediated gene expression via the $G\alpha_{q/11}$ pathway (Heim 2001, Takeda et al, 1997, Yokogami et al. 2000). STAT3 phosphorylation may also be a useful indicator for future functional assays to determine if the KISS1R mutants being tested are truly able to activate $G\alpha_{q/11}$-independent pathways or if the signaling observed is due to residual activation of $G\alpha_{q/11}$-dependent mechanisms.

At this point in our study, we demonstrated that $G\alpha_{q/11}$-independent signaling is involved in both kisspeptin-triggered LH secretion through β-arrestins and L148S-mediated ERK1/2 phosphorylation. Therefore the $G\alpha_{q/11}$-independent pathway may also regulate GnRH secretion and overall fertility. To further investigate the physiological importance of $G\alpha_{q/11}$-independent signaling in reproduction GnRH-neuron specific $G\alpha_{q/11}$ double knockout mice (*Gnaq*$^{d/d}$) were used. We hypothesized that this mouse will display a sub-fertile and not completely infertile phenotype since the endogenous signaling machinery will still be intact to allow partial GnRH release through $G\alpha_{q/11}$-independent mechanisms. We further hypothesized that exogenous kisspeptin stimulation in the *Gnaq*$^{d/d}$ mouse will still be able to trigger KISS1R-dependent GnRH secretion; albeit at a diminished level. To generate the *Gnaq*$^{d/d}$ mouse we used well established floxed and Cre-expressing mice, specifically, the *Gnaq*$^{fl/fl}$*; Gna11*$^{-/-}$ mouse (Offermans et al. 1998, Wettschureck et al. 2001 and Kero et al. 2007) and *Gnrh-Cre* transgenic mouse (Yoon et al. 2005). Both parental strains have been extensively used to characterise the functional importance of other genes in the GnRH neuron and also to identify the role of $G\alpha_{q/11}$ in various specific tissues (Yoon et al. 2005, Skynner et al. 1999 and Wintermantel et al. 2006, Wolfe et al. 2008, Leshan et al. 2009, Offermans et al. 1998, Wettschureck et al. 2001, 2005, Kero et al. 2007 and Breen et al. 2013). Cre recombinase expression and resulting excision of

exon 6 from the *Gnaq* gene is targeted to GnRH neurons only and we showed that this recombination occurs as early as E18, before pubertal onset and after the normal GnRH neuronal migration in development.

We used a variety of reproductive phenotypic indices in order to assess GnRH neuronal function and overall fertility in the $Gnaq^{d/d}$ mice. Established markers for pubertal onset (vaginal opening, preputial separation and anogenital distance) were dependant on exposure to estrogen and testosterone during puberty in females and males respectively (Lapatto et al 2007, Herbison et al. 2008 and Chan et al. 2009, Caligioni 2009, Ojeda and Urbanski 1994 and Nelson et al. 1982, Parakarainen et al. 2005, Novaira 2014). Post puberty we assessed estrous cyclicity, gonadal histology and breeding success to evaluate the significance of $G\alpha_{q/11}$-independent GnRH neuronal function on adult fertility. We found that for both male and female $Gnaq^{d/d}$ mice puberty onset was delayed and adults were sub-fertile. This phenotype closely resembles that of the $Kiss1^{-/-}$ and $Kiss1r^{-/-}$ mice (Seminara et al. 2003, Funes et al. 2003, Lapatto et al. 2007, Kirilov et al. 2013 and Noviara et al. 2014). While females had abnormal estrous cycles, lacked corpora lutea in ovaries and were completely infertile, some males maintained the ability to produce sperm and were able to sire litters in 60% of matings. Overall these results indicate that loss of $G\alpha_{q/11}$ in the GnRH neuron does not completely abolish GnRH secretion and that $G\alpha_{q/11}$-independent mechanisms are capable of maintaining partial fertility in adults.

We then investigated if this partial fertility was due to KISS1R signaling through $G\alpha_{q/11}$-independent mechanisms to trigger kisspeptin-dependent GnRH secretion. While the kisspeptin-triggered LH and FSH responses in $Gnaq^{d/d}$ ovariectomized females were diminished by approximately 70-90% compared to littermate controls, they were still significantly greater than the saline-triggered response by 86% and 98% respectively. $Gnaq^{d/d}$ males followed this pattern for kisspeptin-triggered LH, FSH and testosterone secretion. Taken together, these results indicate that in the absence of $G\alpha_{q/11}$ in the GnRH neuron, kisspeptin is still able to trigger significant yet diminished levels gonadotropin and gonadal hormones and therefore KISS1R can maintain partial levels of fertility through $G\alpha_{q/11}$- independent mechanisms in the GnRH neuron. We therefore have shown

that KISS1R signals via $G\alpha_{q/11}$ independent signaling in the GnRH neuron which is a novel signaling mechanism by which the mouse triggers GnRH secretion.

Although we demonstrated that our $G\alpha_{q/11}$ double knockout is highly specific to the GnRH neuron one alternative explanation for the partial fertility in the *Gnaq*$^{d/d}$ mice is direct action of kisspeptin on the pituitary. *Kiss1* and *Kiss1r* expression have also been detected in the mouse pituitary (Kotani et al 2001, Ohtaki et al 2001) and some studies have suggested that KISS1/KISS1R signaling in the pituitary may directly regulate gonadotropin secretion (Kauffman et al 2007, Richard et al. 2009, Gutierrez-Pascual et al. 2007, Chan et al. 2009/2011, Suzuki et al. 2008 and Witham et al. 2013). Two recent independent studies refuted this by creating GnRH-neuronal specific *Kiss1r* knockout mice which demonstrated that regulation of reproduction by KISS1R solely occurs at the level of the GnRH neuron and while it may play modulatory roles in the pituitary and other tissues, the main site of action is at the GnRH neuron (Kirilov et al 2013 and Novaira et al. 2014). Our results agreed with these latter studies, confirming that *Kiss1/Kiss1r* regulation of reproduction is at the level of GnRH secretion and not due to direct action at the level of the pituitary since a GnRH antagonist (antide) abolished the kisspeptin response.

We also confirmed by real-time PCR that the partial GnRH secretion in the *Gnaq*$^{d/d}$ mice was not due to compensatory gene expression of other genes involved in regulating reproduction at the level of the hypothalamus and pituitary. While expression levels were different between *Gnaq*$^{d/d}$ mice and littermate controls, these were expected and can be explained by differences in the hormonal regulation and milieu along the HPG axis of the *Gnaq*$^{d/d}$ mice. The partial function of the GnRH neuron in the *Gnaq*$^{d/d}$ mice was therefore through $G\alpha_{q/11}$- independent mechanisms and we wanted to investigate if it was possible for the other G-proteins in this family, namely *Gna14* and *Gna15* could be involved in compensatory signaling. Functional studies done by Wacker et al. (2008) demonstrated that KISS1R can signal through $G\alpha_{15}$ to activate ERK1/2. However studies done by the Allen Institute for Brain Science show that $G\alpha_{15}$ is not significantly expressed in the mouse hypothalamus and this is supported by assays on pure cultures of immortalized GnRH neuronal cells such as GT1-7 that show $G\alpha_{15}$ to be weakly expressed (Mellon et

al. 1990). Our results corroborated those from those from the Allan Institute which show non-significant hypothalamic $G\alpha_{14}$ expression. There was also no difference in expression levels between *Gnaq*$^{d/d}$ mice and littermate controls indicating that compensation for the loss of *Gnaq* and *Gna11* through *Gna14* is unlikely. In the absence of known published studies, we conducted a functional assay which showed that unlike $G\alpha_{15}$ KISS1R could not signal through $G\alpha_{14}$ to trigger ERK1/2 phosphorylation. Taken together these results demonstrate that $G\alpha_{q/11}$ independent signaling in the GnRH neuron is most likely the explanation for partial fertility in the *Gnaq*$^{d/d}$ male mice and not compensatory signaling through $G\alpha_{14}$ and $G\alpha_{15}$.

An important question that emerges from our findings is whether KISS1R-coupled $G\alpha_{q/11}$-independent signaling is physiologically relevant in the human population? There is evidence among human patients to suggest that it is. Like L148S, another KISS1R mutant, L120P, may meet the criteria for $G\alpha_{q/11}$-independent signaling based on its pharmacological and molecular characterization (Tenebaum-Rakover et al. 2007). Patients bearing the L120P KISS1R mutation were diagnosed with IHH but also displayed variable gonadotropic deficiency ranging from partial to complete. Interestingly, a L102P-bearing male patient who was examined from ages 12 to 21 was observed to undergo progressive changes in pituitary response from an early pubertal to an almost full pubertal pattern (Tenebaum-Rakover et al. 2007). This led the authors to propose that KISS1R inactivation, as seen with L102P, does not impede pubertal onset but instead delays pubertal maturation of the gonadotropic axis. Based on the current study, we suggest that, mechanistically, gradual pubertal maturation among patients bearing KISS1R mutations like L102P is in part due to $G\alpha_{q/11}$-independent, and in particular β-arrestin-dependent, GnRH secretion (Tenebaum-Rakover et al. 2007).

IHH was once considered a permanent condition, but a growing number of studies over the last two decades have reported on the reversal of this condition among some patients. In their study, Sidhoum, et al. (2014), present clinical, laboratory, neuroendocrine, and neuroradiologic characteristics of 308 patients with IHH. Of these, 44 patients, even some with severe IHH, underwent reversal. Patients exhibiting reversal had a higher frequency of mutations affecting neurokinin B (NKB) signaling compared to patients

with no reversal. Perturbations in NKB activity or signaling have been implicated in GnRH deficiency (Topalaglu et al. 2009, Gianetti et al. 2010 and Noel et al. 2014). The NKB receptor, NK3R, is a $G\alpha_{q/11}$-coupled receptor that appears to signal via β-arrestin (Schmidlin et al. 2003) in addition to PKC (Glidewell-Kenney et al. 2013). Thus, it is possible that NK3R, like Kiss1r, regulates GnRH secretion via β-arrestin and therefore some NK3R mutants that are uncoupled from $G\alpha_{q/11}$ might continue to regulate GnRH secretion via β-arrestin (Noel et al. 2014).

In developing novel molecular strategies to treat diseases, it is critical to develop a detailed mechanistic understanding of the signaling pathways that underlie normal and pathological responses. For example, drugs that trigger β-arrestin-coupled KISS1R signaling with great efficacy may prove useful in treating IHH patients who bear $G\alpha_{q/11}$-uncoupled KISS1R mutants that localize to the cell surface and bind Kp. Such treatment may even be extended to patients bearing KISS1R mutants such as R331X that do not localize efficiently to the cell surface (Pampillo et al. 2009) by developing and using other drugs that can rescue mislocalized receptors (Re et al. 2010 and Janovick et al. 2013). The future development of agonists which potently activate the β-arrestin pathway to trigger KISS1R-dependent GnRH secretion is only possible by first recognizing that KISS1R displays $G\alpha_{q/11}$-independent signaling.

Given that reproductive success is paramount to the survival of any species, it is not surprising to find that there is more than one pathway significantly co-regulating GnRH secretion. Thus, reproductive success, though it might be diminished, can still be achieved in animals bearing certain types of inactivating mutations. The current study describes an important and novel discovery that KISS1R signals and triggers LH secretion via the β-arrestin pathway in the male mouse. We suggest that other GPCRs such as NK3R employ β-arrestin to regulate the reproductive endocrine axis at several levels and that the β-arrestin signaling pathway likely represents a major regulator of fertility. We propose that for some patients, $G\alpha_{q/11}$-independent signaling could account for the phenomenon of IHH reversal and hope that our study will stimulate further investigation which may lead to new approaches in the treatment of IHH and several other reproductive endocrine disorders.

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Chapter 3: Conclusion

3.1 General Summary

Since the discovery of the critical role of the KISS1/KISS1R system in mediating GnRH secretion and thus pubertal onset and fertility, our understanding in the field of reproductive neuroendocrinology has dramatically expanded. Studies have demonstrated that central and peripheral administration of kisspeptin potently stimulates gonadotropin release in a wide variety of mammals and has been used to advance pubertal onset in juvenile rats and monkeys while kisspeptin antagonists were used to supress gonadotropin secretion and delay puberty (Shahab et al. 2005, Keen et al. 2008, Guerriero et al. 2012, Han et al. 2005, Messager et al. 2005, Irwig et al. 2004, Navarro et al. 2004, Gottsch et al. 2004, Ramaswamy et al. 2007, Shahab et al. 2005, Plant et al. 2006, Pineda et al 2010, Roseweir et al. 2009, Millar et al 2010). These responses are conserved in humans where kisspeptin administration in both male and female patients elicits a potent gonadotropin response (Dhillo et al. 2005, Dhillo et al. 2007, George et al. 2011, Chan et al. 2012). Recently, Jayasena et al. (2014) successfully used a single KP-54 injection to stimulate egg maturation for *in vitro* fertilization therapy with sub-fertile women resulting in fertilization, implantation, successful term pregnancy and twelve healthy live births. Acute treatment with kisspeptin has also been used to restore reproductive function in women with hypothalamic amenorrhea and in hypogonadal male patients with type II diabetes (Jayasena et al. 2009, George et al. 2013). Long-term or chronic administration of kisspeptin has been found to result in an initial increase in gonodotropin secretion and pulsatility followed by tachyphylaxis, most likely through the desensitization of KISS1R (Jayasena et al. 2009 and Matsui et al. 2012). This response is clinically useful in treating conditions which improve with lower gonadal steroid levels such as benign prostatic hyperplasia, endometriosis, uterine fibroids and polycystic ovarian syndrome (PCOS) (Pinilla et al. 2012 and Millar et al. 2013).

While many potential therapeutic benefits of the KISS1/KISS1R system have been identified, limited knowledge currently exists on the molecular regulation of this system. The work presented in this dissertation has improved the mechanistic understanding of KISS1R signaling and its physiological role in regulating reproduction. Here we describe

that in addition to the major $G\alpha_{q/11}$-dependent pathway, KISS1R also signals through novel $G\alpha_{q/11}$-independent mechanisms to trigger GnRH secretion and we further provide evidence that these mechanisms involve β-arrestins. Although continuous administration of kisspeptin *in vivo* results in long-term reduced gonadotropin response (Thompson et al. 2006, Messager et al. 2005, Jayasena et al. 2009, Matsui et al. 2012), the mechanism behind this tachyphylaxis was not known until we described a role for GRK2 in mediating the homologous desensitization of KISS1R (Pampillo et al. 2009). In that publication we suggested that β-arrestins may also be involved and here we confirmed that both β-arrestin-1 and -2 potentiate the termination of $G\alpha_{q/11}$-dependent KISS1R signaling. Our group was also the first to reveal that kisspeptin stimulation lead to KISS1R- β-arrestin interaction and subsequent ERK1/2 phosphorylation (Pampillo et al. 2009). Using genetically modified MEFs lacking either $G\alpha_{q/11}$ or β-arrestin we demonstrated that kisspeptin-triggered ERK1/2 activation could be both $G\alpha_{q/11}$-independent and β-arrestin-dependent (Szereszewski et al. 2010). Here, using a $G\alpha_{q/11}$-uncoupled KISS1R mutant, L148S, we confirm that kisspeptin is still able to trigger an ERK1/2 phosphorylation response and that this response is largely mediated by β-arrestin. Using mice lacking either β-arrestin isoform, we are the first to demonstrate the physiological significance of these *in vitro* results where a loss of β-arrestin causes a reduced kisspeptin-stimulated gonadotropin response in male mice (Ahow et al. 2014). Since mice lacking both β-arrestin isoforms are not available we decided to create Gnrh neuron-specific $G\alpha_{q/11}$ double knockout mice in order to investigate the collective effect of $G\alpha_{q/11}$-independent mechanisms on fertility. Our data has provided clear evidence that while mice lacking $G\alpha_{q/11}$ in the Gnrh neuron are subfertile and hypogonadal they still maintain some degree of reproductive function which we attribute to $G\alpha_{q/11}$-independent signaling in the Gnrh neuron. Furthermore, we demonstrate that Kiss1r is able to signal through these $G\alpha_{q/11}$-independent mechanisms since kisspeptin treatment results in a modest yet significant increase in gonadotropin secretion and testosterone in these mice. Overall, the findings presented in this dissertation provide evidence of novel signaling mechanisms downstream of the KISS1/KISS1R system in the GnRH neuron which can be used for development of more effective therapies that involve manipulating the HPG axis.

3.2 Future Directions

As our understanding of the KISS1/KISS1R system continues to grow and technical advances enable more indepth experiments, there are many significant avenues which remain to be investigated.

3.2.1 More comprehensive functional assays and long-term follow up of patients with KISS1R mutations

As mentioned previously, the novel discovery that KISS1R can signal independently of $G\alpha_{q/11}$, leads to the realization that IHH patients bearing KISS1R mutations may not be completely infertile and may in fact recover some degree of fertility later in life (Tenebaum-Rakover et al. 2007). *In vitro* assays performed to evaluate the degree of function affected by mutations in KISS1R use $InsP_3$ as a read-out. However as we have shown, while L148S is incapable of stimulating $InsP_3$ formation and PLCβ mobilization it is still capable of stimulating ERK1/2 phosphorylation and since ERK1/2 phosphorylation is a major event involved in GnRH secretion (Castellano et al. 2006), it is plausible to infer that some degree of GnRH secretion is maintained in patients bearing L148S. Therefore there is a need for more detailed functional assays using ERK1/2 phosphorylation and other read-outs with already known $G\alpha_{q/11}$ –uncoupled mutants such as L102P. Although we have shown that GnRH secretion is sustained in mice lacking $G\alpha_{q/11}$ in the GnRH neuron, long-term follow-up of patients with KISS1R mutations is needed to evaluate the ability of the $G\alpha_{q/11}$-independent mechanisims to attain and maintain fertility in humans.

3.2.2 Distinguish molecular events between the $G\alpha_{q/11}$-dependent and $G\alpha_{q/11}$-independent pathways downstream of KISS1R in the GnRH neuron

While ERK1/2 phosphorylation can be mediated by both $G\alpha_{q/11}$-dependent and independent pathways (Szereszewski et al. 2010), we identified a key signaling molecule, Stat3, that is solely phosphorylated by the $G\alpha_{q/11}$-dependent cascade (Figs. 2.11 and 2.12). We recently demonstrated that kisspeptin stimulates Lif (leukemia inhibitory factor) expression in the uterine epithelia which subsequently leads to activation of the

Lif receptor (gp130) and downstream Stat3 –mediated gene transcription necessary for embryo implantation in the mouse (Calder et al. 2014). Therefore it is not surprising that Stat3 may also play a role in modulating gene transcription downstream of the KISS1/KISS1R system in the hypothalamus. Further studies are necessary to elucidate this response but here we show that Kp-triggered Stat3 phosphorylation is $G\alpha_{q/11}$-specific response since HEK 293 cells transfected with L148S fail to demonstrate this response (Figs. 2.11 and 2.12). This not the first instance where β-arrestin and G-protein pathways differentially regulate factors affecting gene expression. For $AT_{1a}R$, PAR2 and NK1 receptors, β -arrestin-MAPK signalosomes have been described to sequester ERK1/2 in the cytosol, most likely due to the large size of the complex, whereas ERK1/2 that has been phosphorylated by the G-protein pathway is capable of translocating to the nucleus where it triggers Elk-1 driven gene transcription (Luttrell, et al. 2001, DeFea et al. 2000, Luttrell et al. 2010). The stability of the β-arrestin-receptor complex however has a significant role to play in determining the localization and effect of downstream signaling molecules like ERK1/2 specifically, class A GPCRs with more stable β-arrestin interaction tend to demonstrate cytosolic retention of ERK1/2 while some class B receptors like β_2AR and LPA1 have demonstrated ERK1/2 nuclear translocation (Getsy-Palmer et al. 2005 and Shenoy et al. 2006). The overall functional role for this differentiated response is not fully understood but it is thought that it allows the receptor to direct signaling down different pathways since by retaining ERK1/2 in the cytosol more non-nuclear substrates are phosphorylated for example which may include alternative transcription factors that go to the nucleus instead of ERK1/2 (Pearson et al. 2001, Elorza et al. 2000). By distinguishing the $G\alpha_{q/11}$-dependent and independent pathways mediating GnRH secretion we would be better able to develop clinical treatments for specific conditions.

3.2.3 Further elucidate the β-arrestin-mediated KISS1R signaling pathway

We showed that β-arrestin mediates KISS1R homologous desensitization in the presence of continuous kisspeptin administration (Fig. 2.13). We also demonstrated that β-arrestin mediated Gnrh secretion in mice after a single bolus of KP-54 (Fig. 2.4). In order to

harness the therapeutic capacity of kisspeptin we must further understand more about the β-arrestin mediated KISS1R response. For example it may be possible for continuous kisspeptin treatment to be given to trigger KISS1R desensitization resulting in downregulation of the HPG axis (discussed in detail in Chapter 1). Alternatively, in cases where upregulation of the HPG axis is desirious, stimulation with synthetic β-arrestin-biased agonists that stimulate KISS1R to preferentially signal through β-arrestin when the $G\alpha_{q/11}$ pathway to stimulate gonadotropin and gonadal steroid release (discussed in detail in Chapter 1).

While KISS1/KISS1R plays an important role in regulating the HPG axis, there is evidence that it is involved in other organ systems. Therefore improved knowledge of the signaling mechanisms downstream of KISS1R may also be used to treat disease conditions in these systems. KISS1R is expressed in the pancreas (Kotani et al. 2001) and kisspeptin has been shown to have stimulatory effects on insulin secretion (Sonada et al. 2008, Silvestre et al. 2008 and Ravier et al. 2014). Although the mechanism behind this is not fully understood it may provide a novel therapeutic avenue for treatment of diabetes. KISS1 is also highly expressed in the placenta in the first trimester (Bilban et al. 2004, Horikoshi et al. 2003) and KISS1R has been shown to be involved in the placental attachment and invasion process (Taylor et al. 2013). KISS1/KISS1R may therefore be manipulated in novel treatments for placental insufficiency and preeclampsia. Lastly, initially discovered as a metastasis suppressor, (Lee et al. 1999) KISS1R continues to be described in studies as an inhibitor of tumor metastasis (Nash et al. 2007 and Cvetnovic et al. 2013). In addition to manipulating fertility in humans, the Kiss1/Kiss1r system can also be a valuable tool for maximising livestock breeding and propogation of endangered species in zoos and wildlife reserves. Overall, further understanding of the KISS1/KISS1R signaling system is essential for implementation of effective treatments in a wide variety of applications.

3.2.4 Investigate if kisspeptin can restore fertility in *Gnaq*$^{d/d}$ females

We showed that while *Gnaq*$^{d/d}$ females were completely infertile and were not able to ovulate as evidenced by lack of corporea lutea (CL) in the ovaries (Figs. 2.20 and 2.21) they were still able to respond to kisspeptin measured by increased gonadotropin release and were also able to sporadically enter estrus and undergo puberty eventually (Figs. 2.18, 2.19 and 2.22). In patients with impaired $G\alpha_{q/11}$ pathways in the GnRH neuron, determining the therapeutic potential of the $G\alpha_{q/11}$- independent pathway may be very beneficial for rescuing their fertility. Future studies with the *Gnaq*$^{d/d}$ females may involve combining regular superovulation protocols involving HCG and PMSG injections (Calder et al. 2014) with KP-54 administration. Improvement in fertility would be determined by detection of CL in the ovaries and/or successful breeding resulting in birth of full-term litters.

3.2.5 Further confirm kisspeptin triggered response in *Gnaq*$^{d/d}$ mice is as a result of $G\alpha_{q/11}$-independent pathways in GnRH neurons

Both parental lines used in the creation of the *Gnaq*$^{d/d}$ mice were extensively characterised in other publications and shown to result in effective conditional knockout of various genes (discussed in Chapter 2 Results 2.3.4.1 -2.3.4.2). Additionally we performed detailed and extensive experiments which show that Cre recombinase expression is restricted to the hypothalamus and is active as early as embryonic day 18 (E18) (Fig. 2.14). We also used a ROSA26::loxP-Stop-loxP-GFP reporter mouse to further show that the spatial distribution of Cre recombination co-localized strongly with approximately 98% of the Gnrh neuronal population (Fig.2.15). However due to the lack of effective $G\alpha_q$ antibodies for immunoflouresence as well as the scattered spatial distribution of hypothalamic Gnrh neurons we are not able to directly measure the degree of $G\alpha_q$ loss in Gnrh neurons. To further support our data, we can directly measure the gonodatropin response in *Gnaq*$^{d/d}$ mice where kisspeptin is administered intracerebroventricularly (i.c.v) and compare this response to mice pre-treated with GP2A ($G\alpha_q$ specific inhibitor). We would expect that GP2A pre-treatment would not have an effect on kisspeptin-triggered gonadotropin response in the *Gnaq*$^{d/d}$ mice since we

hypothesize that this is due to $G\alpha_{q/11}$ –independent mechanisms. Intracerebroventricular (i.c.v) administration (directly into the third ventricle of the brain) would be important for this study as it sequesters the $G\alpha_q$ specific inhibitor near to the Gnrh neurons and prevents it from affecting the pituitary where Gnrhr signals via $G\alpha_{q/11}$ in gonadotropes to stimulate LH and FSH release.

3.2.6 Elucidate other major molecules involved in the $G\alpha_{q/11}$-independent pathway

We demonstrated that β-arrestin is involved in the $G\alpha_{q/11}$-independent response in Gnrh neurons (Figs. 2.4 and 2.8). However, we do acknowledge that β-arrestin may not be the only molecules involved in the $G\alpha_{q/11}$-independent pathway. Some studies suggest that some GPCRs which couple to the $G\alpha_q$ protein family also tend to couple with and activate $G\alpha_{12}/G\alpha_{13}$ proteins resulting in p115RhoGEF activation (Dagmara et al. 2005, Offermanns review 2003 and Lambert et al. 2008). Kotani et al. (2001) showed that in CHO cells, kisspeptin stimulates stress fibre formation through activating the Rho subfamily of G-proteins and therefore $G\alpha_{12}/G\alpha_{13}$ proteins may be another component in the $G\alpha_{q/11}$-independent response in Gnrh neurons. Other studies have even shown that kisspeptin transactivates the epidermal growth factor receptor (EGFR) in breast cancer cells and thus may also contribute to the $G\alpha_{q/11}$-independent response in the GnRH neuron (Zajac et al. 2011 and Cvetkovic et al. 2013).

3.3 Experimental Limitations

3.3.1 Kiss1r triggers LH secretion via β-arrestin (Figure 2.4)

To test the hypothesis that KISS1R stimulates GnRH secretion via the β-arrestin pathway we used single β-arrestin knockout mice (Fig. 2.4). While a mouse model completely lacking both *Arrb1* (encodes for β-arrestin-1) and *Arrb2* (encodes for β-arrestin-2) would be ideal for testing this hypothesis, full body *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice are perinatal lethal due to pulmonary hypoplasia (Zhang et al. 2010, Conner et al. 1997, Bohn et al. 1998). Floxed β-arrestin mice have not been created yet thereby precluding the creation of Gnrh-neuron specific *Arrb1*$^{-/-}$;*Arrb2*$^{-/-}$ dKO mice.

3.3.2 L148S displays $G\alpha_{q/11}$-independent signaling (Figures 2.5 – 2.12)

To test the hypothesis that L148S, a $G\alpha_{q/11}$ –uncoupled KISS1R mutant, is still able to signal and that this is mediated by β-arrestins we expressed L148S in HEK 293 cells. Previous publications showed that although HEK 293 cells are not representative of the GnRH neuron they do express low levels of KISS1R endogenously and as such provide a strong model in which to investigate molecular signaling mechanisms (Pampillo et al. 2009). Although GnRH neuronal cell cultures are available such as the GT1-7s (used in Fig. 2.13) these cells are very slow to propagate with a doubling time of 36 – 48 hours that is further reduced under antibiotic selection in the creation of shRNA knockdown stable colonies (Mellon et al. 1990). Additionally, similar to other neuronal cultures, GT1-7s are very difficult to transfect requiring chemical reagents such as Lipofectamine in order to have sufficient expression levels. This makes transient expression experiments very expensive and not feasible to perform regularly. New hypothalamic Gnrh neuronal lines have been created and while the GT1-7s are well established and have been used extensively, these newer lines may prove to be less technically challenging for future experiments (Belsham et al. 2004 and Mayer et al. 2009).

Our previous studies utilized $G\alpha_{q/11}$ double knockout MEFs (Szereszewski et al. 2010) to investigate the ability of WT KISS1R to signal independently of $G\alpha_{q/11}$. Here we used a different approach by expressing a $G\alpha_{q/11}$ –uncoupled KISS1R mutant, L148S, in HEK

293 cells to investigate if signaling was still possible. While there is the possibility that L148S-dependent ERK1/2 activation (Figs. 2.5 – 2.8) may be due to undetectable levels of $G\alpha_{q/11}$-dependent signalling we validated with two functional assays: $InsP_3$ formation and PLC mobilization, that L148S is indeed uncoupled from the $G\alpha_{q/11}$ pathway. We could have used $G\alpha_{q/11}$ double knockout MEFs to address this issue of potential residual $G\alpha_{q/11}$ signaling downstream of L148S however this this system also removes $G\alpha_{q/11}$ from all other receptors and signaling pathways, some of which may be involved in the physiological response. Our approach was a further improvement on the previous study since MEFs are derived from mice and therefore not a human based model like the HEK 293 cells and since L148S is an actual KISS1R mutation identified in humans diagnosed with IHH.

3.3.3 KISS1R signals via $G\alpha_{q/11}$ –independent mechanisms to trigger LH secretion and attain and maintain fertility (Figures 2.14- 2.23)

To investigate the role of the $G\alpha_{q/11}$-independent pathway in pubertal onset, fertility and kisspeptin-triggered gonadotropin release, we used mice lacking both *Gnaq* and *Gna11*expression in the Gnrh neuron referred to here as the $Gnaq^{d/d}$ mice (discussed in Chapter 2 Results 2.3.4 – 2.3.5). In order to circumvent the phenotypes associated with global loss of these genes (Chapter 1 Table 1.4), the $Gnaq^{d/d}$ mice bear Gnrh-neuronal specific deletion of *Gnaq* in the context of a global deletion of *Gna11*. In addition to yielding viable mice, this approach renders the advantage of specifically targeting the Gnrh neuron and not other hypothalamic cells. Both parental lines used to create the $Gnaq^{d/d}$ mice have been extensively used in the literature and reports confirm highly effective and specific conditional genetic recombination in each of those studies (Skynner et al. 1999, Yoon et al. 2005, Wintermantel et al. 2006, Kawakowsky et al. 2012, Schmid et al. 2013, Offermanns et al. 1997, Dagmara et al. 2005, Wettschureck et al. 2006, Wettschureck et al. 2007, Kero et al, 2007). Therefore we were fairly certain that we would observe a similar level of *Gnaq* ablation in the Gnrh neuron with the $Gnaq^{d/d}$ mice.

However, due to the lack of commercially available robust $G\alpha_q$ antibodies and the scattered distribution of the Gnrh neuronal population throughout the hypothalamus, we

were unable to directly demonstrate the loss of $G\alpha_q$ in all Gnrh neurons. To address this we investigated the spatial and temporal activation of Cre recombinase using RT-PCR and ROSA26::loxP-Stop-loxP-GFP reporter mice. We found that *Cre recombinase* expression and action were specific to the hypothalamus of the *Gnaq*$^{d/d}$ mice and not expressed elsewhere along the HPG axis (Fig. 2.14). While we only examined the female HPG axis (Fig. 2.14D) we would expect that if *Gnrh-Cre* was expressed in the male pituitary or testis that fertility would be further decreased. We see the opposite however since male *Gnaq*$^{d/d}$ mice are more fertile than females thereby providing further evidence for the hypothalamic specificity of *Gnrh- Cre recombinase* expression. We also found that *Cre recombinase* activity occoured as early as E18 and strongly colocalized with at least 97% of Gnrh neurons (Fig. 2.15) thus confirming existing studies with this *Gnrh-Cre* mouse strain (Skynner et al. 1999, Yoon et al. 2005 and Wintermantel et al. 2006). Mayer and Boehm (2011) demonstrated that only 10% of the entire Gnrh population is enough to sustain pubertal onset and fertility. Since the *Gnaq*$^{d/d}$ mice demonstrate a distinct phenotype with significant delays in pubertal onset and infertility, it would be fair to deduce that genetic recombination and ablation of *Gnaq* was more than 90% effective in Gnrh neurons. In addition to being a highly efficient and specific system, *Gnrh-Cre* is activated very early in development so that there is sufficient time to allow for complete enzymatic recombination of Gnaq prior to puberty (7 -8 weeks in this case).

Overall, while we were not able to directly demonstrate the loss of $G\alpha_q$ in all Gnrh neurons we are confident that efficient ablation of *Gnaq* and *Gna11* occoured in the majority of Gnrh neurons and is therefore an ideal model for these studies.

3.4 Final Conclusions

Our study is the first to report on novel $G\alpha_{q/11}$-independent pathways downstream of KISS1R in the GnRH neuron and to describe the role of β-arrestin in mediating this pathway. We also identified β-arrestin is a molecule involved in the rapid homologous desensitization of KISS1R. In addition to improving our understanding of the molecular regulation of the KISS1/KISS1R system, these results also highlight the physiological importance of these signaling pathways on fertility and reproductive capacity. It is our hope that the work presented here will fuel further research and development of more effective treatment strategies for disorders involving HPG axis dysregulation.

3.5 References

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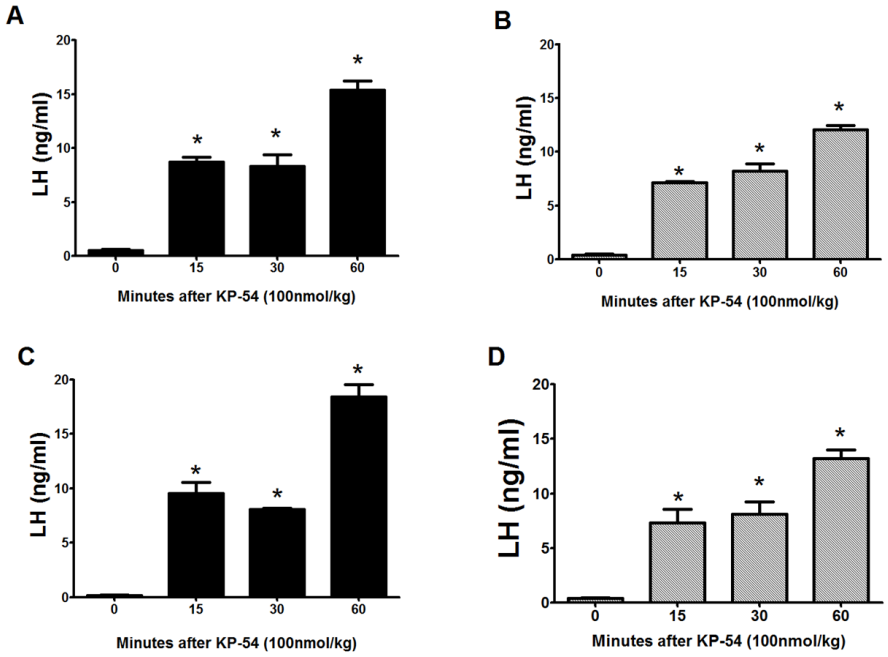
Appendix 1: Time-course for KP-54 triggered LH secretion in β-arrestin deficient mice

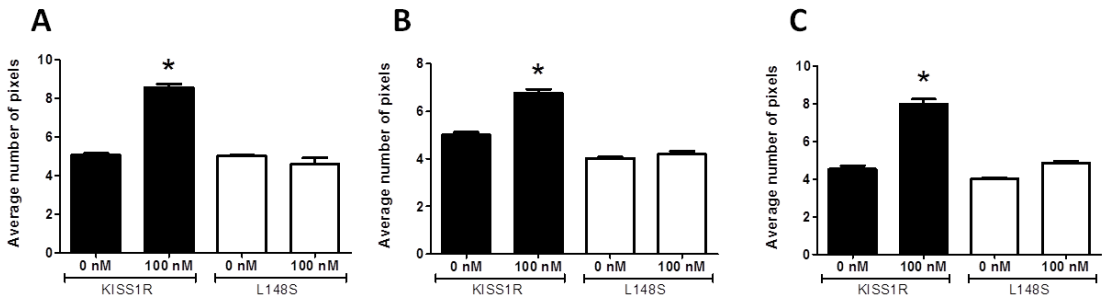


Appendix 1: Both *Arrb1*$^{-/-}$ or *Arrb2*$^{-/-}$ mice along with littermate controls demonstrate maximal LH release 60 min after a single KP-54 injection.

Serum LH levels were measured by RIA. Mice were either left untreated or treated with 100 nmol KP-54/kg body weight and blood was collected 15, 30 and 60 min later. KP-54-triggered LH levels in all mice were significantly higher (*P < 0.001) compared to their respective untreated controls. For all genotypes 60 min following KP-54 administration resulted in the most robust serum LH response. For (A) *Arrb1*$^{+/+}$ and (B) *Arrb1*$^{-/-}$ mice and for (C) *Arrb2*$^{+/+}$ and (D) *Arrb2*$^{-/-}$ mice, bars represent a mean of 3-6 mice for each time-point[6]. Only male mice were used to preclude the effects of estrous cycle variation on GnRH secretion in females. Error bars represent SEM.

[6] Because *Arrb1* and *Arrb2* mice were generated in different strains we evaluated the WT littermates individually even though genotypically they were identical

Appendix 2: Signaling molecules differentially regulated by the $G\alpha_{q/11}$-independent pathway



Appendix 2: Other molecules which are differentially phosphorylated by the $G\alpha_{q/11}$-independent pathway downstream of L148S.

Densitometric analysis of specific signaling molecules on the Human Phospho-Kinase Array in Figure 2.11 (A) p38 (B) CREB and (C) RSK1/2/3[7]. After KP-10 treatment, KISS1R expressing HEK 293 cells showed a significant increase in p38, CREB and RSK1/2/3 phosphorylation (*P < 0.05) while cells expressing L148S failed to demonstrate any significant response. Densitometric analysis represents the mean of 3 independent experiments where mean pixel intensity is normalized to reference controls on array in Figure 2.11. Error bars represent SEM.

[7] Absense of activation of STAT3 in Fig. 2.11 as well as CREB and RSK1/2/3 suggest that the $G\alpha_{q/11}$-independent pathway downstream of L148S may not mediate changes in gene expression since these are all transcriptional factors.

Appendix 3: Animal Protocol Ethics Approval

Appendix 3: Animal Protocol Ethics Approval Form

AUP Number: 2008-017-03

PI Name: Babwah, Andy V

AUP Title: **An In Vivo Analysis Of Kisspeptin/gpr54 (kiss1r) Signaling In Human Reproduction**

Approval Date: 10/15/2012

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Submitted by: Copeman, Laura

on behalf of the Animal Use Subcommittee University Council on Animal Care

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Format Electronic

Portion chapter/article

Title or numeric reference of the portion(s) Introduction, Materials and Methods, Results, Discussion, References, Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7, Figure 8.

Title of the article or chapter the portion is from KISS1R Signals Independently of Gq/11 and Triggers LH Secretion via the B-Arrestin Pathway in the Male Mouse

Editor of portion(s) Endocrine Society

Author of portion(s) Maryse Ahow, Le Min, Macarena Pampillo, Connor Nash, Junping Wen, Kathleen Soltis, Rona S. Carroll, Christine A. Glidewell-Kenney, Pamela L. Mellon, Moshmi Bhattacharya, Stuart A. Tobet, Ursula B. Kaiser, and Andy V. Babwah

Volume of serial or monograph 155

Page range of the portion None

Publication date of portion Published online August 27 2014

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Creation of copies for the disabled no

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For distribution to United States and Canada

In the following language(s) Original language of publication

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The requesting person/organization is: Maryse R Ahow

Order reference number None

Author/Editor Maryse R. Ahow

The standard identifier Maryse R. Ahow 2014

Title Pathway Specific Signaling and its Impact on Fertility: A focus on the Kisspeptin Receptor, Kiss1r

Publisher University of Western Ontario

Expected publication date Nov 2014

Estimated size (pages) 214

Total (may include CCC user fee) 0.00 USD

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Ahow MR and Babwah A., 2011 β-arrestin mediates KISS1R desensitization. Poster presents at *Paul Harding Research Day*, U.W.O.