THE GNRH RECEPTOR-ASSOCIATED MEMBRANE RAFT PROTEOME IN

MOUSE GONADOTROPE CELLS

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THE GONADOTROPIN-RELEASING HORMONE RECEPTOR AND

ASSOCIATED MEMBRANE MICRODOMAIN

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Gonadotropin releasing hormone (GnRH) is the central hormone of reproduction in
vertebrates. This hormone is secreted from the hypothalamus in response to
environmental, steroid hormone feedback and other stimuli in a pulsatile manner and
travels via the hypophyseal portal vasculature to the anterior pituitary. GnRH binding
to its receptor on the surface of pituitary gonadotropes stimulates the release of the
gonadotropin hormones: luteinizing hormone (LH) and follicle stimulating hormone
(FSH), heterodimers of the common α subunit with the hormone-specific β subunits.
In addition to secretion of gonadotropin hormones, GnRH stimulates the transcription
of the gonadotropin subunit genes and that of its own receptor (GnRHR). The GnRHR
has been shown in recent years to be a constitutive occupant of membrane raft
microdomains within the plasma membrane. GnRHR association with these
microdomains appears to be required for the initiation of downstream signaling
processes within the GnRH signaling network including activation of the mitogen-
activated protein kinase, extracellular signal regulated kinase (ERK). GnRHR-induced
ERK activation is absolutely required for gonadotropin subunit gene expression and
fertility in mice. In this dissertation the components of the GnRHR-associated
membrane raft microdomain are explored providing insight into how the receptor

might be connected to membrane microdomains, the actin cytoskeletal network, and the initiation of downstream transcriptional events. These studies introduce the flotillin/reggie proteins and β catenin as novel members of the GnRHR-associated membrane raft proteome in addition to identifying a list of proteins for future studies.

BIOGRAPHICAL SKETCH

Krystal H. Allen was born in Panorama City, California in February of 1983. She developed a love of animals and science while growing up between the high desert of the Antelope Valley and the suburbs of Los Angeles. Krystal was always very driven to succeed in her studies; she completed her bachelor's degree in animal science in three years and was honored as a President's Council Scholar and a University Scholar (formerly Kellogg Scholar), two of the highest honors awarded at her undergraduate institution. She graduated *summa cum laude* as the co-valedictorian of the College of Agriculture from the California State Polytechnic University, Pomona in 2004. Krystal was also very active in her college community holding officer positions in the Pre-Veterinary, Ag Council, and Ag Ambassadors clubs and earning the Student Leader of the Year award for the College of Agriculture in 2004. When faced with the difficult decision of where to go to graduate school, she boldly chose to apply to the highly selective dual DVM/PhD combined degree program at the highest ranked veterinary college in the U.S., Cornell University. Krystal was accepted into the dual degree program at Cornell in the fall of 2004 and has continued to distinguish herself as a leader in her community. She participated in the Veterinary Leadership Program in 2005, and was a founding member of the Graduate students, Residents and Post-docs Experiencing Synergy (GRAPES) club of Biomedical Sciences in 2007. In 2010 she graduated from the veterinary portion of her program earning a Doctor of Veterinary Medicine degree. Krystal then focused all of her efforts on completing her thesis research, the results of which are presented in this dissertation.

This dissertation is dedicated to all of the people who have helped me to succeed and become the woman I am today.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
αGSU	glycoprotein subunit alpha
LH	luteinizing hormone
FSH	follicle stimulating hormone
ERK	extracellular signal regulated kinase
TGFβ	transforming growth factor beta
mRNA	messenger ribonucleic acid
GPCR	G-protein coupled receptor
TMD	transmembrane domain
IP3	inositol 1,4,5-trisphosphate
DAG	diacylglycerol
Ca2+	calcium ion
AA	arachidonic acid
PKC	protein kinase C
MAPK	mitogen activated protein kinase
PLC	phospholipase C
SAPK/JNK	stress activated protein kinase/ Jun N-terminal kinase
Elk-1	E twenty-six-like transcription factor-1
Egr-1	early growth factor-1
Ets	E twenty-six

SRE	serum response element
Raf-1	rapidly accelerated fibrosarcoma-1
Ras	rat sarcoma
VGCC	voltage-gated calcium channel
Pyk2	proline-rich tyrosine kinase
MEK	MAPK/ERK Kinase
MKK-7	dual specificity MAP kinase kinase-7
PAK-1	p21 activated kinase
AP-1	activating protein-1
c-Jun	jun proto oncogene
c-Fos	Finkel-Biskis-Jinkins murine osteogenic sarcoma virus
ATF	activating transcription factor
MKP	MAP kinase phosphatase
DUSP	dual specificity phosphatase
Wnt	wingless
APC	adenomatous polyposis coli
GSK-3β	glycogen synthase kinase-3beta
TCF/LEF	T cell factor/lymphoid enhancer factor
siRNA	small interfering RNA
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP family Verprolin-homologous protein
FAK	focal adhesion kinase
c-Src	cellular-sarcoma

ARP   actin-related protein

DRM   detergent-resistant membrane

FLOT   flotillin

Chapter 1: Literature Review

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is the central hormone of reproduction in vertebrates. This hormone is secreted from the hypothalamus in response to environmental, steroid hormone feedback and other stimuli in a pulsatile manner and travels via the hypophyseal portal vasculature to the anterior pituitary. A small subset of cells (5-8%) within the anterior pituitary expresses the four signature genes associated with gonadotropes: the GnRH receptor (GnRHR), the common α glycoprotein subunit (αGSU), the luteinizing hormone β subunit, and the follicle stimulating hormone β subunit. GnRH binding to its receptor on the surface of pituitary gonadotropes stimulates a variety of intracellular signaling events culminating in the release of the gonadotropin hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH), heterodimers of the common α subunit with the hormone-specific β subunits. In addition to secretion of gonadotropin hormones, GnRH also stimulates the transcription of the gonadotropin subunit genes as well as stimulating the transcription of its own receptor. The GnRHR has been shown in recent years to be a constitutive occupant of membrane raft microdomains within the plasma membrane. GnRHR association with these microdomains underlies a functional necessity for the initiation of some downstream signaling processes within the GnRH signaling network including activation of the mitogen-activated protein kinase, extracellular signal regulated kinase (ERK). GnRHR-induced ERK activation is absolutely required for gonadotropin subunit gene expression and fertility in mice.

AN OVERVIEW OF THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Gonadotropin releasing hormone (GnRH) is a decapeptide hormone that is central to successful reproductive function in most vertebrate species and all mammals. Neurons which secrete GnRH are initially located within the olfactory epithelium, but early in development these neurons migrate to the hypothalamus where they colonize primarily the Preoptic-septal area, and to a lesser extent the Mediobasal and Arcuate nucleus areas [1-5]. Meanwhile, the cells that will make up the anterior pituitary (adenohypophysis) begin as ectodermal cells in the pharynx from which an evagination known as Rathke's pouch expands to meet an evagination from the diencephalon which will become the posterior pituitary (neurohypophysis) [6, 7]. The anterior and posterior pituitary lobes will fuse to form the pituitary gland. Despite being a single gland, the two halves are distinct with the anterior pituitary consisting of endocrine cells while the posterior pit is composed of neuronal axons to "cell bodies" within distinct hypothalamic nuclei of the hypothalamus [8-11]. Unlike the neurohypophysis, the anterior pituitary is derived from epithelial cells and does not have a direct axonal connection to the hypothalamus. Instead a unique vascular system carries blood from capillary loops in the median eminence into long portal veins, and finally into sinusoids which bathe the anterior pituitary in blood carrying hormones secreted by the hypothalamus. This is known as the hypophyseal portal system and it allows the anterior pituitary to experience concentrations of hypothalamic hormones that are much higher than those seen by the rest of the body through systemic circulation [12-14].

There are five endocrine cell types present in the anterior pituitary including somatotropes, corticotropes, lactotropes, thyrotropes and gonadotropes [14]. GnRH is secreted from neurons in the hypothalamus in response to physiologic stimuli and is carried by the hypophyseal portal circulation to the anterior pituitary. In the anterior pituitary, GnRH binds to its receptor (GnRHR) on the surface of gonadotrope cells where it stimulates, among other things, the release of the glycoprotein hormones luteinizing hormone (LH), and follicle stimulating hormone (FSH) [15, 16]. Gonadotrope cells of the anterior pituitary respond to varied amplitude and frequency of GnRH by secreting FSH and/or LH into systemic circulation where these hormones exert their effects primarily upon the gonads [17] (**Figure 1**). It appears that GnRH pulse frequency plays a role in which of the gonadotropins are secreted with higher frequency pulses of GnRH preferentially triggering LH release and lower frequency pulses leading to FSH secretion [18]. Similarly, increasing pulse frequency of GnRH is correlated with increased LHβ transcription and slower frequency GnRH release is associated with increased FSHβ gene transcription [19-22].

In addition to GnRH, other factors help to regulate the production and release of FSH including activins, inhibins, and follistatin. Activins and inhibins are members of the transforming growth factor β (TGFβ) family which exert their influence primarily through autocrine and paracrine secretion at all levels of the reproductive axis [23, 24]. Activins are secreted locally and bind to their receptor on the surface of gonadotrope cells to help establish and maintain a permissive environment for the action of GnRH on FSH production while GnRH itself is capable of modulating activin/inhibin mRNA levels [20, 25, 26]. Inhibins on the other hand bind their own receptor (betaglycan) and act by antagonizing the actions of activins on FSH synthesis and

Figure1 Overview of the Hypothalamic-Pituitary-Gondadal Axis: Neurons in the hypothalamus secrete GnRH in response to physiologic stimuli. The GnRH peptide is carried by the hypophyseal portal system to the anterior pituitary where it binds to its receptor on the surface of gonadotrope cells. Gonadotrope cells respond to GnRH stimulation with pulsatile release of LH and FSH. Activin and Follistatin are also released by cells in the anterior pituitary, providing autocrine and paracrine feedback. The gonadotropin hormones (LH and FSH) reach the gonads via systemic circulation and stimulate spermatogenesis in the male and follicular development or ovulation in the female. Additionally, the gonads are stimulated to produce the sex steroid hormones as well as other factors including inhibin and activin. These hormones can circulate to provide feedback to the hypothalamus and pituitary as well as stimulate the development and maintenance of secondary sex characteristics.

Figure 1

External and Internal cues

Hypothalamus

GnRH

Activin, Follistatin

Ant. Pituitary

Post. Pituitary

Inhibins

Sex Steroids

LH   FSH
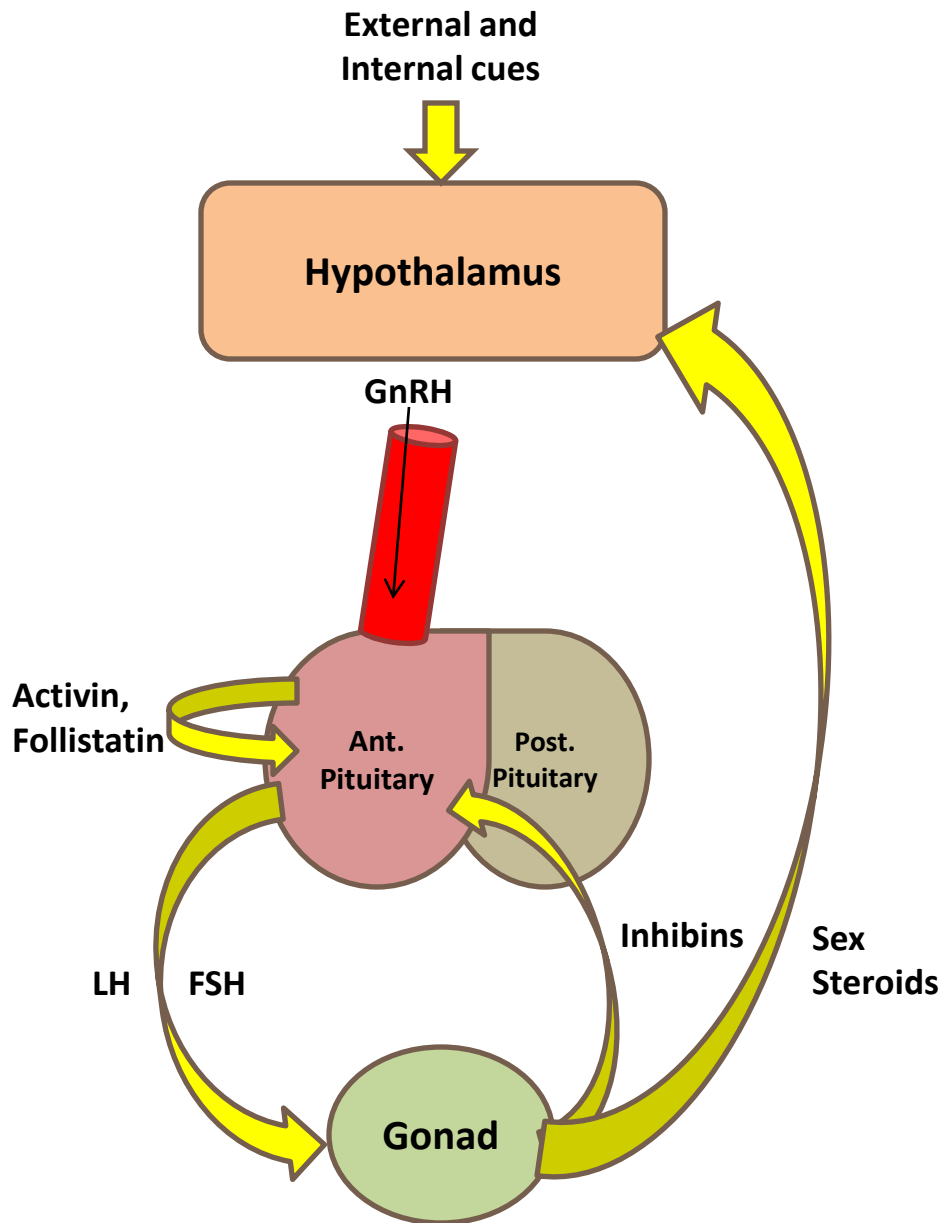
Gonad

secretion [27-29]. In contrast to activins, endocrine secretion of inhibins from the gonads appears to play a more prominent role in its modulation of pituitary gonadotropes, although inhibin is produced locally within the anterior pituitary [23, 24, 30, 31]. Meanwhile follistatins are activin-binding proteins capable of "bio-neutralizing" activins, masking the binding site for its receptor [32-35]. Activins are also capable of inducing the expression of follistatin [36] thereby establishing a "self-limiting reciprocal feedback loop" [27]. From the work that has been done so far, it is clear that regulation of FSH synthesis and secretion from the anterior pituitary is complex, dynamic, and under the influence of a variety of factors. Interestingly, it has recently been shown that activins can also regulate LHβ gene expression under certain conditions [37, 38], suggesting that LH production and release may not be as straightforward as previously thought.

In females, FSH stimulates follicular growth and maturation early in the follicular phase of the menstrual and estrous cycle, while LH stimulates growth during the pre-ovulatory phase, then induces ovulation and the formation of a corpus luteum [39-41]. In the male, FSH acts on Sertoli cells to stimulate spermatogenesis, while LH acts primarily on Leydig cells regulating the production and release of androgens [39, 42, 43]. The gonads also release steroid hormones such as estrogen, progesterone or testosterone which provide mostly negative feedback to the hypothalamus and pituitary allowing for control of reproductive timing and success [44].

THE GnRH RECEPTOR

6

In most vertebrates studied at least two, but often three forms of GnRH are present [45-56]. The GnRH first identified in the hypothalamus in mammals was termed GnRH I, with a subsequently discovered GnRH in chickens being termed GnRH II [45, 57, 58]. Interestingly it is GnRH II which has been most conserved from bony fish to humans suggesting that this form of GnRH serves an important evolutionary role [59]. A third form of GnRH was discovered in salmon and termed GnRH III. At present this form of GnRH has only been identified in teleost fish [60]. The existence of three GnRH forms hints that there should be three receptors, one for each form of GnRH. Indeed three types of GnRHRs have been identified, but most were named in order of discovery or based on pharmacologic characteristics [59]. Millar and coworkers examined the different types of GnRHR discovered in species to date and have suggested a more systematic classification into type I, II, or III categories based on phylogenetic relatedness and sequence similarity [59]. Under this system, the type I GnRHRs have the highest affinity for GnRH I, although they are also capable of binding GnRH II. Meanwhile the type II GnRHR is highly selective for GnRH II and does not bind GnRH I in most cases. Interestingly, the type II GnRHR sequence in humans, chimpanzees, cows and sheep contains a frameshift mutation resulting in an internal stop codon and no functional receptor has been identified in these species [59, 61]. Considering the strong evolutionary conservation of GnRH II, it seems that in these species the type I GnRHR must bind and mediate the effects of GnRH II [59, 62]. The type III GnRHR has been identified in the bullfrog and several types of teleost fish, but not in mammals [59, 63].

The complementary DNA encoding the mammalian type I GnRHR was first cloned from mouse mRNA in 1992 [64, 65]. Analysis of the cDNA revealed the mammalian GnRHR to be a seven transmembrane spanning domain G-protein coupled receptor (GPCR) with a very short

7

intracellular carboxyl-terminal domain [65-67]. In other GPCRs, the C-terminal tail is typically the site of agonist-induced receptor phosphorylation and β-arrestin binding to target the receptor for internalization. As might be expected the type I GnRHR, which lacks this tail, does not rapidly desensitize. Neither does the type I GnRHR appear to bind β-arrestin or internalize via a dynamin-dependent mechanism [68-72]. For this reason the mammalian type-I GnRHR has been called a naturally occurring desensitization and internalization resistant mutant [72]. Interestingly the type II GnRHR, which occurs in mammals as well as other vertebrates, does possess a C-terminal tail and is capable of rapid homologous desensitization, binding of β-arrestin and internalization via a dynamin-dependent mechanism unlike its type I mammalian counterpart [70-73]. Indeed, when the C-terminal tail of the catfish type II GnRHR was added to the type I rat GnRHR, the chimeric receptor was then shown to rapidly desensitize pointing to the absence of the C-terminal tail as the reason for the type I receptor's slow desensitization kinetics [70]. These findings point to the type I mammalian GnRHR as unique, not only among GPCRs, but also within the family of other GnRHRs. For the purposes of this chapter we will focus on the type I mammalian GnRHR expressed in pituitary gonadotropes, referring to it simply as GnRHR.

The GnRHR is also distinguished by alterations in several key amino acid residues. The first of these relates to the highly conserved D and N residues located in transmembrane domain (TMD) 2 and TMD7 of other GPCRs, respectively. In the GnRHR these residues are effectively reversed relative to their orientation in other GPCRs with N replacing D at position 87 in TMD2 and D replacing N at position 318 within TMD7. Thus, the GnRHR appears to display a naturally occurring reciprocal mutation of these 2 highly conserved residues. Mutational studies

have revealed N^{87} to be critical for ligand binding and signaling while D^{318} appears to be necessary for coupling to inositol 1,4,5-trisphosphate (IP_3) production [74, 75]======. Another unusual feature of the GnRHR is a modified DRY motif at the junction of TMD3 and the second intracellular loop. In most other GPCRs this motif is critical for proper initiation of intracellular signaling. However in the GnRHR, S^{140} replaces the highly conserved Y residue. Mutational studies to replace S140 with Y demonstrated increased the affinity of ligand binding and increased the rate of internalization. In these studies G-protein coupling did not appear to be effected [77].

GnRHR-ASSOCIATED SIGNALING

The GnRHR, like other GPCRs, changes conformation upon ligand binding which results in dissociation of the β and γ subunits from the α subunit of its associated heterotrimeric G-protein. In pituitary gonadotropes this most often involves the $G\alpha_{q/11}$ subunit [78], although other Gα subunits have been implicated in other cell types. For example, it appears that the GnRHR couples to $G\alpha_s$, $G\alpha_q$, and $G\alpha_i$ in hypothalamic neurons and to $G\alpha_i$ in prostate cancer cell lines [79-81]. In gonadotrope cells the activated, GTP-bound, $G\alpha_{q/11}$ then initiates activation of phospholipase C (PLC) β, which, in turn, elaborates the second messengers Inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Increases in IP_3 triggers rapid release of Ca^{2+} from intracellular stores, while DAG leads to activation of PKC isoforms and subsequently results in activation of voltage-gated calcium channels and a more sustained Ca^{2+} influx from the extracellular environment [82-87]. Protein kinase C, Ca^{2+}, and arachidonic acid have been shown to participate in GnRH-stimulated release of the gonadotropin hormones from secretory vesicles [88, 89]. Additionally activation of PKC and Ca^{2+} influx from both intra- and

9

extracellular stores have been shown to be required for mitogen-activated protein kinase (MAPK) activation in rat pituitary cells in primary culture and mouse gonadotrope-derived αT3-1 cells [90, 91].

Mitogen-activated protein kinase pathways represent a highly conserved family of kinases which participate in intracellular signaling cascades in response to a variety of extracellular stimuli. In the basic model for MAPK activation, a MAP kinase kinase kinase (MAP3K) phosphorylates a MAP kinase kinase (MAP2K) which then phosphorylates a MAPK [92]. The GnRHR has been shown to activate all four of the classical mammalian MAPK cascades in response to agonist binding: extracellular-regulated signal kinase (ERK), stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), p38 MAPK, and big MAPK (BMK/ERK5) [82, 90, 91, 93-96]. Once activated, MAP kinases phosphorylate a number of substrates within the cell including transcription factors, cytoskeletal components, and other enzymes or signaling molecules [97-100]. A hallmark trait of the MAPK family of enzymes is their ability to translocate to the nucleus and activate transcription factors [95, 101]. For example, GnRH stimulation of αT3-1 cells has been shown to result in phosphorylation and translocation of ERK to the nucleus. In the nucleus ERK then phosphorylates and activates the ternary factor, Elk-1, which binds to serum response elements (SREs) within the immediate early gene, Egr-1, promoter and induces expression of Egr-1 [102, 103].

In gonadotrope cells, the mechanisms leading to activation of the MAPKs, ERK and JNK, have been extensively studied with somewhat less attention being paid to the p38MAPK and

BMK/ERK5 pathways. Occupancy of the GnRHR by agonist has been shown to lead to ERK phosphorylation and activation in gonadotrope cell lines in a mechanism that is dependent upon PKC and Ca^{2+} in addition to other factors [82, 91]. In αT3-1 cells, several PKC isoforms exist; among them PKCα, PKCδ, PKCζ, and PKCε have been shown to be activated in response to GnRH treatment [104-106]. Interestingly, chronic treatment with phorbol ester has been shown to specifically deplete PKCα and PKCε in αT3-1 cells and such depletion reduces GnRH-induced ERK activation [105, 107, 108]. Among these PKC isoforms, PKCε has also been implicated in ERK activation in other cell types by forming a signaling module involving Ras and Raf-1 [109] pointing to this as potentially the PKC isozyme involved in GnRH-induced activation of ERK. Classically, PKC has been suggested to directly phosphorylate Raf-1 (c-Raf) kinase, which is thought to be upstream of ERK activation by MEK1/2. Recent studies in our lab have paradoxically shown that pharmacologic inhibition or pituitary specific genetic knock-out of Raf-1 do not inhibit GnRH-induced ERK phosphorylation; however, overexpression of a constitutively active Raf-1 construct does result in ERK phosphorylation [110]. These studies confirm that Raf-1 may be capable of initiating a cascade which results in ERK phosphorylation, but cast doubt as to whether this role is essential or required in the context of pituitary gonadotropes. It is entirely possible that Raf-1 is one of multiple pathways simultaneously activated by GnRH treatment which all lead to ERK activation.

Stimulation of gonadotrope cells with GnRH agonists has also been shown to induce a biphasic calcium response. In the first phase, a rapid, initial increase in intracellular Ca^{2+} is triggered by IP3 binding to its receptor on the endoplasmic reticulum resulting in release of Ca^{2+} from intracellular stores. Later, DAG activates PKC and stimulates a more sustained rise in

intracellular Ca^{2+} concentration via voltage-gated calcium channels [reviewed by [111]].

Interestingly, it is this second phase of Ca^{2+} influx from the extracellular environment which our lab has previously shown to be required for GnRH-induced ERK, but not JNK, phosphorylation [91]. The mechanism by which extracellular Ca^{2+} influx is detected by the ERK pathway is just beginning to be elucidated. Previous efforts in our laboratory have shown that GnRH treatment of gonadotrope cells results in calcium-loading of the calcium sensing-protein, calmodulin and further demonstrated that calmodulin itself was required for ERK activation [102]. More recently our laboratory and others have shown that calcium-sensitive proline-rich tyrosine kinase (Pyk2) may represent an enzymatic link between calcium influx, calmodulin and ERK activation in response to GnRH treatment in gonadotrope cells [112, 113]; however, additional studies will be required to fully elucidate this mechanism.

In contrast to the role of extracellular calcium described above, Ca^{2+} influx from intracellular stores was shown to be important for JNK phosphorylation while ERK phosphorylation remained unaffected by the disruption of this calcium pool suggesting that these two MAPK pathways might be functionally compartmentalized within the cell [91]. In addition to calcium, the JNK pathway in αT3-1 cells has been shown to be mediated by protein tyrosine kinases, such as c-src, and the small G-protein, cdc42 [91, 96]. In contrast to the findings of Levi and colleagues [96], our group has demonstrated that GnRH-induced JNK phosphorylation is independent of DAG-dependent PKC isoforms [91], thus the role of PKC isozymes in GnRH-induced JNK activation remains controversial. As c-src is thought to be activated downstream of PKC activation, these findings suggest that perhaps c-src is activated by other means, that c-src is not essential to JNK activation, or that a phorbol ester-insensitive PKC isoform is involved [91]. Our group has also shown that expression of dominant negative MKK7 and dominant

Figure 2 The GnRHR-associated signaling network in gonadotrope cells: GnRH exerts its effect on gonadotrope cells by binding to its receptor, GnRHR. The binding of agonist induces a conformational change resulting in the dissociation of the α subunit from the β and γ subunits of the G-protein associated with the receptor. The active Gα then stimulates PLC to elaborate the second messengers IP3 and DAG which go on to initiate downstream signaling pathways culminating in the initiation of MAPK signaling cascades. Active MAPKs such as ERK and JNK then translocate to the nucleus where they activate transcription factors including Elk-1 initiating transcription of immediate early genes like Egr-1. Many of these immediate early genes are themselves transcription factors which further stimulate the transcription of secondary and tertiary genes like the LHβ subunit gene.

Figure 2

14
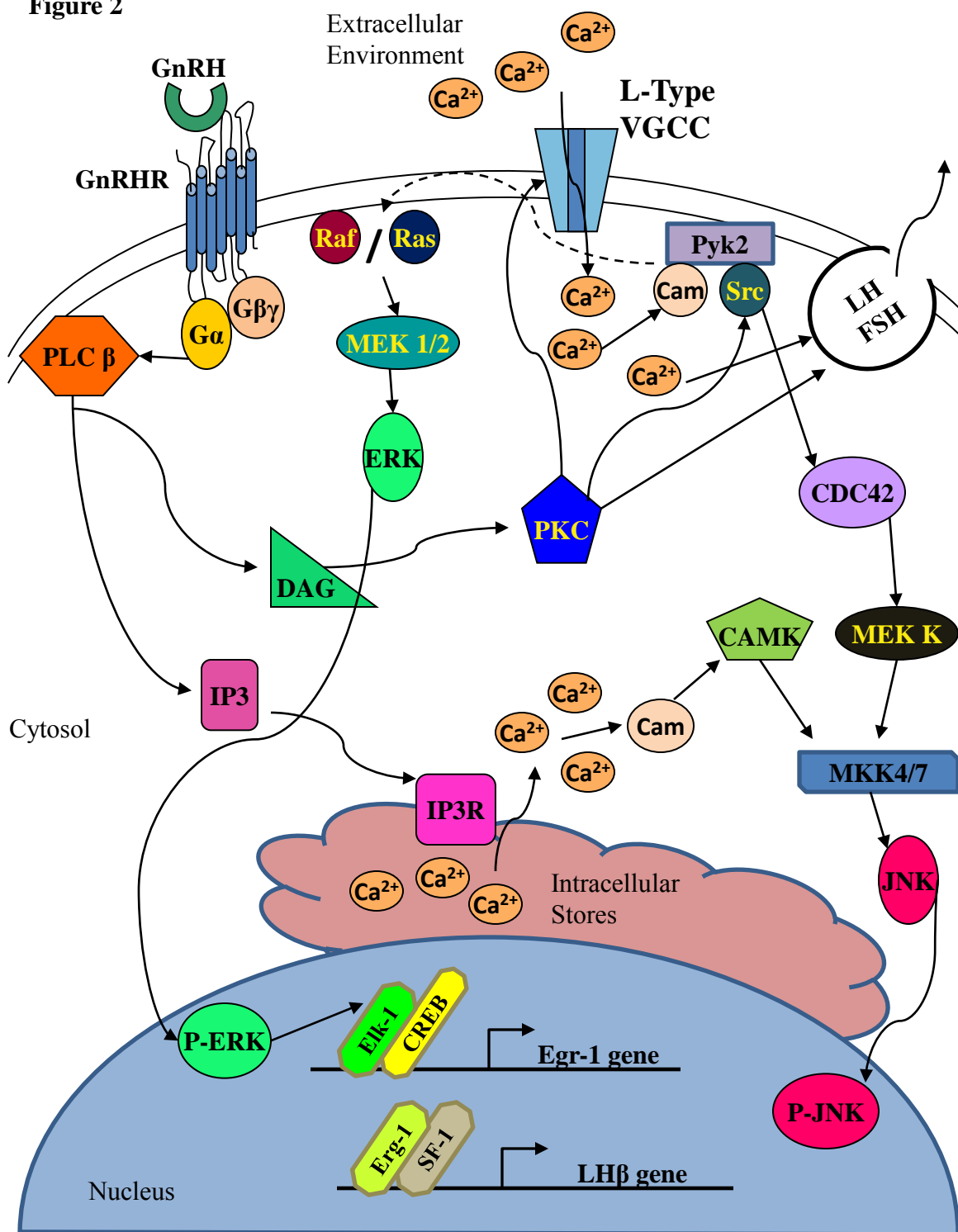
negative PAK 1 both reduced GnRH agonist-induced JNK phosphorylation confirming that these proteins are involved in GnRH signaling to JNK [91] (**Figure 2**).

GnRH-INDUCED GENE TRANSCRIPTION

The involvement of MAPKs in gonadotropin subunit gene expression in response to GnRH treatment remains controversial. Many different groups have published apparently contradictory results regarding which MAPK members are involved in the activation of transcription factors leading to the regulation of GnRH-responsive genes [114], with essentially all of these studies carried out in vitro using gonadotrope-derived cell lines. At least 75 genes have been shown to be responsive to GnRH treatment of gonadotrope cells [115]. These genes can generally be organized into immediate early genes, secondary genes and tertiary genes. Immediate early genes are characterized by their rapid activation kinetics without the need for new protein synthesis indicating that their transcription is triggered by modification/activation of existing transcription factors [116, 117]. The immediate early genes primarily function to participate in the transcription of secondary and tertiary genes [118]. The levels of many of these immediate early genes in unstimulated cells are often undetectable, but they can be rapidly transcribed upon stimulation of the cell and resultant PKC activation [119].

The immediate early genes involved in GnRH-mediated transcription include members of the activating protein-1 (AP-1) family of transcription factors (c-Jun, c-Fos, ATF-1, -2, -3) as well as members of the Ets family of transcription factors such as Elk-1 [120, 121]. Once transcribed, the immediate early genes may be activated by one or more MAPK, may form homo- or

heterodimers and then bind to their targets on the promoter regions of secondary and tertiary genes to initiate transcription. In general, it appears that JNK is responsible for phosphorylating and activating c-Jun and ATF family members which may then go on to activate transcription of the LHβ or αGSU glycoprotein subunit gene promoters [122-124]. Similarly, ERK is implicated in activation of c-Fos and Elk-1 leading to the transcription of the LHβ and αGSU genes [121, 122]. However both ERK and JNK have been implicated as activating either c-Fos or c-Jun resulting in transcription of the FSHβ gene [94, 125, 126]. Further p38MAPK and ERK, but not JNK, have been suggested as upstream activators of Egr-1 which assists in the transcription of the LHβ promoter [127]. It seems that the organization of transcriptional modules downstream of MAPK cascades might not follow a simple one-to-one association and instead might constitute a network of signaling molecules capable of activating interconnected networks of transcription factors with differing affinities that regulate themselves on the basis of their collective permissive or repressive micro-environments. For example, both ERK and JNK may be capable of phosphorylating and activating Elk-1, but ERK is essential for regulation of Egr-1 transcription downstream of Elk-1. Meanwhile JNK plays only a minor role in Egr-1 regulation possibly setting the baseline tone but not the GnRH-induced response [103].

Interestingly, the MAP kinase phosphatases, MKP-1 and -2 (also known as DUSP 1 and 4), are also transcribed as part of the immediate early gene response in gonadotrope cells [128, 129]. These dual-specificity phosphatases selectively down-regulate MAPK activity by dephosphorylating both the Thr and Tyr residues necessary for MAPK activation [130]. So it appears that ERK and JNK activation of transcription factors also induces transcription of MKP-1 and -2 which are capable of dephosphorylating and inactivating ERK and JNK [103, 128].

This further demonstrates the complexity of the GnRH-induced gene transcription network by adding a mechanism for intracellular negative feedback on MAPK activity through the activation of dual-specificity phosphatases.

Deeper into the gene network we find the tertiary genes whose transcriptional response times may range from hours to days due in part to the fact that these genes are dependent upon newly synthesized transcription factors. For example, the LHβ subunit promoter requires a combination of pre-existing and newly synthesized transcription factors for induction. In this case, the immediate early gene, Egr-1 must first be up-regulated, then Egr-1 and SF-1 must bind the LHβ promoter along with other factors before LHβ can be transcribed under the specific influence of GnRH [129]. Interestingly Egr-1 was shown to be essential for the production of LHβ with the Egr-1 knock-out mouse being infertile and failing to produce the LHβ subunit [131, 132]. Similarly work in our lab has generated a pituitary specific conditional double ERK knock-out mouse in which ERK was shown to be essential for fertility in female, but not male mice, due to deficiencies in LH production [133]. As ERK has previously been shown to be crucial for relaying the GnRH stimulus to Egr-1 gene transcription in αT3-1 cells [103], it appears that a similar need for ERK exists *in vivo*.

Adding interesting complexity to this system is the relatively recent finding that β catenin might also be involved in mediating the GnRH-induced transcriptional responses. β Catenin is a member of the armadillo gene family of proteins classically known for its ability to bind E-cadherin and as a signaling molecule in the Wnt/Frizzled pathway [134-136]. In classical

17

Wnt/Frizzled signaling, levels of β catenin in the cytosol are kept low by a destruction complex: adenomatous polyposis coli (APC), Axin and glycogen synthase kinase-3β (GSK3β). In this complex, GSK3β phosphorylates and targets β catenin for degradation. Upon the binding of Wnt to its receptor Frizzled on the cell surface, a cascade of intracellular signaling is initiated in which GSK3β is phosphorylated on serine 9, which results in its inactivation. The inactivation of GSK3β prevents the phosphorylation and targeting of β catenin for destruction. Subsequently levels of β catenin in the cell increase, leading to its translocation to the nucleus where it acts as a transcriptional coactivator [137]. β catenin typically associates with T cell factor (TCF)/lymphoid enhancer factors (LEF) to promote the transcription of Wnt target genes including many immediate early genes such as c-Jun [138].

Recently, β catenin was found to participate in transcriptional coactivation of the LHβ gene in pituitary-derived LβT2 cells in response to GnRH stimulation [139]. In these studies, siRNA knock down of β catenin was shown to reduce basal and attenuate GnRH-induced LHβ promoter activity. Further, overexpression of a constitutively active mutant of β catenin along with overexpression of Egr-1 and SF-1 were shown to enhance the transcriptional activity of the LHβ promoter even in the absence of stimulation by GnRH. The s concluded that β catenin was required for achieving the maximal transcriptional response of the LHβ promoter to stimulation by GnRH agonists [139]. In another study, β catenin was shown to be capable of translocating to the nucleus in response to GnRH treatment of both LβT2 cells, and HEK293 cells transfected with the GnRHR. Interestingly this same group was able to show that GnRH treatment resulted in phosphoinhibition of GSK3β in HEK cells, but they were unable to show this in LβT2 cells [140]. Further supporting the role of β catenin is the discovery that LHβ gene expression levels

were reduced in embryonic pituitary glands devoid of β catenin [141]. These findings support the inclusion of β catenin within the transcriptional network activated in response to GnRH treatment in pituitary gonadotrope cells.

GnRHR-MEDIATED SIGNALING AND THE ACTIN CYTOSKELETON

Recently, it was reported that GnRH agonist treatment is capable of inducing changes in cell topography and morphology in pituitary cells and αT3-1 cells. These changes were evident after as little as 1 minute of treatment and after 10 minutes prominent stress fibers, indicative of actin remodeling, were detectable [142]. These observations are not without precedent as HEK 293 cells expressing exogenous GnRHR were also found to show marked changes in cell morphology and adhesion in response to GnRH treatment [143]. Additionally several GPCR agonists have been shown to promote activation of Rho family GTPases which are known to be involved in dynamic reorganization of actin [144]. Under basal conditions, the majority of Rho in the cytosol is bound to guanine nucleotide dissociation inhibitors (GDIs) [145] but upon stimulation, activated Rho translocates to the membrane to interact with specific targets [144]. The mechanism of activation of Rho by GPCRs remains unclear, but it is thought to involve receptor coupling to Gα 12/13 or Gαq/11. Dutt demonstrated that overexpression of constitutively active mutant Gαq family members was sufficient to induce Rho activation and stimulate Rho-dependent cellular responses including actin stress fiber formation [146]. Activated Rho GTPases are capable of activating WASP/WAVE proteins and diaphanous related formins, which are two forms of actin nucleation promoting factors [144, 147].

19

It would seem that cells expressing the GnRHR are not only capable of inducing cytoskeletal reorganization they are dependent upon it for the initiation of downstream signaling events. In HEK293 cells stably expressing GnRHR, pre-treatment with cytochalasin D (to depolymerize actin) or Latrunculin B (to prevent *de novo* actin polymerization) prevented GnRH-induced phosphorylation of ERK 1/2, c-src, and focal adhesion kinase (FAK). In these studies a dominant negative Rac-1, but not Rho A, was found to prevent GnRH-stimulated phosphorylation of FAK leading the authors to conclude that GnRH-induced activation of Rac-1 was necessary for signaling to ERK via FAK and c-src [143]. Interestingly these authors did not find ERK phosphorylation to be downstream of PKC or PLCβ activation. However in another study, stabilization of actin polymerization using Jasplakinolide treatment in αT3-1 cells endogenously expressing GnRHR prevented GnRH-induced ERK phosphorylation yet phorbol ester treatment was still able to activate ERK [142].

Recent studies in our lab have shown that the actin scaffolding protein, cortactin, was phosphorylated in response to GnRH treatment in αT3-1 cells. Curiously, in these experiments, knock down of cortactin using siRNA was able to reduce the appearance of lamellipodia and membrane ruffles in response to GnRH treatment, yet this did not appear to have any effect on ERK phosphorylation. However, the blunted actin kinetics did result in the loss of secretory granule mobilization and a reduction in common glycoprotein α subunit secretion following multiple pulses of GnRH [Navratil, Allen, Roberson unpublished manuscript]. Similarly studies in LβT2 cells also demonstrated that disruption of the actin cytoskeletal network prevented GnRH-induced secretion of LH [142]. Taken together these studies suggest that gonadotropes

require an intact and dynamic actin cytoskeleton to coordinate the secretion of the gonadotropin hormones and possibly for other intracellular signaling events.

THE GnRHR AND MEMBRANE RAFT MICRODOMAINS

The plasma membrane makes life possible and allows for a cell to regulate its internal environment, yet it must be permeable, adaptable and still maintain biophysical integrity. In spite of its biological importance, we are only beginning to understand how this fundamental structure is generated and maintained in a dynamic state. One of the first steps towards elucidating the organization of the plasma membrane came from the "Fluid Mosaic" model proposed by Singer and Nicolson. In this model, the plasma membrane is seen as a fully homogenous, two-dimensional, liquid structure in which all membrane proteins and lipids were equally distributed and freely-diffuse throughout a uniform plane [148]. As our understanding of the plasma membrane evolved, so too did our model for how constituents within the membrane were organized. Studies began to reveal that there were barriers to diffusion even between regions of the plasma membrane and that heterogeneities might exist. For example, Spiegel and coworkers observed that differences existed in the distribution of endogenous gangliosides between the apical and basolateral membranes of rat renal cortex cells [149]. These differences were proposed to be maintained by differential sorting or insertion of membrane proteins or by tight junctions involved in maintaining polarity in epithelial cells [149, 150]. The "lipid raft" hypothesis was first presented in 1992 and was based on the idea that complexes of lipids that were poorly solubilized by non-ionic detergents (like Triton X-100) would "float" during sucrose gradient fractionation due to low buoyant density and that these potentially represented pre-

formed complexes present in the cell prior to detergent extraction [151]. The idea that such complexes or domains consisting of lipids and proteins could exist and further that these might represent functional platforms coordinating cellular processes soon took hold. These so-called membrane rafts were soon defined as small (10-200 nm) dynamic microdomains, enriched in cholesterol and sphingolipids [152]. There are several ways membrane rafts are thought to form in living cells including as a result of the differential miscibility of lipids which naturally allows lipids with highly acylated, tightly packed side chains to preferentially associate with each other or with cholesterol, as a result of complex protein-lipid interactions, or as a result of protein-protein interactions which "trap" lipids in a given region [153-155]. Additionally studies using single particle tracking revealed that proteins did not move by random Brownian motion across long distances, but did within small domains, infrequently "hopping" between domains [156-158]. This led to the proposal that actin filaments act as "fences" to trap proteins within small regions [156, 157, 159]. The observation that lipids followed similar diffusion restrictions led to the extension of this model to include cytoskeletal-anchored transmembrane proteins as "pickets" which serve to trap proteins and lipids within such regions [159-161]. Membrane rafts are hypothesized to serve an organizational role within the plasma membrane acting as scaffolds to compartmentalize or facilitate signal transduction, endocytosis, or to prevent cross-talk between the various proteins and lipids that make up the plasma membrane of a cell [152, 162, 163].

Perhaps because membrane rafts can be easily, albeit crudely, isolated based on density by sucrose gradient fractionation in the presence of a non-ionic detergent such as Triton X-100, many labs began to isolate and characterize them. The resulting low density detergent-resistant

membranes (DRMs) were often taken to represent the membrane raft compartment. For this reason the membrane raft hypothesis was criticized as being overly simplistic, or as superimposing biological significance on a biochemical artifact. After all, not all things that co-purify with DRMs represent members of membrane raft microdomains in living cells under physiologic conditions [164]. Other early approaches to determine whether membrane raft microdomains were of biological relevance included the use of methyl-β-cyclodextrin to extract cholesterol from the plasma membrane [165]. This method was predicted to disrupt membrane rafts due to their higher composition of cholesterol altering their buoyant density and function [165]. Indeed, cholesterol depletion does result in the disassociation of many proteins from detergent resistant/low density membrane fractions [166]. Unfortunately removal of cholesterol using cyclodextrins can also have a variety of other effects including loss of morphology, rounding and loss of viability [166]. In addition to these, methyl-β-cyclodextrin has also been shown to remove phospholipids from membranes [167], and may interact with membrane proteins [166]. As a result, studies involving cholesterol depletion were often discounted as being too harsh, or producing global effects on cellular function and viability that may not be related to membrane rafts [166, 168].

EVIDENCE FOR THE EXISTENCE OF MEMBRANE RAFTS

Experiments in model membranes have shown that different species of lipids do indeed have different biophysical affinities for one another. In model membranes, ternary mixtures of sphingomyelin, unsaturated phosphadidylcholine, and cholesterol have been shown to partition into cholesterol and sphingolipid rich, liquid ordered (Lo), and cholesterol poor, liquid

disordered (Ld), phases over a wide variety of temperatures [169, 170]. Subsequent biophysical

experiments comparing model membranes to plasma membrane vesicles and to reconstituted

plasma membrane vesicles along with detergent resistant membrane fractionation from both

cellular membranes and model membranes have borne out that these Lo domains are likely to

exist in living cells and that detergent-resistant membrane fractionation is a reliable way to

isolate them [reviewed by [171]]. Other techniques including transmission electron microscopy

on plasma membrane sheets has shown that most or all plasma membrane proteins are clustered

in cholesterol enriched "islands" separated by relatively protein-free spaces. In these studies, the

authors noted that some "islands" contained raft marker proteins while others did not, but in

either case they were all found to be associated with cytoskeletal proteins on the inner leaflet

[154]. These studies suggest that actin participates in forming, maintaining or stabilizing

membrane microdomains. Further support for this hypothesis was found by Chichili and

Rodgers who determined that actin organizes membrane raft proteins using fluorescence

resonance energy transfer (FRET) techniques to determine that raft marker proteins were in

proximity to actin cytoskeletal components [155]. As mentioned previously, single particle

tracking has revealed that molecules in the membrane do not move as predicted by a "random

Brownian walk", but rather move by Brownian motion within finite domains with rare transitions

between domains [156-158]. Taken together the studies listed here along with others strongly

suggests that membrane rafts exist in living cells under physiologic conditions.

THE GnRHR AND MEMBRANE RAFTS

We have previously demonstrated that the GnRHR constitutively and exclusively localizes to low density membrane raft microdomains [99, 172, 173]. Accompanying the GnRHR in membrane raft compartments are several components of its signaling network including $G\alpha_{q/11}$, c-Raf kinase, and ERK [172-174]. Considering that these components are all constitutively present within the membrane raft compartment it seems reasonable to expect that the GnRHR and several members of its signaling network might constitute a pre-formed platform poised and ready to rapidly and specifically conduct the GnRH signal as soon as agonist binds the receptor. We have also shown ERK activation in response to GnRH to be dependent upon an intact membrane raft microdomain [172, 173]. Similar to what was observed for actin perturbation [142], activation of ERK could still be achieved with phorbol ester treatment despite the perturbation of membrane raft microdomains [173]. It is clear from these studies that something is missing in our understanding of how ERK becomes activated in response to GnRH stimulation. Precisely how the GnRH signal is relayed between receptor binding and PKC/ERK activation, and why this would require an intact actin cytoskeleton or how membrane raft microdomains might be involved remain to be determined. It appears that some factor (or factors) is missing from our current model of GnRH-induced intracellular signaling including ERK activation. We hypothesized that such a factor would be found in association with the GnRHR within membrane raft microdomains. In order to identify a list of candidate proteins which might be involved in GnRHR-associated signaling within pituitary gonadotrope cells we employed a combination of sub-cellular fractionation and immunoprecipitation to generate samples for Mass Spectrometry analysis. This approach allows us to examine the low density membrane raft compartment while taking advantage of more selective antibody-antigen interactions to add specificity and relevance to our list of proteins. In this dissertation, I further

go on to use complementary techniques to validate that a selection of the proteins we identified represent true participants within the GnRHR-associated membrane raft proteome and potentially within the GnRHR-induced transcriptional network.

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Chapter 2: The Gonadotropin Releasing Hormone Receptor Associates with Flotillins/Reggies 1 and 2 within Membrane Raft Microdomains in Gonadotrope Cells.

SUMMARY

The gonadotropin releasing hormone receptor (GnRHR) is expressed in pituitary gonadotropes and is a unique member of the heptahelical G-protein coupled receptor superfamily. The GnRHR has been shown to partition uniquely into membrane raft microdomains in order to facilitate productive signaling to a number of signaling pathways including the actin cytoskeleton upon stimulation with GnRH. Here we demonstrate that the flotillin/reggie proteins are found in association with the GnRHR in membrane raft microdomains using biochemical, immunoprecipitation and imaging approaches in mouse pituitary and clonal αT3-1 cells. GnRH administration to αT3-1 cells appears to reorganize the spatial distribution of Flotillins 1 and 2 in a manner consistent with actin reorganization. To define the protein composition of the GnRHR-associated membrane raft microdomain in an unbiased manner, we used immunoprecipitation approaches followed by mass spectrometry to identify proteins associated with the raft compartment containing the GnRHR and flotillins. These studies revealed a complex protein mixture within the raft compartment potentially representing a number of important signaling pathways. This approach was validated using a group of raft-associated proteins associated with the actin cytoskeleton known to be induced by GnRH. Our studies support the importance of protein complexes within the raft that are necessary to productive GnRH signaling within the gonadotrope.

53

INTRODUCTION

The gonadotropin releasing hormone receptor (GnRHR) is expressed in pituitary gonadotropes and is a member of the heptahelical G-protein coupled receptor (GPCR) superfamily [1, 2]. Within this family, the GnRHR is unique in that it lacks a cytoplasmic carboxyl terminal tail, which would otherwise be involved in receptor desensitization and internalization. For this reason, the GnRHR has been called a naturally occurring desensitization resistant mutant [3, 4]. The GnRHR further distinguishes itself by being an exclusive and constituent resident of membrane raft microdomains within the plasma membrane, unlike many other GPCRs which only partition into raft domains following activation or receptor dimerization [5]. The GnRHR binds to its ligand gonadotropin releasing hormone (GnRH) resulting in dissociation of the heterotrimeric G-protein associated with the receptor and initiation of several intracellular signaling cascades resulting in calcium influx from both extracellular and intracellular pools, the activation of several MAP Kinase pathways including those leading to extracellular signal regulated kinase (ERK) 1 and 2 and c-Jun N-terminal kinase (JNK) [6]. Currently the mechanisms by which the GnRHR and many members of its signaling network are able to partition into the membrane raft compartment, even in the absence of stimulation, are unknown.

Membrane rafts are thought to be small (10-200 nm), dynamic microdomains within the plasma membrane that are enriched in cholesterol and sphingolipids [7]. There are several ways membrane rafts are thought to form in living cells including as a result of the differential miscibility of lipids which naturally allows lipids with highly acylated, tightly packed side chains to preferentially associate with each other or with cholesterol, as a result of complex protein-lipid

54

interactions, or as a result of protein-protein interactions which "trap" lipids in a given region of the membrane [8-10]. Membrane rafts are hypothesized to serve an organizational role within the plasma membrane acting as scaffolds to compartmentalize or facilitate signal transduction, endocytosis, or to prevent cross-talk between the various proteins and lipids that make up the plasma membrane of a cell [7, 11, 12]. Membrane rafts can be easily, albeit crudely, isolated by sucrose gradient fractionation in the presence of a non-ionic detergent such as Triton X-100. The resulting low density detergent-resistant membranes (DRMs) are often taken to represent the membrane raft compartment although it should be noted that not all things that co-purify with DRMs represent members of membrane raft microdomains in living cells under physiologic conditions [13]. We have recently demonstrated a role and requirement for the raft compartment and productive signaling through the GnRHR [5, 6, 14].

Flotillin-1 and flotillin-2 (also known as reggie-2/reggie-1, respectively) are widely expressed, highly conserved membrane raft microdomain-associated proteins [15, 16] capable of forming stable homo- and hetero-tetramers [17]. Flotillin proteins are constitutively associated with membrane raft domains in several cell types [16, 18, 19] and are capable of forming preassembled platforms in living cells under physiologic conditions [20] and serve as useful markers of membrane raft compartments in some situations. Although their precise function is not yet known, flotillins are thought to participate in membrane receptor-mediated signaling, membrane raft-associated endocytosis, phagocytosis and regulation of neurite outgrowth, cytoskeletal organization, and scaffolding of molecular processes [21].

Recently flotillin-1 was identified through a yeast two-hybrid screen as a binding partner for the G-protein subunit, $G\alpha_{q/11}$ [22], the same G-protein subunit which participates in GnRHR-associated signaling in pituitary gonadotropes. Given that flotillins are thought to participate in signaling events and may act as a scaffold for recruitment of proteins into membrane raft microdomains, we hypothesized that flotillins interact with the GnRHR or other members of its signaling network. Our group has previously identified other molecules involved in GnRHR signaling as inhabitants of the membrane raft compartment including Raf-1 kinase, calmodulin and ERK isoforms [5, 14]; however, the full characterization of the raft compartment containing the GnRHR has not be fully elucidated. In order to determine with certainty what other proteins may also inhabit the membrane raft compartment linked to the GnRHR, we turned to mass spectroscopy (MS) and a proteomic analysis. Mass spectroscopy is a powerful tool presenting the unique opportunity to probe a sample set from an unbiased standpoint allowing for the identification of a multitude of targets including those not previously identified.

Here we show that the flotillin proteins are present in membrane raft microdomains in pituitary gonadotrope cells along with the GnRHR. Further we demonstrate that both flotillin-1 and flotillin-2 are found in association with the GnRHR within DRMs and that all three co-localize at the plasma membrane in gonadotrope-derived αT3-1 cells. We have used out ability to specifically isolate DRM containing the GnRHR to define and begin to validate the GnRHR-associated membrane proteome in gonadotrope cells.

MATERIAL AND METHODS

Cells, Antibodies, and Chemicals

αT3-1 cells, an immortalized mouse gonadotrope cell line (generously provided by Dr. Pamela Mellon, University of California, San Diego), were cultured as described previously [5]. Briefly, αT3-1 cells were maintained in high glucose (4.5 g/L) DMEM containing 2 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and 1 X nonessential amino acids and 10% FBS. Cells were grown in 5% CO_2 in air at 37°C in a humidified environment. Anti-HA antibodies were from Cell Signaling Technology (Danvers, MA). Anti-Flotillin-1 and Anti-Flotillin-2 antibodies were from BD Biosciences (San Jose, CA). Anti-LH antibody was a gift from National Institutes of Health (National Hormone Peptide Program, A.F. Parlow, Torrance, CA; NIDDK). The GnRHR antibody was raised in a rabbit against 20 amino acids of the second extracellular loop (aa193-aa212) of the ovine GnRHR. This sequence shows no overlap with any other receptor or peptide. Pre-immune rabbit serum (NRS) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies and Texas Red-streptavidin used for immunohistochemistry were from Jackson Immunoresearch (West Grove, PA). Buserelin (des-GLY10 [D-Ser(t-But)6]-LH-RH Ethylamide; referred to as GnRHa) and all other chemicals were obtained from Sigma. In all experiments, GnRHa was used at 10 nM. Glass bottom microwell dishes for confocal studies were obtained from Mat-Tek (Ashland, MA). PEI (Polyethylenimine) transfection reagent was purchased from Polysciences (Warrington, PA). Tissue-Tech OCT compound was from Miles Inc. (Elkhart, IN).

Tissue Preparation and Immunohistochemistry

All animal studies were completed in accordance with the Cornell University Institutional Animal Care and Use Committee. Animals were killed by CO_2 asphyxiation and whole pituitaries were dissected free and fixed in 4% paraformaldehyde overnight. Following fixation,

pituitaries were embedded in paraffin and sectioned (5 µm). Pituitary sections were

deparaffinized in xylene, and rehydrated through ethanol dilution series to distilled H_2O. Epitopes

were unmasked by boiling in 10 mM citric acid for 10 min. Slides were blocked and incubated

with an antibody specific for either flotillin 1 or flotillin 2 for 1 hour at room temperature. Slides

were then washed with PBS and exposed to an anti-LHβ antibody (1:1250) for 1 hour at room

temperature. For visualization, slides were incubated for thirty minutes at 37° C with Texas Red-

conjugated anti-guinea pig IgG secondary antibodies (1:500) to detect LHβ and Alexa-488

conjugated mouse IgG to detect either flotillin 1 or flotillin 2. Sections were then washed in PBS,

stained with DAPI, mounted and imaged using a Zeiss LSM 510 confocal microscope.

Preparation of Cell Lysates and Immunoblotting

After the indicated treatments, cells were washed twice in cold PBS, and scraped into cold

radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM

NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate,

2 mM EDTA, 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM

benzamidine. Lysates were cleared by centrifugation and protein concentrations of the lysates

were determined by Bradford assay. Protein samples were boiled for 5 minutes in SDS load

buffer, resolved by SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose

membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk (NFDM) or

1X Casein in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then

incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies.

Protein bands were visualized using enhanced chemiluminescence according to the

manufacturer's instructions (PerkinElmer, Boston, MA). Rather than onto film,

chemiluminescence was detected using a BioRad Chemidoc™ XRS+ System and pictures analyzed using the Image Lab™ Software from BioRad (Hercules, CA).

Cell Fractionations

Detergent-resistant, low-density membrane fractions were prepared essentially as described previously [5]. Briefly, αT3-1 cells were grown to 60-70% confluence in 15 cm^2 dishes. Following the treatments indicated, cells (~1.5 x 10^8 per dish) were washed twice in cold PBS, and scraped into PBS containing 2 mM sodium vanadate, 0.25 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 20mM β-glycerophosphate. Cells were pelleted by centrifugation and then resuspended in MES buffer (MBS) containing 25 mM MES, pH6.5, 130 mM NaCl, and protease inhibitors to a final volume of 400 μL. The samples were adjusted to a final concentration of 0.1% Triton X-100 and incubated on ice for 10 minutes. Following dounce homogenization (20 strokes), the samples were mixed with an equal volume of 90% sucrose, placed in a 5 mL ultracentrifuge tube, and overlaid with a discontinuous gradient of sucrose in MBS consisting of 35% (3.7 mL) and 5% (500 μL) layers. The gradients were centrifuged at 116,000 x g in a SW55Ti rotor for 20 hours at 4˚C. Low-density detergent resistant membranes were visible as a band of flocculent material at the 35-5% interface. Fractions (500 μL) were collected starting from the top of the gradient. Total Membrane pellets were prepared as described previously by [14].

Male B6/129 mice (8-20 weeks of age) were euthanized by CO$_2$ asphyxiation. For whole pituitary fractionations, pituitaries (n=10) were collected and suspended in 400 μL cold MBS

containing 0.1% Triton X-100, homogenized in a glass dounce (20 strokes), and subjected to discontinuous sucrose density centrifugation as described above for preparation of detergent-resistant low-density membranes. Animal use and experimental protocols for these studies were approved by the Cornell University Institutional Animal Care and Use Committee.

Silver Staining of Polyacrylamide Gels

Samples were prepared as for immunoblotting as described above except that the SDS polyacrylamide gels were fixed in 5 gel-volumes of ethanol:glacial acetic acid:water (30:10:60) for 12 hours at room temperature, followed by two 30 minute washes in 30% ethanol. The gel was then washed twice for 10 minutes in 10 gel-volumes of deionized water, followed by a 30 minute incubation in 5 gel-volumes of a 0.1% $AgNO_3$ solution (freshly diluted in deionized water) at room temperature. The gel was then rinsed for 20 seconds on each side with a steady stream of deionized water. The stain was developed by adding 5 gel-volumes of an aqueous solution of 2.5% sodium carbonate and 0.02% formaldehyde and incubating at room temperature until the desired contrast was achieved. The staining reaction was stopped by washing the gel in 1% acetic acid for a few minutes followed by rinsing with deionized water.

Plasmids and Transfections

The plasmids containing Flotillin-1-GFP and Flotillin-2-GFP constructs were kindly provided by Dr. Ben Nichols (Medical Research Council (MRC) Laboratory of Molecular Biology, Cambridge, UK). The plasmid containing the Flotillin-1-Cherry fluorescent construct was kindly provided by Dr. Jeremie Rossy (Department of Pathology, University of Bern, Bern, Switzerland). And the plasmid containing the HA-GnRHR construct has been described

previously [23]. All plasmids were transfected into αT3-1 cells using PEI as follows: 500,000

αT3-1 cells were plated onto 6-well dishes the night before transfection, for each well of a 6-well

plate, 1μg of plasmid DNA along with 3 μg of PEI reagent were added to 200 μL of serum-free

media and incubated at room temperature for 15 minutes. The suspension was then added to the

culture media for a period of 4 hours. After transfection, the cells were washed and returned to

growth medium (10%FBS in high glucose DMEM) for 48 hours. For transient transfections,

cells were collected following 48 hours of incubation. For the generation of stable cell lines the

transfected cells were grown in neomycin selection media containing geneticin (Invitrogen,

Carlsbad, CA) at 500μg/mL of 10% FBS high glucose DMEM beginning at 48 hours post-

transfection and continuing for a period of at least 3 weeks.

Confocal microscopy

αT3-1 cells grown on glass bottom microwell dishes were transiently transfected with a

Flotillin-2 construct with a GFP fluorophore linked to the C-terminus and a Flotillin-1 construct

with an mCherry fluorophore linked to the C-terminus for 48 hours. Cells were fixed in 4%

paraformaldehyde for 10 minutes at room temperature and then imaged. Imaging was done

utilizing the 63X oil objective and the 488nm and 543nm laser lines of a Zeiss LSM 510 Meta

confocal microscope. The Cherry and GFP signals were acquired using multi-track mode and no

crosstalk between Cherry and GFP was observed. For studies involving the GnRH Receptor a

construct consisting of a triple HA-tag fused to the N-terminal region of the GnRH Receptor in a

plasmid containing a neomycin selection cassette was transfected into αT3-1 cells. Cells were

grown under neomycin selection conditions (500mg/mL of 10%FBS high glucose DMEM) for a

period of 3 weeks to ensure stable expression. This stable cell line was then grown on glass

bottom microwell dishes and cells were transiently transfected with either a Flotillin-1 or Flotillin-2 construct with a GFP fluorophore linked to the C-terminus for 48 hours. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were blocked in PBS containing 3% BSA and then incubated with Rabbit monoclonal anti-HA antibody overnight at 4°C. The following day, cells were washed and incubated with a Biotin-labeled goat anti-rabbit secondary antibody for 1 hour at room temperature. Finally cells were labeled with Streptavidin-Texas Red for 1 hour at room temperature. Phalloidin staining was performed as previously described [24]. Cells were then imaged by confocal laser scanning microscopy (CLSM) as above.

Immunoprecipitation

Aliquots (400 µL) of fractions containing suspensions of low-density membranes, or sucrose alone, were diluted in an equal volume of PBS and adjusted to 0.01% Triton X-100. Anti-GnRHR immune serum (IS, 25 µL), anti-Flotillin-1 antibody, anti-Flotillin-2 antibody, or non-immune rabbit serum (NRS, 25 µL), were added, and the samples were rocked for 1 hour at 4°C. Protein A/G agarose beads (30 µL) were added and the samples were rocked overnight at 4°C. Beads were then washed 3 times in PBS with 0.01% Triton X-100, resuspended in 60 µL of SDS load buffer, and boiled.

Samples used for further analysis by Mass Spectrometry were immunoprecipitated using the Pierce Crosslink IP kit purchased from Thermo Fisher Scientific (Rockford, IL) rather than using the above method. Anti-GnRHR or anti-flotillin-1 antibodies were crosslinked to protein A/G agarose beads according to the manufacturer's instructions. Samples were pre-cleared using pre-

immune rabbit serum and the control agarose resin supplied with the kit. Pre-cleared samples were then incubated with antibody-crosslinked protein A/G beads on a rocker overnight at 4˚C followed by immunoprecipitation and sample recovery according to the manufacturer's instructions. Eluted sample was then boiled in SDS loading buffer and resolved using SDS PAGE. Protein concentration in the eluted sample was measured using the Bradford Assay.

In-gel Digestion and Extraction for NanoLC MS/MS and Mass Spectrometry Data Analysis

Preparation of SDS-resolved samples for in-gel digestion, extraction and subsequent NanoLC followed by tandem MS and data analysis was performed essentially as described previously [25] with minor modifications. Briefly, whole cell detergent resistant membrane raft fractions, or immunoprecipitated proteins were resolved by 10% SDS PAGE, then stained using SYPRO®ruby (Invitrogen, Carlsbad, CA). Bands of proteins were then selected and excised manually for further processing before being subjected to in-gel digestion with trypsin. Mascot version 2.3 was used to identify peptides and proteins using the mouse genome as a reference (mouse_refseq_20070725.fasta) and using one decoy database with a 99% confidence interval. All detected peptides were also strictly validated by two criteria: Mascot expectation value < 0.01 and Mascot ion score > 36 for whole floating fraction and >25 for the Immunoprecipitated samples. The decoy database search in Mascot search engine allows us to estimate false discovery rate for detected tryptic peptides, which yielded 1-3% for each sample submitted. After the additional filters described above were applied, the peptide false discovery rate decreased significantly down to 0.8%. In each case, all proteins identified in the false discovery analysis (in decoy database search) were inferred by a single peptide hit. To be included in our lists we required that each protein be represented by 2 or more unique peptides.

Pathway Analysis

Lists of peptides identified in the Mass Spectrometry analyses were subjected to pathway analysis using the Ingenuity® Pathway Analysis software package from Ingenuity® Systems (Redwood City, CA). For Ingenuity®, the proteins were identified using their GI accession number and the calculated Exponentially Modified Protein Abundance Index (emPAI) number was used as a crude estimate of relative abundance within the sample set.
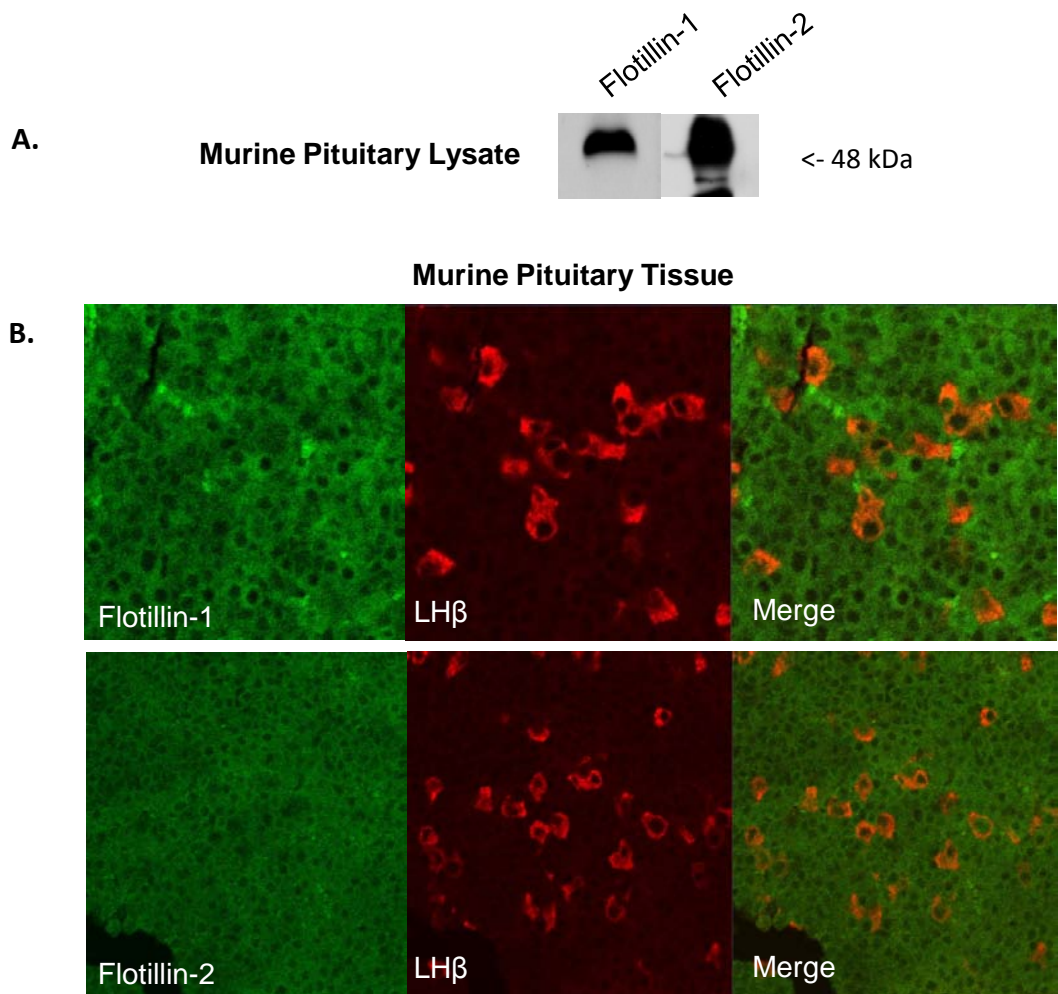
RESULTS

Flotillins 1 and 2 are present in mouse gonadotrope cells

To address the question of whether flotillins might be participating in the membrane raft microdomain into which the GnRHR partitions in pituitary gonadotrope cells, we initially sought to determine whether flotillins-1 and -2 were present in the gonadotrope. For these experiments, whole murine pituitary lysates were obtained and analyzed by Western blot. The blots were probed with mouse monoclonal antibodies directed against flotillin-1 and flotillin-2. Flotillin-1 and -2 immunoreactivity were detected in whole murine pituitary lysate **(Figure 1A)**. As gonadotrope cells only represent 5-8% of cells in the anterior pituitary in mice, we next examined whether flotillins are expressed specifically in gonadotrope cells or in other pituitary cell lineages. Sections of murine pituitaries were co-labeled with anti-flotillin-1 or anti-flotillin-2 and anti-LHβ as a marker of gonadotrope cells **(Figure 1B)**. Confocal laser scanning microscopy was performed and revealed strong immunoreactivity for flotillin-1 and flotillin-2 throughout the cells of the anterior pituitary. As expected, only a small sub-set of these cells stained positive for LHβ; however, the merged image reveals flotillin-1 and flotillin-2 co-localize with LHβ immunoreactivity in gonadotropes.

64

Figure 1. Flotillins 1 and 2 are present in mouse gonadotrope cells.
A. Whole murine pituitary lysates were resolved by SDS-PAGE followed by western blot analysis. The blots were probed for flotillin-1 and flotillin-2. **B.** Fixed sections of murine pituitaries were probed simultaneously with anti-flotillin-1 or anti-flotillin-2 and anti-LHβ antibodies. Fluorochrome-conjugated secondary antibodies (flotillins with FITC; LHβ with Texas Red) were then applied to the sections. Confocal laser scanning microscopy imaging was performed to observe the sections.

Figure 1

A.

Murine Pituitary Lysate

Flotillin-1   Flotillin-2

<- 48 kDa

Murine Pituitary Tissue

B.

Flotillin-1   LHβ   Merge

Flotillin-2   LHβ   Merge

66

Flotillins 1 and 2 are present in the detergent-resistant membrane raft compartment in mouse gonadotrope cells and αT3-1 cells

We next examined whether flotillins-1 and -2 were localized constitutively and exclusively to membrane raft microdomains in pituitary gonadotrope cells. For this, detergent-resistant membrane fractions were resolved using SDS-PAGE and the blots were probed with anti-flotillin-1 or anti-flotillin-2 (**Figure 2A**). Flotillin-1 and -2 were detected in the low density membrane raft fractions, but not in the high density fractions suggesting that the flotillins do partition into detergent-resistant membrane raft domains in mouse pituitary cells. Similar results were obtained in αT3-1 cells, an immortalized pituitary gonadotrope cell line, (**Figure 2B**) and support the use of this cell line in subsequent experiments. To determine whether our sucrose gradient fractionations sufficiently reduced the complexity of our samples compared to whole cell lysate or the non-detergent-resistant membrane fractions (deep fractions), we resolved representative samples from whole cell lysate from αT3-1 cells, total membrane pellet, the low density detergent resistant membrane fraction and the deep fraction by SDS PAGE and subjected the gel to staining with silver salts (**Figure 2C**). These images confirm that there are noticeably fewer proteins present in the low density fractions when compared with either whole cell lysate or the deep fraction.

Flotillins colocalize within GM1 positive domains at the plasma membrane
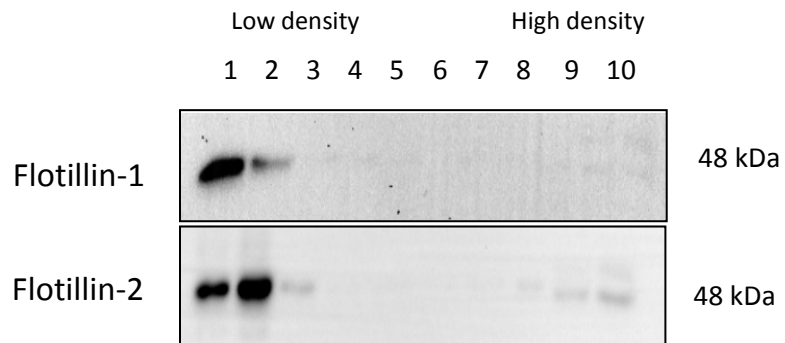
We then asked whether flotillin-1 and flotillin-2 are present within the membrane raft microdomain of intact cells. To address this question we used Alexa 594-conjugated cholera

67

Figure 2. Flotillins are present in membrane rat fractions. Whole pituitaries (**A**.) and αT3-1 cells (**B.**) were lysed and placed in a discontinuous sucrose gradient. Following centrifugation, ten fractions beginning from the top of the gradient were obtained. The fractions were then resolved by SDS-PAGE and analyzed by Western Blot using anti-flotillin-1 or anti-flotillin-2 antibodies. (**C.**) Silver stained images showing the protein present in whole cell lysate, isolated total membrane, low density fraction 1, or high density fraction 10.

Figure 2

A.   **Pituitary Lysate**

Low density   High density

1 2 3 4 5 6 7 8 9 10

Flotillin-1   48 kDa

Flotillin-2   48 kDa

B.   **αT3-1 Lysate**

Low density   High density

1 2 3 4 5 6 7 8 9 10

Flotillin-1   48 kDa

Flotillin-2   48 kDa

C.

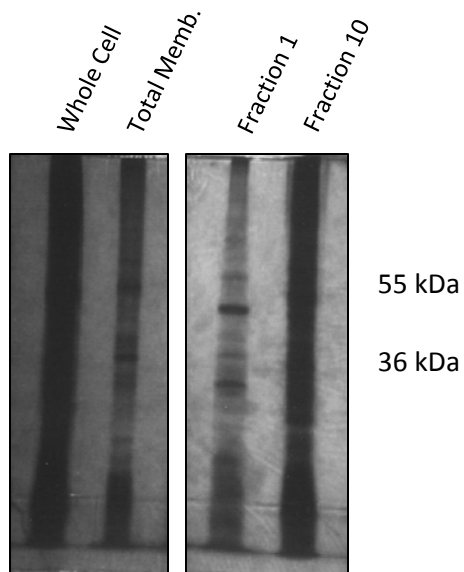Whole Cell   Total Memb.   Fraction 1   Fraction 10

55 kDa

36 kDa
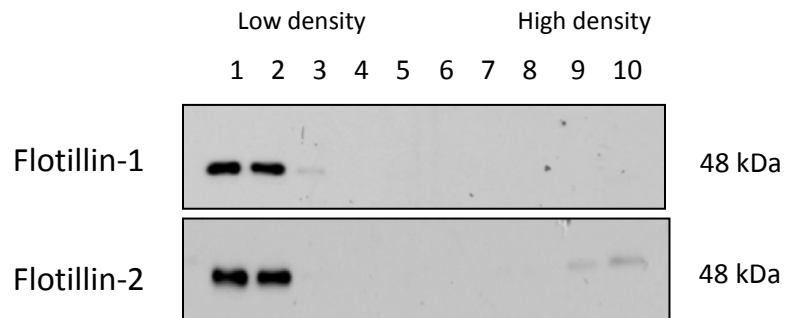
Figure 3. Flotillins 1 and 2 colocalize within GM1-positive domains at the plasma membrane. A. αT3-1 cells that were transiently transfected with flotillin-1-GFP and subsequently stained with Alexa 594-conjugated CtxB. The confocal image displays flotillin-1 protein in green and ganglioside GM1 in red. Colocalization results in a yellow color. **B.** αT3-1 cells were transiently transfected with Flotillin 1-cherry, and Flotillin 2-GFP. The transfected cells were then fixed and imaged using laser scanning confocal microscopy.

Figure 3

Flotillin 1-GFP   **CtxB Alexa-594**   **Merge**

B.

Flotillin 2-GFP   **Flotillin 1-Cherry**   **Merge**

71
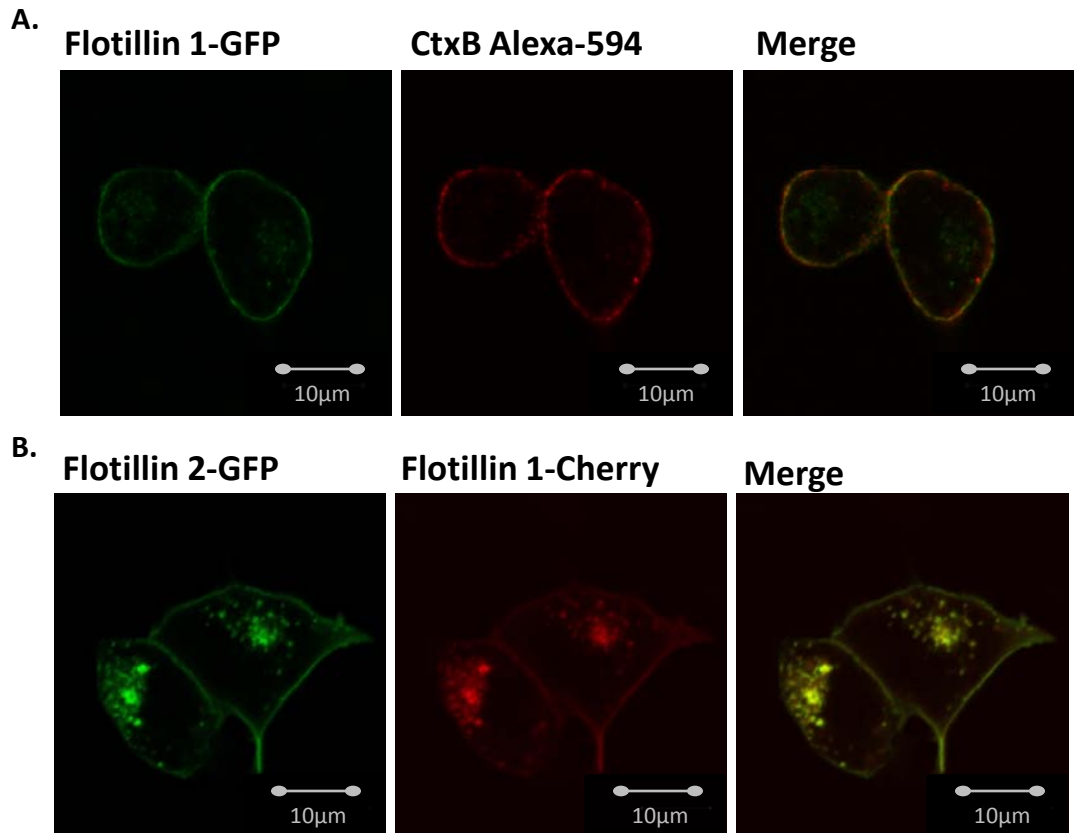
toxin B to mark ganglioside GM1-positive membrane raft microdomains in αT3-1 cells

transiently transfected with a green fluorescent protein (GFP) labeled flotillin-1 **(Figure 3A)**.

Here the merged image reveals the flotillin-1 fluorescent signal to colocalize within GM1-

positive domains. Next we questioned whether the two flotillin proteins define the same or

separate domains by transiently co-transfecting flotillin-1-cherry and flotillin-2-GFP **(Figure**

3B). In this case confocal microscopy revealed that the two flotillins colocalize with each other

and, by extension, with GM1 within membrane rafts in αT3-1 cells.

Flotillin 1 and 2 redistribute following treatment with GnRH agonist (GnRHa)

It has previously been shown that αT3-1 cells alter their morphology in response to treatment

with GnRH agonists [Navratil 2007]; therefore, we reasoned that this might have an effect on

flotillin distribution and localization at the plasma membrane. To determine whether this was

the case, αT3-1 cells were transiently transfected with flotillin-1-GFP and then were either

untreated or exposed to the pharmacologic GnRH super-agonist (GnRHa), Buserelin, at a

concentration of 10nM for 15 minutes prior to fixation and imaging **(Figure 4 A and B)**.

Similarly, αT3-1 cells were either untreated or exposed to GnRHa for 15 minutes before being

fixed and stained for phalloidin **(Figure 4 C and D)** as a control showing actin reorganization in

response to GnRHa treatment. These images demonstrate that flotillin-1 redistributes within the

membrane and enriches at lamellapodia and filopodia following GnRHa treatment. A similar

pattern was seen with flotillin 2 GFP and redistribution to membrane regions associated with

filopodial formation **(Figure 5)**. Interestingly, the images captured also appear to show that

flotillin 2 forms dynamic aggregates within the plasma membrane.

Figure 4. Flotillin-1 redistribution following GnRHa. A. αT3-1 cells were transiently transfected with flotillin-1-GFP and subsequently fixed. The cells were then imaged by confocal laser scanning microscopy. In the image, flotillin-1 appears as a green color. **B.** The αT3-1 cells in this image were exposed to 10 nM Buserelin for 15 min prior to fixation. The arrows point to the redistribution of flotillin-1 around the membrane. **C.** Untreated αT3-1 cells were fixed and then stained with Alexa 594-phalloidin prior to imaging. Actin appears as a red color in the images shown. **D.** αT3-1 cells were exposed to 10 nM Buserelin for 15 min prior to fixation and then stained as in panel C.

Figure 4

A.

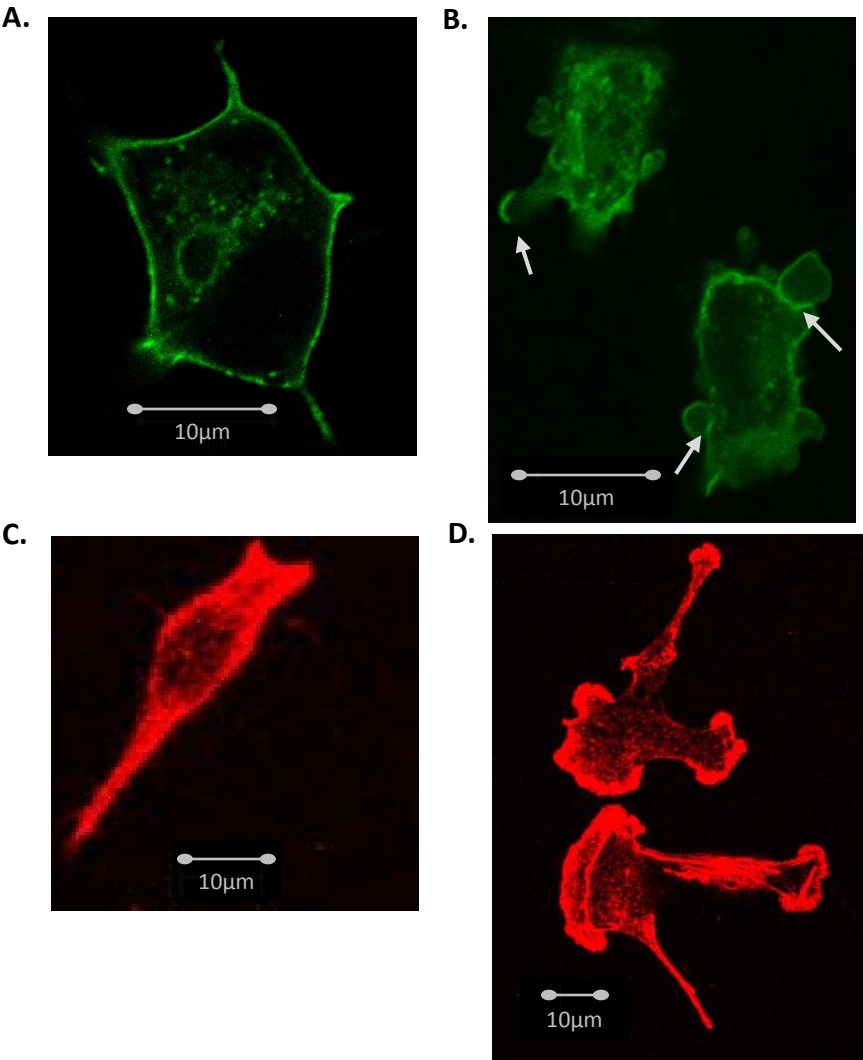B.

10μm

10μm

C.

D.

10μm

10μm

74

Figure 5. Flotillin-2 redistribution following GnRHa. αT3-1 cells were transiently transfected with flotillin-2-GFP and live cells were imaged by confocal laser scanning microscopy. In the image, flotillin-2 appears as a green color. The cell in these images was exposed to 10 nM Buserelin while a live-action video was captured. The above panel shows the cell at rest (untreated), at 3, 5, and 7 minutes post exposure to GnRHa. The arrows point to the redistribution of flotillin-2 around the membrane near areas of process extention/retraction.

Figure 5
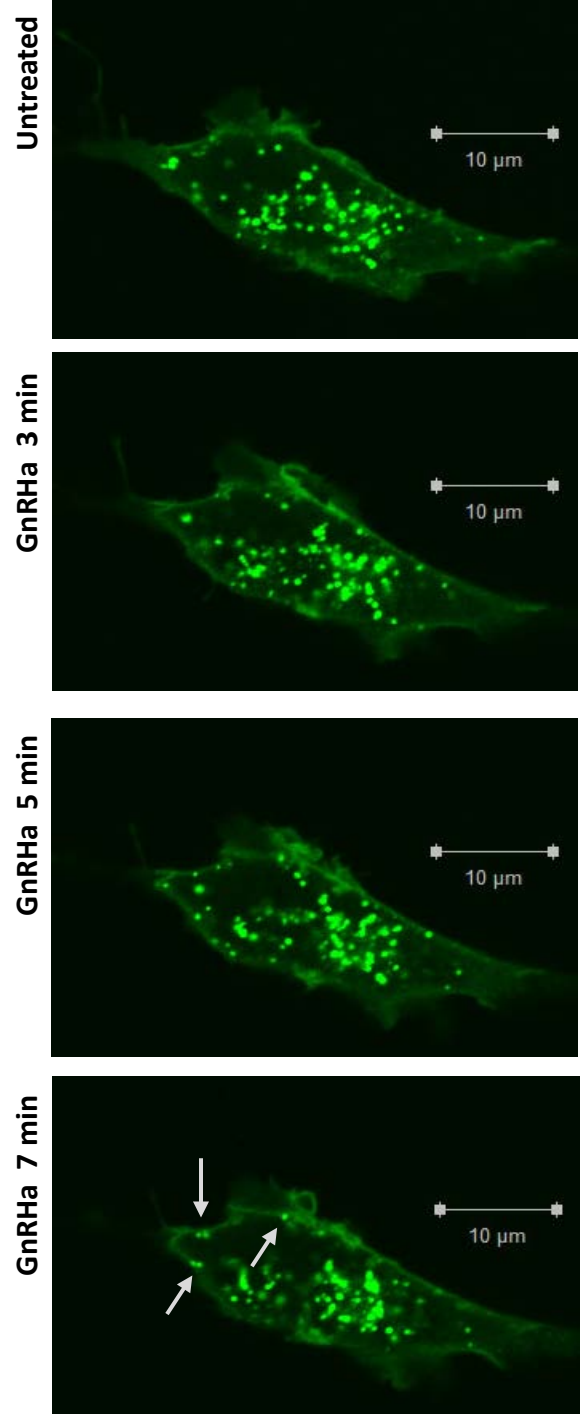
Flotillins 1 and 2 coimmunoprecipitate with the GnRH Receptor from membrane raft fractions

Next we tested the hypothesis that the GnRHR and Flotillin occupy the same membrane microdomain in pituitary gonadotropes. To test this hypothesis, immunoprecipitation of the GnRHR from membrane raft fractions from αT3-1 cells was used. Both flotillin 1 and flotillin 2 co-immunoprecipitated with the GnRHR **(Figure 6A)**. We then performed the reciprocal immunoprecipitation using antibodies targeting either flotillin-1 or flotillin-2. The results provide clear evidence that the GnRHR co-immunoprecipitated with both of the flotillins independent of GnRH agonist treatment **(Figure 6B)**.

Flotillins-1 and -2 colocalize with the GnRHR

Understanding that sucrose gradient fractionation might not be an ideal methodology to determine whether a functional interaction exists between two proteins in a cell under physiologic conditions, we sought to determine whether the flotillin proteins and the GnRHR interact at the membrane in intact gonadotrope-derived αT3-1 cells. A stable cell line expressing the murine GnRHR tagged with an HA epitope was transiently transfected with flotillin-1-GFP or flotillin-2-GFP. Image analysis provides important evidence for overlapping expression domains for the GnRHR and each flotillin **(Figure 7A and B)**.

Figure 6. Immunoprecipitation (IP) of Flotillin 1, Flotillin 2 or GnRHR followed by immunobloting: Lipid raft fractions collected from αT3-1 cells that were exposed to 0 (untreated) or 10nM Buserelin for 15 min. were incubated with anti-GnRHR (**A**), anti-Flotillin 2, or anti-Flotillin 1 (**B**) antibody for 1 hour at 4°C and then to protein A/G agarose beads overnight at 4°C. Beads (IPs) were then washed and resuspended in SDS loading buffer, boiled, resolved using SDS-PAGE, and then probed by Western Blot (IB) as indicated.

Figure 6

A.

IP: GnRHR | Input

Untreated | Buserelin | Untreated | Buserelin

IB: Flotillin-1   <-48 kDa

IB: Flotillin-2   <-48kDa

B.

IP: FLOT 1 | Input

Untreated | Buserelin | Untreated | Buserelin

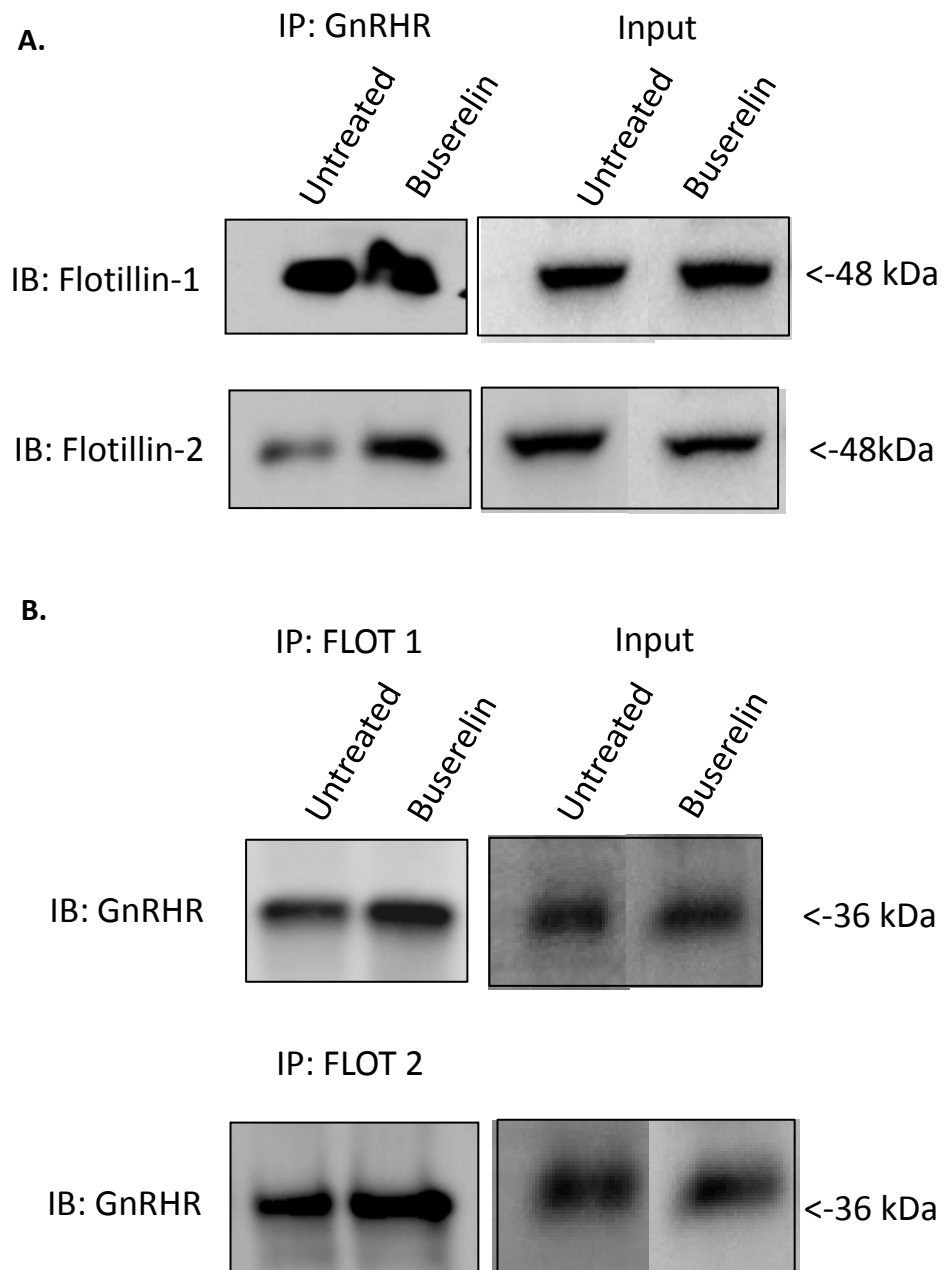IB: GnRHR   <-36 kDa

IP: FLOT 2

IB: GnRHR   <-36 kDa

79

Figure 7. Flotillins 1 and 2 colocalize with the GnRHR at the plasma membrane. A. Cells were stably transfected with HA-GnRHR-BAD then transiently transfected with Flotillin 1-GFP or Flotillin 2-GFP, fixed and stained with rabbit anti-HA antibody and followed with anti-rabbit Texas Red conjugated antibody. Cells were then imaged using laser scanning confocal microscopy. **B.** Cells were stably transfected with HA-GnRHR-BAD then transiently transfected with Flotillin 1- GFP, fixed and stained with rabbit anti-HA antibody followed with anti-rabbit Texas Red conjugated antibody. Cells were then imaged using a z-stack series. The images represent every 4th image from the z stack moving left to right.

Figure 7

A.

Flot 1- GFP · GnRHR Texas Red · Merge

Flot 2- GFP

B.

81
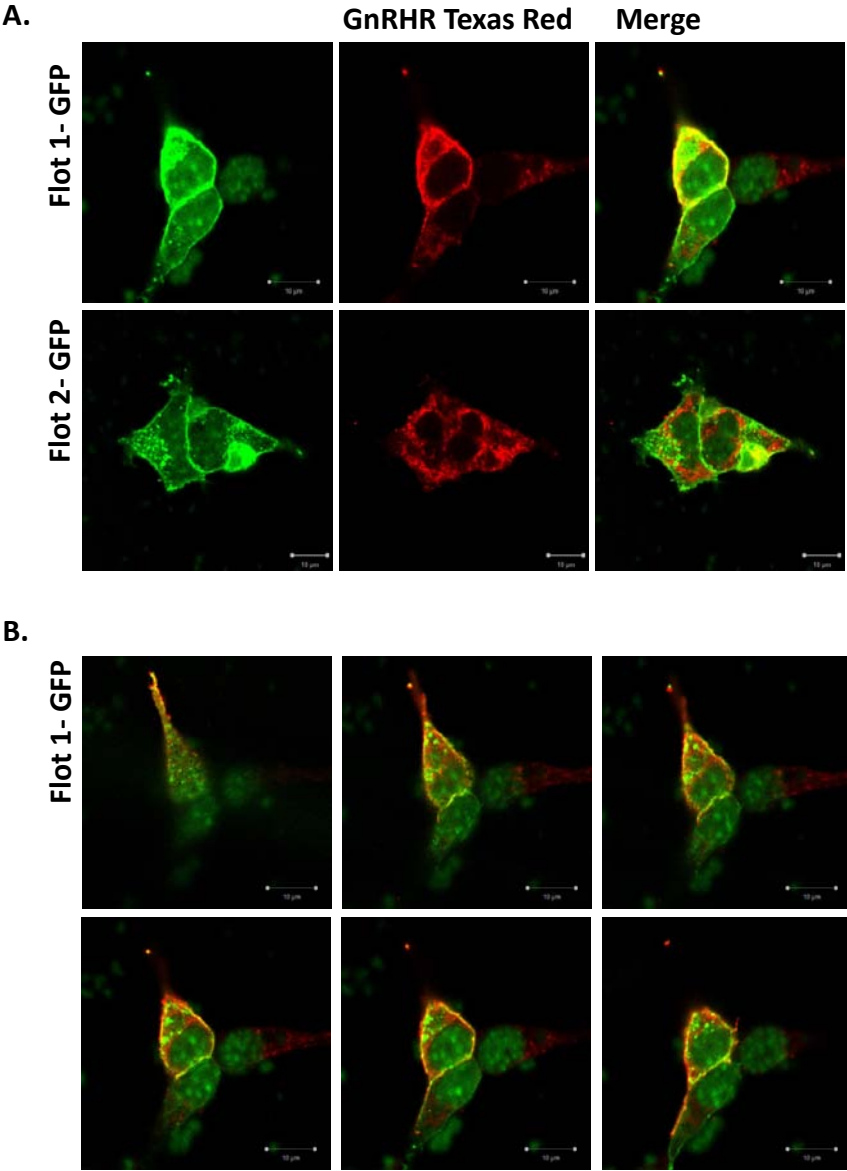
The membrane raft proteome in αT3-1 cells

Evidence presented thus far supports the conclusion that the GnRHR and flotillin proteins co-localize within similar, if not identical, membrane microdomains which led us to hypothesis that such microdomains also include other members of the GnRHR signaling network. In the past, we and others have shown $G\alpha_{q/11}$, c-Raf kinase, and ERK to be constitutive residents of detergent-resistant membrane raft microdomains [5, 14, 26]. Here we sought to further define the membrane raft proteome using mass spectrometry. Sucrose gradient fractionation in the presence of Triton X-100 was performed on three independent samples and only the raft fraction from each replicate was resolved on a single SDS polyacrylamide gel. This gel was then subjected to silver staining which revealed the raft fractions to be substantially less complex than either the deep or whole cell fractions (see **Figure 2C**). Further the three independent raft fractions all showed remarkably similar staining patterns. In collaboration with the Cornell Life Sciences Core Laboratories, our group decided to attempt a mass spectrometry analysis of one of these raft fractions without further resolution via a second gel dimension. An aliquot of the raft fraction was run on a 10% SDS PAGE mini gel and stained with SYPRO®ruby. Eleven gel bands were manually identified (**Figure 8**) and excised from the gel and then subjected to gel-based tandem Mass Spectrometry (MS). The resultant MS data were analyzed by the MASCOT 2.3 software against the NCBI mouse genome database and revealed 2,032 unique proteins. This list of proteins was then analyzed using the Ingenuity® program which demonstrated a great

Figure 8 One Dimensional Gel Images Stained with Sypro®Ruby: Whole cell lysates from αT3-1 cells were subjected to sucrose gradient fractionation. The fraction representing the low density detergent-resistant membrane was then resolved by one dimensional SDS PAGE and stained by Sypro®Ruby. The next lane represents the proteins immunoprecipitated out of the detergent-resistant membrane raft fraction resolved by one dimensional SDS PAGE and stained with Sypro®Ruby. The entire lanes from each of these gels were divided into bands based on staining intensity patterns and these bands were then manually excised and processed for Mass Spectrometry. For reference the molecular weight marker is included and labeled with molecular weights in kiloDaltons.
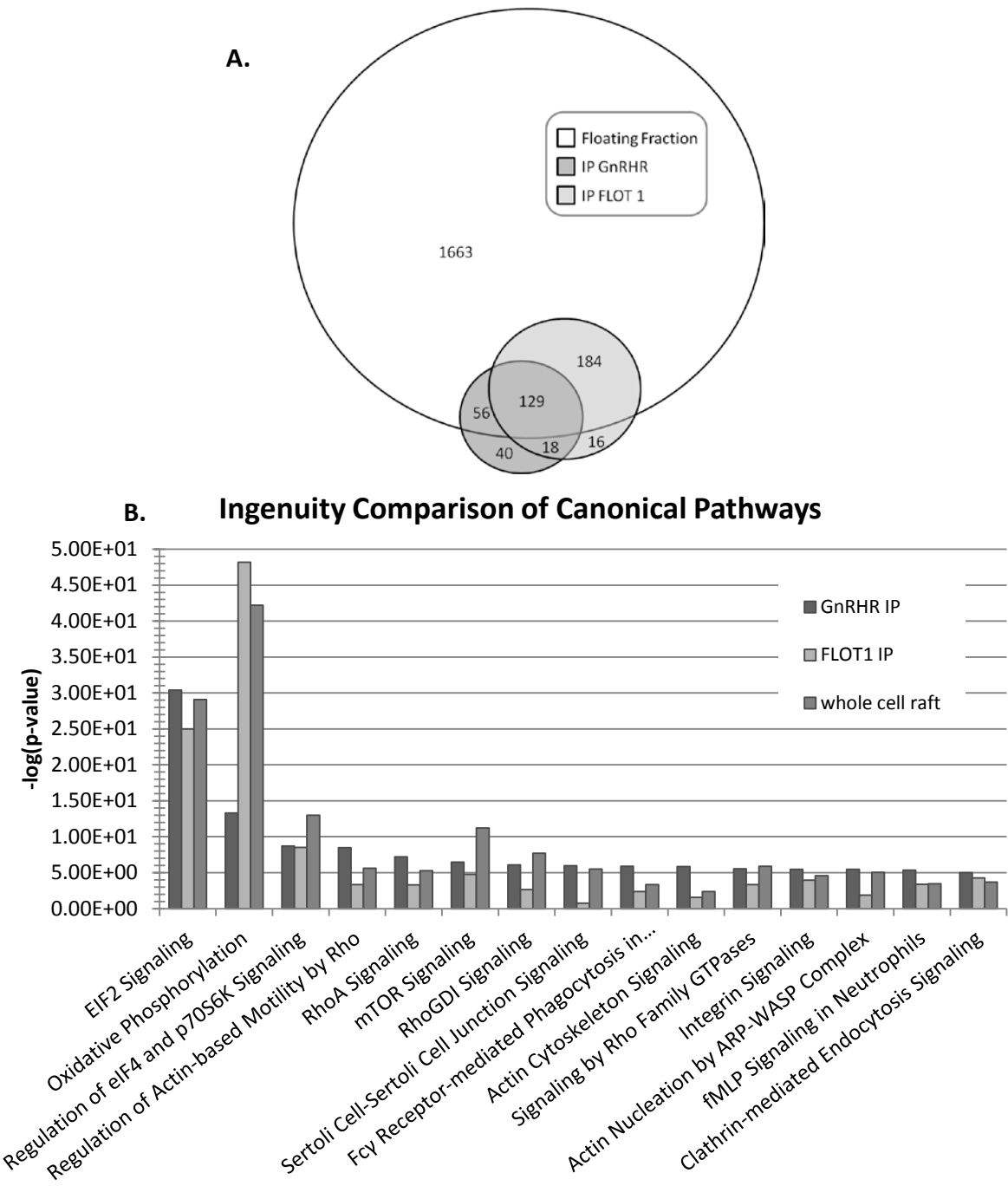
Figure 8
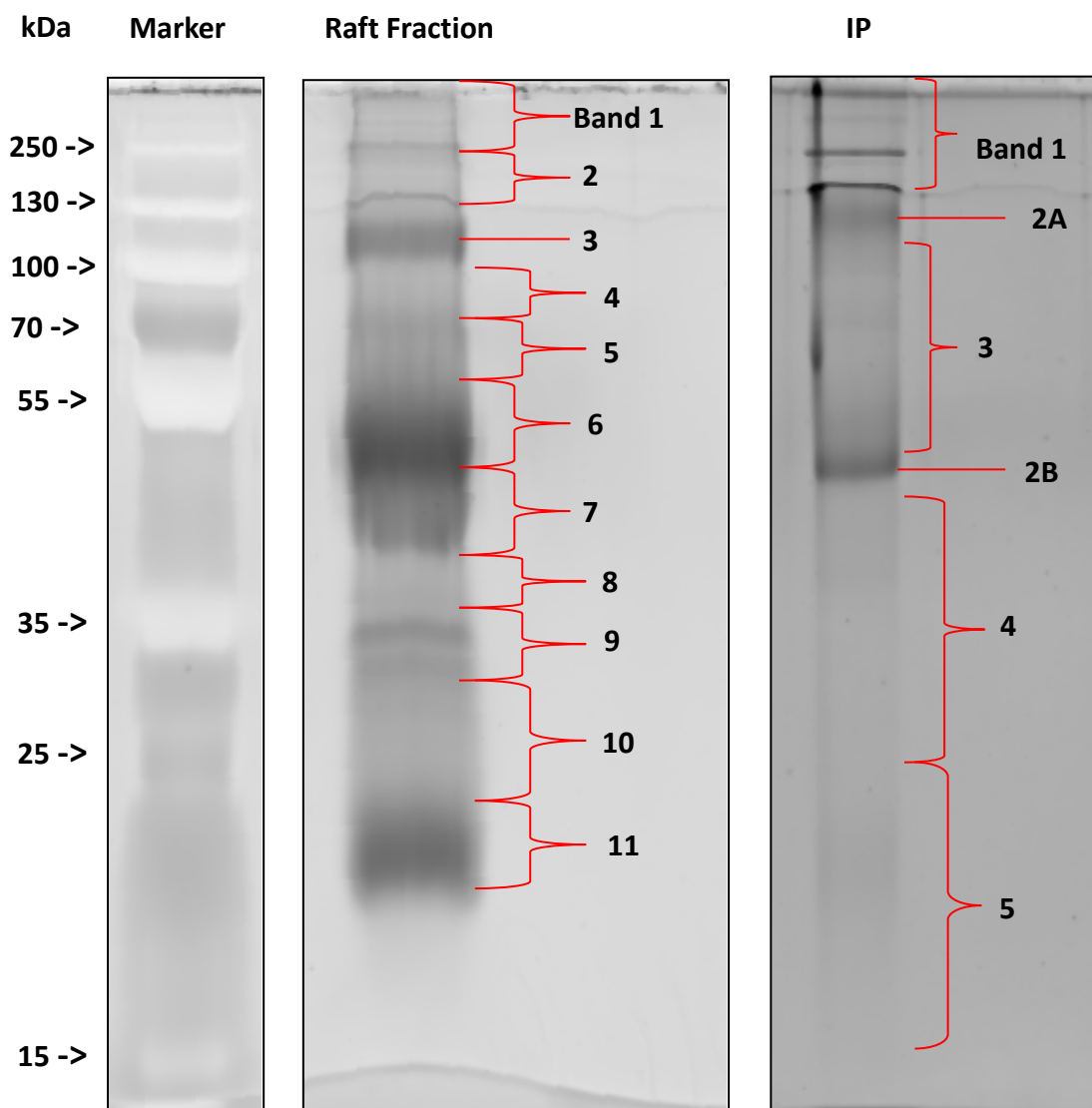
variety of proteins predicted to be involved in many, diverse, cellular processes. These data also revealed markers of endoplasmic reticulum and mitochondria suggesting that the complexity of these samples was the result of proteins not specifically associated with the plasma membrane.

As our primary objective was to determine the membrane raft proteome in which the GnRHR participates and other proteins that might interact with the GnRHR in that context, we decided that our first MS run had not been selective enough. We therefore decided to move forward using immunoprecipitation to isolate what we expected to be relevant proteins out of detergent resistant membrane fractions allowing us to better focus on the specific proteins we predicted would have a high likelihood of participating in the specific membrane raft microdomain in which the GnRHR is resident. For these experiments, detergent resistant membranes were prepared and subjected to immunoprecipitation using antibodies cross-linked to agarose beads to prevent contamination of the mass spectrometry sample with the antibodies used to precipitate proteins of interest. In separate experiments, we targeted either the GnRHR or flotillin-1 using antibodies directed against each of these proteins. The resulting samples were then prepared for gel-based tandem MS as above with the exception that only 5-7 bands were manually excised from each gel (**Figure 8**). From these experiments, we identified 243 unique proteins by immunoprecipitating the GnRHR and 347 unique proteins by immunoprecipitating flotillin-1. We then analyzed the data sets from each of the three mass spectrometry experiments to determine which proteins were shared in common between and among the different methods **(Figure 9A)**. This analysis revealed 129 proteins to be present in all three isolations. We then used Ingenuity® to compare the three populations of proteins and to predict the canonical pathways represented, a portion of this analysis is shown in **Figure 9B**.

Figure 9. Summary of mass spectrometry findings. A. Venn Diagram showing proteins identified by mass spectrometry. Numbers inside the figure represent the number of proteins identified by mass spectrometry for each approach with areas of overlap indicating shared proteins present in both or all the indicated approaches. **B.** The list of proteins identified via tandem MS all three sample preparation strategies was input into the Ingenuity® Pathway Analysis software and a Comparison Analysis was run. This figure shows the first 15 canonical pathways in which identified proteins from each strategy are predicted to be involved and the relative probabilities that the proteins occurring in our analyses were there by chance rather than representing a valid pathway (p-value). In order to more easily visualize the differences between p-values among these pathways we took the negative log transformation of the p-value in order to maintain the upright orientation of our bar graph while maximizing the visual distance between values.

Figure 9

A.

Floating Fraction
IP GnRHR
IP FLOT 1

1663

184

129

56

18   16

40   18

B.   **Ingenuity Comparison of Canonical Pathways**

Pathway analysis reveals a short-list of proteins involved in several key pathways

The Ingenuity® analysis of proteins present in all three approaches revealed several canonical pathways of interest. When we compared the proteins involved in each of these pathways, a core set of 11 proteins linked to signaling through the actin pathway continued to recur **(Table 1)**. We then sought to validate a sub-set of these proteins to determine whether they could be identified within the detergent-resistant membrane raft compartment by traditional biochemical means. In these experiments, detergent-resistant fractions were prepared as above and resolved by SDS PAGE. Blots were then probed with antibodies directed against ARP3, actin, cortactin or phosphorylated cortactin **(Figure 10)**. These studies reveal that each of these proteins reside constitutively, but not exclusively within detergent-resistant membrane fractions. Further, all three proteins co-localized with the GnRHR and Flotillin 1 in the same raft fraction, thus validating our MS studies.

DISCUSSION

The plasma membrane is arguably the most important part of any living cell. It separates the internal from the external milieu and makes homeostasis possible. In spite of its central importance, there is still much we do not know about how the plasma membrane is organized or how this organization impacts cellular behavior in response to hormones. It is clear that the plasma membrane is a heterologous mixture of lipids, proteins, and other molecules, but how are

**Table 1.   Proteins Identified in 11 of the First 15 Cannonical Ingenuity®
Pathways Shared by 3 Independent Mass Spectrometry Analyses**

Gene	Accession Number	Protein ID [Mus musculus]
GNB1	GI 6680045	guanine nucleotide-binding protein, beta-1 subunit
ACTR3*	GI 23956222	ARP3 actin-related protein 3 homolog
MYL6	GI 33620739	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle
ARPC5L	GI 21312654	actin related protein 2/3 complex, subunit 5-like
MYL12B	GI 21728376	myosin light chain, regulatory B
ACTB*	GI 6671509	actin, beta, cytoplasmic
ARPC4*	GI 13386054	actin related protein 2/3 complex, subunit 4
VIM	GI 31982755	vimentin
ARPC3*	GI 9790141	actin related protein 2/3 complex, subunit 3
CTTN*	GI 75677414	cortactin
HSPA8	GI 31981690	heat shock protein 8

Figure 10. ARP3, Actin and Cortactin are present in membrane raft fractions.
αT3-1 cells were either untreated or exposed to 10 nM Buserelin (GnRHa) for 15 min prior to lysis and placement into a discontinuous sucrose gradient. Following centrifugation, ten fractions starting from the top of the gradient were obtained. The fractions were resolved by SDS-PAGE analysis and analyzed by Western Blot using anti-beta Actin, anti-ARP3, anti-Cortactin of anti-phospho (p)- Cortactin antibodies as indicated. Membrane raft fractions blotted for the GnRHR and for Flotillin 1 are shown as a control to indicate the raft fractions.

Figure 10
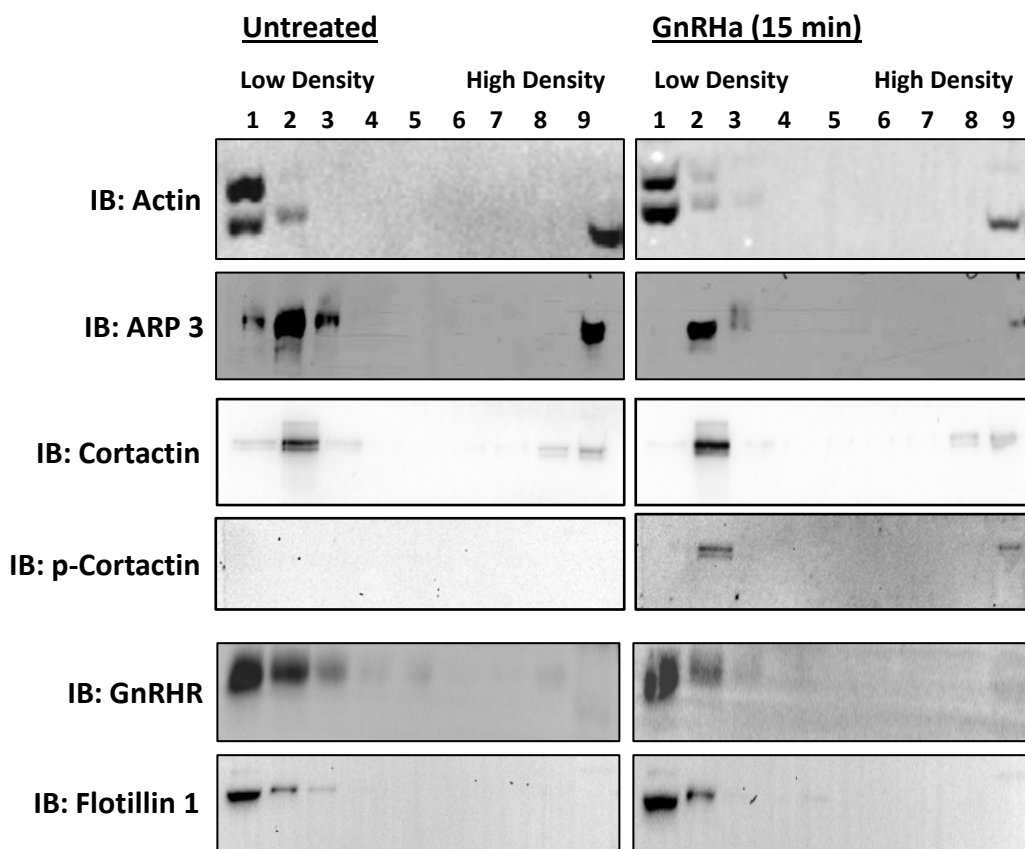
these molecules are organized? The "lipid raft" hypothesis presented a novel, albeit

controversial, idea that cells may be taking advantage of the properties of different lipids,

especially cholesterol, to partition the cell membrane into discrete domains which were resistant

to solubilization with low ionic strength detergents and thus "floated" into low density fractions

during sucrose gradient centrifugation [7, 27]. This hypothesis has been criticized as a mere

artifact of placing cell membranes in the presence of detergent and low temperatures, which do

not occur under physiologic conditions [28]. Recently new techniques including single particle

tracking, double immunogold electron microscopy, transmission electron microscopy, FRET,

and Confocal Laser Scanning Microscopy have provided evidence for the existence of membrane

microdomains in living cells under physiologic conditions [9, 18, 19, 29]. While detergent-

resistant membrane fractionation remains an easy way to isolate components of membrane raft

microdomains, it is now clear that this technique alone cannot be taken to represent

physiologically relevant compartments. For this reason we employed a variety of strategies in

our current studies linking the raft marker, flotillin specifically to the membrane compartment in

which the GnRHR localizes.

We have previously demonstrated that the GnRHR partitions into membrane raft compartments

in gonadotrope cells [5, 6, 14]. Our current studies have shown both flotillin 1 and flotillin 2 to

be present in cells of multiple lineages within the mouse anterior pituitary, within gonadotrope

cells, and in a gonadotrope-derived cell line, αT3-1 cells. In agreement with other cell and tissue

types [21], we demonstrated that both flotillins partition into detergent-resistant membrane raft

fractions in mouse gonadotrope cells. We have further shown, in intact cells, that both flotillin 1

and 2 are found within discrete membrane microdomains in association with both the ganglioside

GM1, and the GnRHR. We also demonstrate that both of the flotillin proteins co-immunoprecipitate with the GnRHR. These data suggest that the GnRHR and both flotillin proteins exist within the same membrane microdomain in association with each other and imply that they may be part of a larger membrane raft-associated complex.

Here we employed mass spectrometry to help us define the membrane raft-associated complex our traditional molecular biology data suggested may exist. We began our proteomic exploration with a less-stringent detergent-resistant membrane-based approach to generate a large, potentially less-accurate, set of detergent-resistant membrane proteins identified through mass spectrometry. This strategy yielded a large number of proteins which pathway analysis predicted to be involved in many, diverse cellular processes providing us with a useful overview of the potential targets that could be involved in membrane rafts in gonadotropes. We then followed this study with a more selective immunoprecipitation-based approach and analyzed the proteins present in all of our approaches in order to reveal a smaller, more selective, list of proteins which we expect to be physiologically relevant in our system. Ingenuity® pathway analysis comparing the proteins present in all three of our MS approaches revealed strong representation of several canonical pathways including EIF-2 signaling, oxidative phosphorylation, EIF-4 signaling, and regulation of actin-based motility by Rho GTPases.

The identification of the canonical pathway involving regulation of actin-based motility by Rho GTPases was of particular interest in our system because it has previously been shown that stimulation of gonadotrope cells with GnRH can induce robust actin cytoskeletal reorganization,

and that such reorganization is upstream of MAPK activation by not only the GnRHR, but also other GPCRs [24, 30, 31]. These studies include the observation that GnRH-dependent engagement of the actin cytoskeleton in gonadotropes is clearly linked to secretion of gonadotropin subunits, a key component of normal reproduction and fertility (AM Navratil and MS Roberson, unpublished data). Additionally, flotillin 2 has been shown to associate with the actin cytoskeleton in several cell types [29]. Here we have shown that flotillin 1 and 2 are capable of forming discrete, punctate domains of high fluorescent intensity which rearrange themselves and appear to coalesce to filopodia in response to GnRH stimulation. Others have shown that flotillins are capable of forming homo- and hetero-tetramers [17], and exist in visible, preassembled platforms[20], consistent with what we observed. This is suggestive of a role for flotillins as a signaling scaffold onto which signaling intermediates may assemble to facilitate rapid, efficient, compartmentalized signaling to the actin cytoskeleton in response to agonist stimulation. This role is further supported by our identification of actin, ARP3, cortactin and phosphorylated cortactin within raft fractions in αT3-1 cells. It may indeed be the case that the ability for rapid secretory responses with engagement of the GnRHR requires these protein complexes within the raft compartment.

The flotillin proteins have also been suggested as defining their own clathrin-independent, dynamin-independent endocytic pathway especially in cells that lack caveolin expression [32, 33]. Additionally, flotillin proteins have been implicated in the formation of membrane raft-associated signal transduction complexes, including src family kinases, and involving inactivation and degradation via endolysosomes [19]. Flotillin-1 was recently shown to be required for endocytosis of the dopamine transporter [34]. Given that the GnRHR internalizes

and desensitizes in an unconventional manner [4] and that αT3-1 cells lack caveolin [5], it seems reasonable to postulate that flotillins may be involved in GnRHR internalization in gonadotrope cells. Our finding that flotillin 1 was capable of responding to GnRHa stimulation by redistributing itself coincident with actin cytoskeletal reorganization appears to support this conclusion.

We have used a variety of techniques to positively determine that flotillin 1 and 2 and the GnRHR are constitutive members of a membrane microdomain which is dynamic, associated with GM1 and actin and which is consistent with what have been called membrane raft microdomains by others. These studies support the conclusion that the GnRHR is resident within a distinct subpopulation of membrane microdomains defined by the presence of flotillin. As the flotillin proteins are more abundantly expressed than the GnRHR, it would seem important to point out that it is unlikely that all flotillin-positive domains would contain the GnRHR; however it appears that all GnRHR-positive domains do contain flotillins 1 and 2. These studies begin to define a membrane compartment/complex(es) that is physiologically and functionally relevant to gonadotrope behavior. Future studies will aim to further define the membrane microdomain in which the GnRHR and flotillin are residents and to explore the role(s) of flotillin 1 and 2 in the context of pituitary gonadotrope function and the control of reproduction.

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Chapter 3: Beta catenin is a functional participant of the GnRH receptor associated membrane raft proteome.

SUMMARY

The gonadotropin releasing hormone receptor (GnRHR) is a unique member of the G-protein-coupled receptor superfamily in that it localizes to detergent-resistant membrane rafts within the plasma membrane of gonadotropes in an exclusive and constitutive manner independent of receptor-ligand interactions. Using proteomic analyses of specific immunoprecipitated (IP) protein complexes from membrane rafts, we previously identified 129 unique peptides within this membrane compartment which can be isolated along with GnRHR and flotillin 1. Pathway analysis of these data provides evidence that a number of these peptides are linked to known signaling pathways and cellular processes. Unique among the peptides within the membrane raft compartment was β catenin. β catenin is present constitutively but not exclusively in rafts from IP studies linked to the GnRHR and flotillin 1. In αT3-1 cells, GnRH administration resulted in a rapid translocation of β catenin to the nuclear compartment putatively independent of phosphorylation of GSK3β, a known modulator of β catenin activity. We hypothesized that β catenin likely regulates key gene transcriptional events linked to activation of the GnRHR in gonadotropes. In order to test this hypothesis, we examined the ability of β catenin to regulate the immediate early gene, Nur77; an orphan nuclear receptor known to be regulated by GnRH as an immediate early gene in gonadotropes. Overexpression of β catenin in αT3-1 cells induced Nur77 promoter activity is a sequence- and promoter-specific manner. The combined actions of β catenin and GnRH resulted in a synergistic activation of the Nur77 promoter suggesting important combinatorial action of GnRH signaling with β catenin. These studies support the

conclusion that membrane raft-localized β catenin may play a critical role in the expression of immediate early gene transcription induced by GnRH in gonadotropes.

INTRODUCTION

The gonadotropin releasing hormone receptor (GnRHR) is a unique member of the rhodopsin-like G-protein coupled receptor (GPCR) family. Since it was first characterized in 1992 [1, 2], the GnRHR has received much attention from the scientific community as a central pharmaceutical target for fertility control in mammals. Despite this level of importance, there is still much to learn about the molecular mechanisms regulating and regulated by this receptor. The GnRHR lacks an intracellular C-terminal tail which, in other GPCRs, would normally be the site of β arrestin-directed internalization [3, 4]. This predicts that the GnRHR is not phosphorylated and internalized via the cannonical β arrestin- mediated pathway [5, 6]. The GnRHR does desensitize and is internalized, yet the mechanism it uses to accomplish this is still unknown. In addition to its non-canonical desensitization/internalization kinetics, the receptor also is a constitutive resident of membrane raft microdomains independent of its activation state [7-9]. This differs from many other GPCRs which only partition into membrane raft domains following ligand binding or receptor dimerization [10, 11]. Membrane raft microdomains are small (10-200 nm), highly dynamic regions of the plasma membrane enriched in sphingolipids and cholesterol and are thought to be important compartments for organizing signaling pathways induced by receptor occupancy [12].

A large body of literature suggests that the GnRHR binds its agonist, gonadotropin releasing

hormone (GnRH), at the surface of gonadotrope cells and stimulates the dissociation of

heterotrimeric G-protein subunits. This leads to activation of phospholipase C (PLC), production

of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), and stimulation of protein kinase

C (PKC) in combination with calcium (Ca^{2+}) influx from both intra- and extra-cellular stores,

and the initiation of several intracellular signaling cascades including the mitogen activated

protein (MAP) kinase pathways (recently reviewed in detail by [9]). The GnRHR is dependent

upon its localization within an intact membrane raft microdomain for its ability to induce some

of these signaling events. For example, treatment of cells with methyl-β-cyclodextrin prevented

GnRH agonist-dependent phosphorylation of the MAP kinase, extracellular regulated signal

kinase (ERK) [7, 9]. Similarly, the GnRHR also requires an unperturbed actin cytoskeletal

network as pharmacological stabilization of actin polymerization prevented GnRH-induced ERK

phosphorylation [13]. Interestingly, both of these effects could be by-passed by direct

stimulation of PKC which did result in efficient induction of ERK phosphorylation [8, 13].

From these findings it seems clear that our current knowledge of GnRH-induction of

downstream signaling is incomplete. We hypothesized that other factors must be involved and

that these would be found in association with the GnRHR within membrane raft microdomains.

In a recent study (Chapter 2 of this dissertation), our lab used a combination of detergent-

resistant membrane fractionation and immunoprecipitation of both the GnRHR and a membrane

raft marker protein, flotillin 1, to generate three sample sets that were subjected to tandem mass

spectrometry in an effort to define the membrane raft proteome. We then analyzed the protein

pathways that were common to all three of these datasets using the Ingenuity® Pathway Analysis

software to help us identify relevant targets for further study and validation. Interestingly, β

catenin was identified as an interacting protein within the membrane raft using immunoprecipitation of both the GnRHR and flotillin 1.

β catenin is a protein known for its ability to bind E-cadherin and as a signaling molecule in the Wnt/Frizzled signaling pathway. In canonical wnt/Frizzled signaling, levels of β catenin in the cytosol are kept low by a destruction complex, APC, Axin and GSK3β. In this complex, GSK3β phosphorylates and targets β catenin for ubiquitination and degradation. Upon the binding of Wnt to its receptor Frizzled on the cell surface, a cascade of intracellular signaling is initiated in which GSK3β is phosphorylated on serine 9 and inactivated. The inactivation of GSK3β allows levels of β catenin in the cell to increase, leading to its translocation to the nucleus where it acts as a transcriptional coactivator on Wnt-responsive genes [14]. Recently, β catenin has been shown to participate in transcriptional coactivation of gonadotropin subunit genes in pituitary-derived LβT2 cells in response to GnRH stimulation [15]. Thus, β catenin may be playing a key role in pituitary gonadotrope function; however, relatively little is known regarding how the GnRH signaling pathway integrates β catenin activity.

Here we used the Ingenuity® software to identify β catenin as potential member of the GnRHR-associated membrane raft microdomain. The present studies confirm that β catenin is present in detergent-resistant membrane fractions in the gonadotrope-derived αT3-1 cell line. We further validate that β catenin is capable of co-immunoprecipitating with the GnRHR in membrane rafts. Wild type β catenin rapidly translocates to the nucleus following stimulation by GnRH, where it is capable of acting as a transcriptional activator on the Nur77 promoter, an immediate early gene known to be transactivated in response to GnRH stimulation. These studies support the

conclusion that signaling to β catenin appears to play a role in the integration of immediate early gene responses induced by GnRH.

MATERIALS AND METHODS

Cells, Antibodies, and Chemicals

αT3-1 cells, an immortalized mouse gonadotrope cell line (generously provided by Dr. Pamela Mellon, University of California, San Diego), were cultured as described previously [7]. Briefly, αT3-1 cells were maintained in high glucose (4.5 g/L) DMEM containing 2 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and 1 X nonessential amino acids and 10% FBS (referred to as growth media). Cells were grown in 5% CO_2 in air at 37°C in a humidified environment. Anti- β catenin antibody was obtained from Cell Signaling Technology (Danvers, MA). Anti-Flotillin 1 and Anti-Flotillin 2 antibodies were from BD Biosciences (San Jose, CA). The GnRHR antibody was raised in a rabbit against 20 amino acids of the second extracellular loop (aa193-aa212) of the ovine GnRHR, and has been described previously [8]. Nonimmune rabbit serum (NRS) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase conjugated secondary antibodies were purchased from BioRad (Hercules, CA). Buserelin (des-GLY10 [D-Ser(t-But)6]-LH-RH Ethylamide; referred to as GnRHa) and all other chemicals were obtained from Sigma. In all experiments, GnRHa was used at 10 nM. Glass bottom microwell dishes for confocal studies were obtained from Mat-Tek (Ashland, MA). PEI (Polyethylenimine) transfection reagent was purchased from Polysciences (Warrington, PA).

Pathway Analysis

105

Lists of peptides identified by Mass Spectroscopy were subjected to pathway analysis using the Ingenuity® Pathway Analysis software package from Ingenuity® Systems (Redwood City, CA). For Ingenuity®, the proteins were identified using their GI accession number and the calculated exponentially modified Protein Abundance Index (emPAI) number was used as a crude estimate of relative abundance within the sample set.

Cell Fractionations

Detergent-resistant, low-density membrane fractions were prepared essentially as described previously [7]. Briefly, αT3-1 cells were grown to 60-70% confluence in 15 cm2 dishes. Following the treatments indicated, cells (~1.5 x 108 per dish) were washed twice in cold PBS, and scraped into PBS with protease inhibitors. Cells were pelleted by centrifugation and then resuspended in MES buffer (MBS) containing 25 mM MES, pH6.5, 130 mM NaCl, and protease inhibitors to a final volume of 400 μL. The samples were adjusted to a final concentration of 0.1% Triton X-100 and incubated on ice for 10 minutes. Following dounce homogenization (20 strokes), the samples were mixed with an equal volume of 90% sucrose, placed in a 5 mL ultracentrifuge tube, and overlaid with a discontinuous gradient of sucrose in MBS consisting of 35% (3.7 mL) and 5% (500 μL) layers. The gradients were centrifuged at 116,000 x g in a SW55Ti rotor for 20 hours at 4˚C. Low-density detergent resistant membranes were visible as a band of flocculent material at the 35-5% interface. Fractions (500 μL) were collected starting from the top of the gradient.

Immunoprecipitation

Immunoprecipitation of proteins was carried out essentially as described by Bliss [8]. Briefly, aliquots (400 μL) of fractions containing suspensions of low-density membranes, or sucrose alone, were diluted in an equal volume of PBS and adjusted to 0.01% Triton X-100. Anti-GnRHR immune serum (IS, 25 μL), anti-Flotillin-1 antibody, anti-Flotillin-2 antibody, or non-immune rabbit serum (NRS, 25 μL), were added, and the samples were rocked for 1 hour at 4°C. Protein A/G agarose beads (30 μL) were added and the samples were rocked overnight at 4°C. Beads were then washed 3 times in PBS with 0.01% Triton X-100, resuspended in 60 μL of SDS load buffer, and boiled.

Confocal microscopy

αT3-1 cells grown on glass bottom microwell dishes were transiently transfected with a plasmid containing wild-type mouse beta catenin linked to a GFP fluorophore at the C-terminus for 48 hours. Transfected cells were then serum starved for 2 hours followed by treatment with 10nM Buserelin for the time indicated, then washed in cold PBS twice. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then imaged. Imaging was done utilizing the 63X oil objective and the 488nm laser line of a Zeiss LSM 510 Meta confocal microscope.

Plasmids and Transfections

The plasmids containing the wild-type mouse β catenin-GFP construct was a generous gift from Dr. James Nelson (Stanford University School of Medicine, Stanford, CA) described in [16]. Transient transfections into αT3-1 cells were accomplished using PEI as follows: 500,000 αT3-1 cells were plated onto 6-well dishes the night before transfection, for each well of a 6-well plate

1μg of plasmid DNA along with 3 μg of PEI reagent were added to 200 μL of serum-free media and incubated at room temperature for 15 minutes, then the suspension was added to the culture media over cells for a period of 4 hours. After transfection the cells were washed and returned to growth medium (10%FBS in high glucose DMEM) for 48 hours. For transient transfections cells were collected following 48 hours of incubation.

Luciferase Assays

The luciferase reporter plasmids used consisted of the mouse promoter regions (described as no. of base-pairs upstream of the ATG start site) of either the GnRHR gene or the orphan nuclear receptor, Nur77 gene fused upstream of a luciferase reporter. For the Nur77 gene promoter, a series of promoter deletion fragments were prepared using PCR and cloned into the luciferase reporter construct. The night before transfection, 500,000 αT3-1 cells were plated onto 6-well dishes. Each well of cells were co-transfected with 1μg of one of the luciferase reporter plasmids along with the indicated dose of wild-type β catenin plasmid DNA, the total amount of transfected DNA was held constant by addition of empty pAVT vector. PEI reagent was added to the DNA mixture in a 3:1 ratio of PEI to DNA in 200μL serum free DMEM, the solution was then mixed and incubated at room temperature for 15 minutes. The PEI-DNA suspension was then added to cells. Cells were transfected for a period of 6 hours, the media was changed and the cells were allowed to recover in growth medium for 4 hours, followed by treatment with 10nM Buserelin (GnRHa) in serum free DMEM for 6 hours. Cells were collected into 250μL of Cell Culture Lysis Buffer from Promega (Madison, WI), and then added to Luciferase Assay Reagent from Promega (Madison, WI) and relative light units measured on a luminometer according to the manufacturer's instructions.

Statistical Analysis

All data are presented as means +/- SE, with analysis by paired t test (proc: t test) (SAS Institute, Cary, NC). Differences were accepted as significant at $p < 0.05$.

RESULTS

Ingenuity® Pathway Analysis of biological functions of peptides identified within the membrane raft proteome.

We have previously used mass spectrometry to describe the GnRHR-associated membrane raft proteome using three approaches: isolating the detergent-resistant membrane fraction, immunoprecipitation the GnRHR, and immunoprecipitation of the raft marker protein, Flotillin 1. Here we used the Ingenuity® Pathway Analysis software to compare the biological function networks common to all three of these MS approaches. The top 9 biological functional networks with the highest significance scores are shown in **Figure 1**. To more easily visualize the differences in significance scores, the negative log of the p-value for each pathway was used rather than the p-value. The asterisk (*****) denotes functional networks in which the common feature of β catenin as a participant in key pathways. β catenin was present in five of the top nine pathways identified by this analysis.

Figure 1. Mass Spectrometry analyses of membrane rafts associated with the GnRHR reveal common pathways. We have previously used mass spectrometry to describe the GnRHR-associated membrane raft proteome using three approaches: i. isolating the detergent-resistant membrane fraction, ii. immunoprecipitation the GnRHR, and iii. immunoprecipitation of the raft marker protein, Flotillin 1. Here we show an Ingenuity® Pathway analysis comparing the functional biological networks common to all three of the above MS approaches. The asterisk (✱) denotes functional networks in which β catenin is predicted to participate.
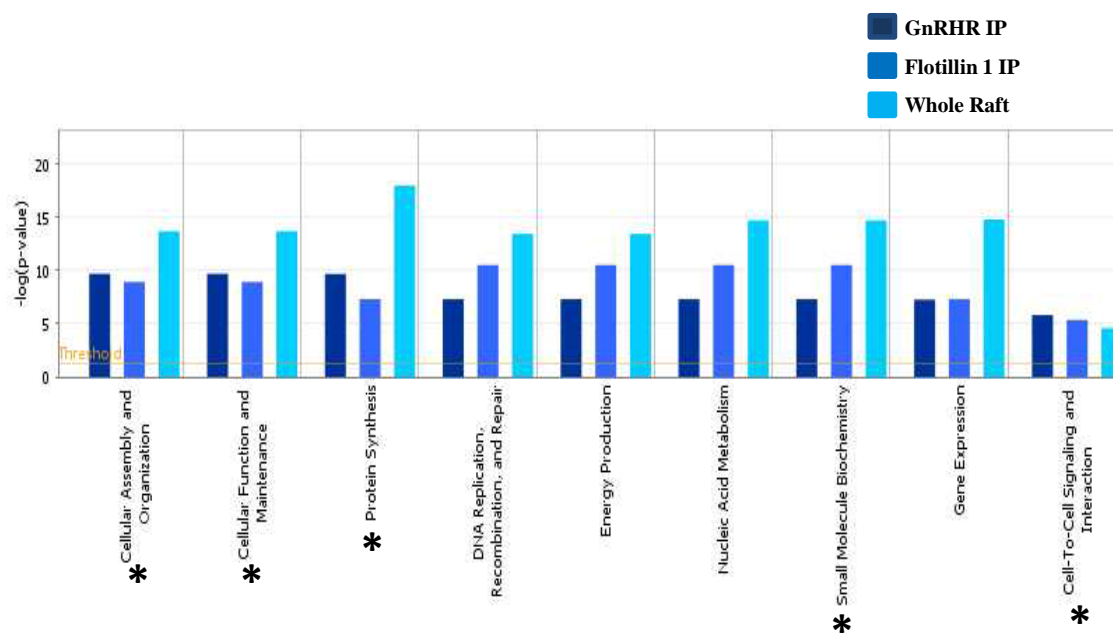
Figure 1

Figure 2. Ingenuity® Cell-to-Cell Signaling Network. Ingenuity® Pathway Analysis identified a Cell-to-Cell Signaling Pathway in which 19 of the 129 proteins identified in our three MS analyses appeared. This figure shows the Ingenuity® Network Map. Grey coloring identifies proteins which appeared in our MS dataset, while white coloring identifies proteins which participate in the network, but were not identified from our dataset.

Figure 2

Table 1: Cell-to-Cell Signaling Network Proteins from three MS Analyses

Abbr.	Accession Number	Protein Name	Abbr.	Accession Number	Protein Name
CTNNA1	6753294	catenin alpha 1	RPL11	13385408	ribosomal protein L11
CTNNB1	6671684	catenin, beta	RPL22	6677775	ribosomal protein L22
EEF1A2	6681273	eukaryotic translation elongation factor 1 alpha 2	RPL23	12584986	ribosomal protein L23
EEF1D	54287684	eukaryotic translation elongation factor 1 delta isoform b	RPL24	18250296	ribosomal protein L24
G3BP1	6680045	guanine nucleotide-binding protein, beta-1 subunit	RPL27	8567400	ribosomal protein L27
JUP	28395018	junction plakoglobin	RPL31	16716589	ribosomal protein L31
MRPL12	22164792	mitochondrial ribosomal protein L12	RPL35	13385044	ribosomal protein L35
Ncl	84875537	nucleolin	RPL12P2	83745120	ribosomal protein, large P2
PABPC1	31560656	poly A binding protein, cytoplasmic 1	RPL22L1	13386010	ribosomal protein L22 like 1
RBMX	6755296	RNA binding motif protein, X-linked			

We next explored the pathways which Ingenuity® Analysis identified from the proteins present in each of our three MS analyses. The three MS approaches outlined above shared 129 proteins in common and 19 of these were predicted to participate in a single network identified by Ingenuity as involving cell-to-cell signaling **(Figure 2)**. Proteins shaded grey were present in our dataset while those in white are network participants which were not identified by our analyses. **Table 1** gives the names, abbreviations and accession numbers of the 19 proteins identified within this cell-to-cell signaling network which occur in our list of 129 shared proteins. For the sake of simplicity, proteins which are predicted to participate in this network, but which were not identified in our MS dataset were not included in Table 1.

β Catenin partitions into membrane raft domains in αT3-1 cells.

We sought to validate the finding of β catenin in our MS studies using biochemical methods. We prepared membrane raft fractions from αT3-1 cells as described above, collecting ten fractions beginning at the top (low buoyant density) of the gradient. Fractions were then resolved by SDS PAGE and immunoblotted for β catenin and Flotillin 1 as a marker of membrane rafts. As shown in **Figure 3,** β catenin partitions into the low density membrane raft fractions, although its participation in the raft complex is not exclusive as it was also found in the high density fraction. The presence of β catenin within the raft fraction was further validated using reciprocal immunoprecipitations using antibodies against the GnRHR and Flotillin 1 confirm that β catenin is directly associated with GnRHR/Flotillin 1-containing rafts **(Figure 4)**. Since the GnRHR changes conformation following the binding of GnRH, cells were exposed to 10nM Buserelin (GnRHa) to examine the possibility that raft association may be altered

Figure 3. β catenin partitions into membrane raft domain in gonadotrope cells.
αT3-1 cells were lysed and resolved in a discontinuous sucrose gradient. Ten fractions
starting from the top of the gradient were obtained following centrifugation. The
fractions were resolved by SDS-PAGE followed by Western Blot analysis using an
anti- β catenin antibody.

116

Figure 3

	Low Density								High Density	

IB: β Catenin   1 2 3 4 5 6 7 8 9 10   <-100 kDa
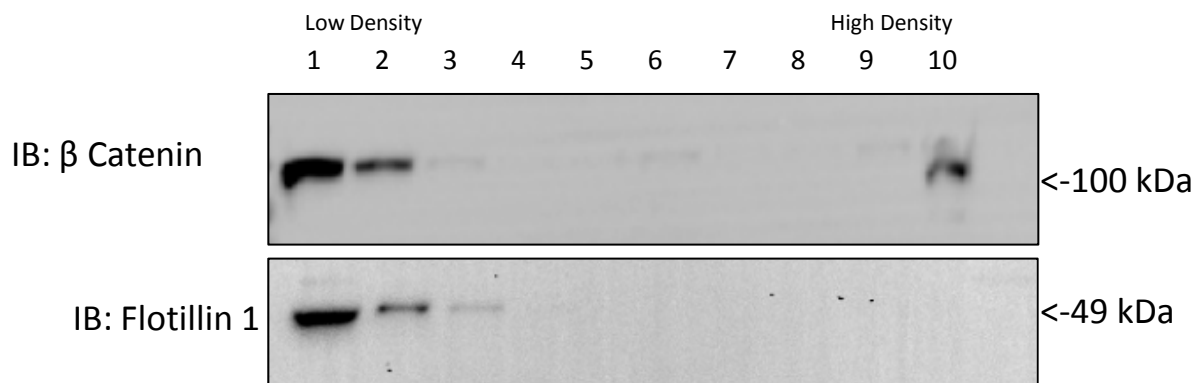
IB: Flotillin 1   <-49 kDa

117

Figure 4. β catenin co-immunoprecipitates with the GnRHR from membrane raft fractions. Lipid raft fractions obtained from αT3-1 cells that were exposed to 0 (untreated) or 10 nM Buserelin for 15 minutes were incubated with anti-GnRHR or anti- β catenin antibody (IP) for 1 hour at 4°C and then exposed to protein A/G agarose beads overnight at 4°C. Beads were then resuspended in SDS loading buffer, boiled, resolved by SDS-PAGE and probed (IB) as indicated.

Figure 4
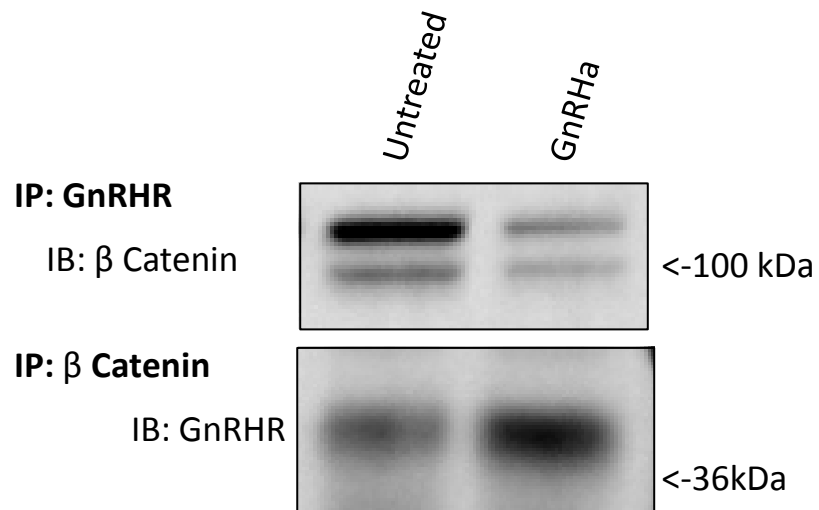
following GnRHR activation. Following GnRHa administration, β catenin was present in immunoprecipates using the GnRHR and Flotillin 1 antibodies, albeit at varying levels. Over the course of multiple studies, differences in the levels of β catenin in these immunoprecipitation experiments were well-within normal experimental variation. These studies support the conclusion that β catenin is present in GnRHR- and Flotillin 1-containing rafts as predicted by the mass spectrometry studies.

Wild type β catenin translocates to the nucleus following GnRHa stimulation in αT3-1 cells independent of GSK3 β phosphorylation state.

In other cell types, β catenin has been shown to translocate to the nucleus in response to treatment with wnt family members.   We sought to determine whether this might occur in αT3-1 cells following treatment with the GnRH agonist, Buserelin. In this experiment, cells were transiently transfected with a plasmid containing wild type mouse β catenin linked to a GFP fluorophore. Forty-eight hours later, transfected cells were serum starved for 2 hours, then either left untreated or treated with Buserelin for 15 minutes. Following treatment, cells were fixed and imaged using confocal laser scanning microscopy. Representative images for β catenin-GFP are shown in **Figure 5A**. These studies suggest that wild type β catenin is distributed diffusely throughout the cell in the absence of GnRHa treatment. Following 15 minutes of GnRHa treatment, β catenin translocated to the cell's nucleus. For the Wnt pathway, the behavior of β catenin is dependent in part upon Wnt-induced phosphorylation of serine 9 on GSK3β. We hypothesized that GnRH-induced modification of GSK3β may help explain the nuclear translocation of β catenin in αT3-1 cells. Interestingly, we find no evidence of GnRH-induced

Figure 5. β catenin translocates to the nucleus following GnRHa stimulation independent of GSK3β phosphorylation. **A.** αT3-1 cells were transiently transfected with expression vectors containing wild type β catenin-GFP. Transfected cells were treated with either vehicle or Buserelin (GnRHa) for 15 minutes, then fixed and imaged using laser scanning confocal microscopy. Magnification bar is set to 10μm. **B.** αT3-1 cells were cultured in the absence or presence of Buserelin (GnRHa; 10 nM) for the times indicated. Cell lysates were resolved by SDS-PAGE and Western blot analysis was used to determine the phosphorylation state of GSK3β and ERKs.
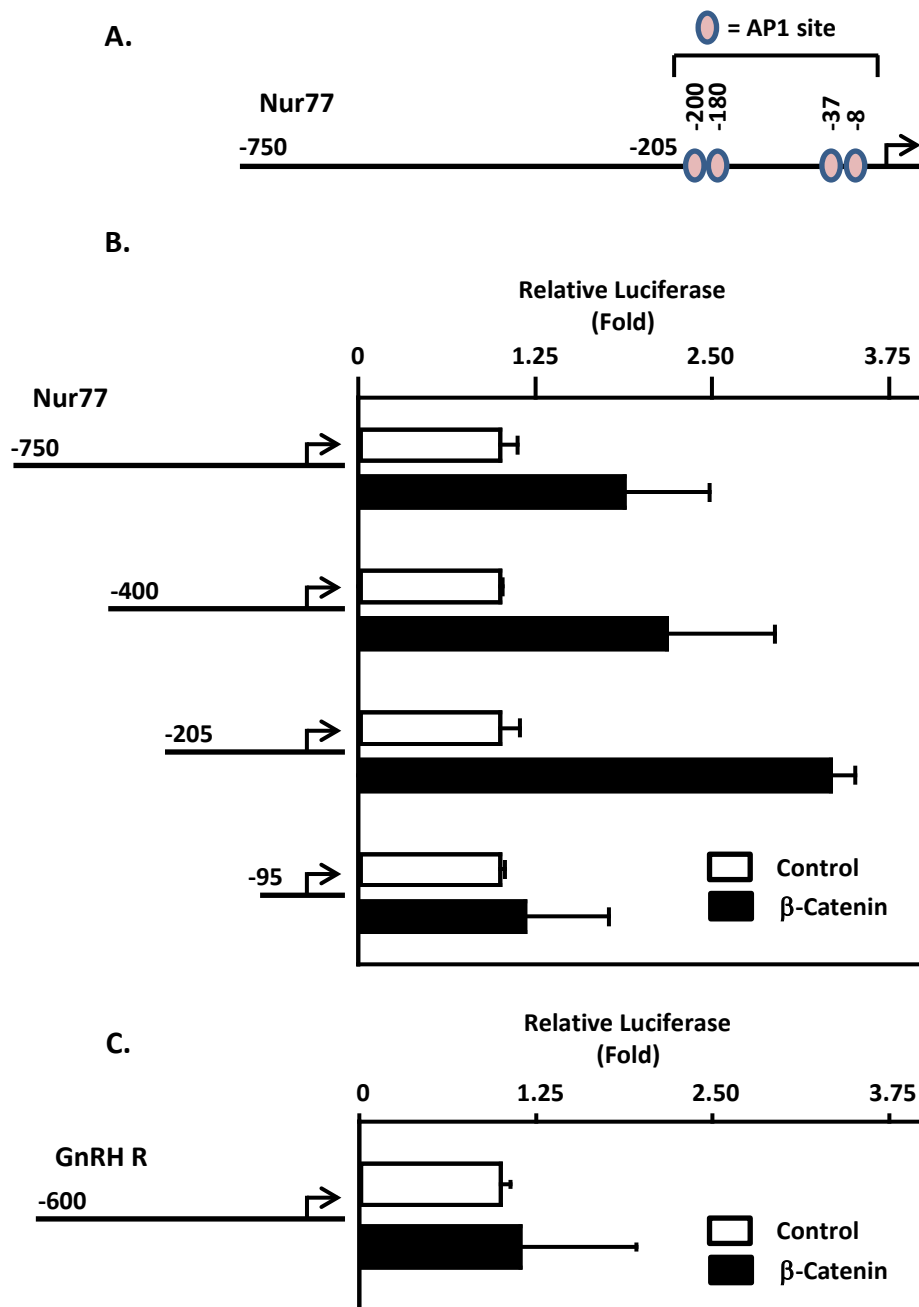
Figure 5

A.

B.
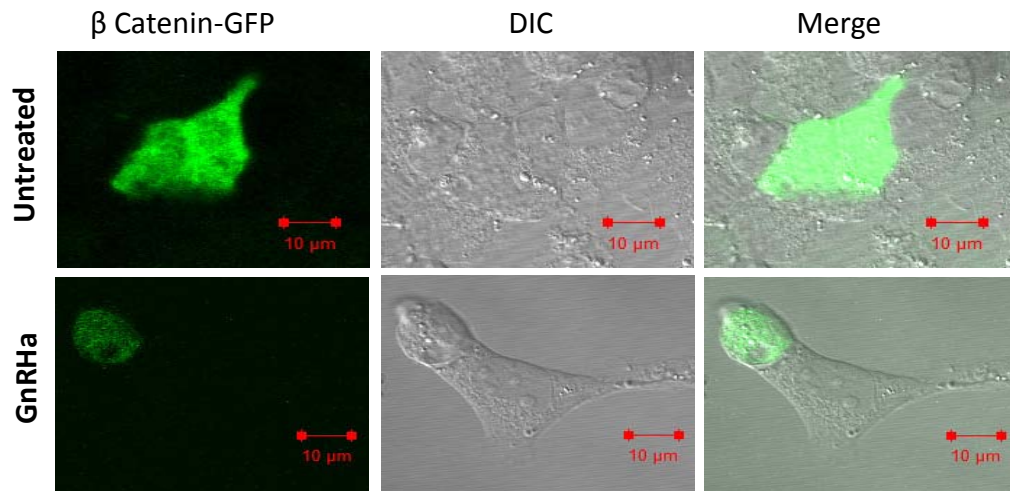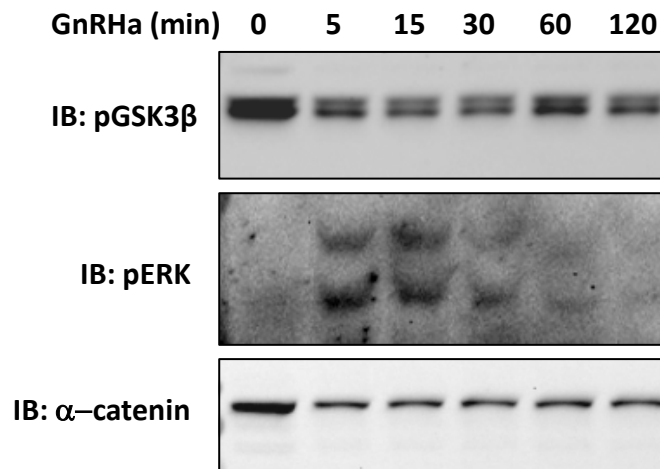
changes in GSK3β phosphorylation state **(Figure 5B)**. In contrast, GnRH induces a robust

phosphorylation of ERK isoforms suggesting that the GnRH signaling pathway was engaged in

these experiments. These studies provide evidence that GnRHa-induced nuclear translocation of

β catenin occurs independent of serine 9 phosphorylation of GSK3 β.

Identification of promoter sequences within the 5' flanking sequence of the Nur77 gene that

are required for β catenin- and GnRH-induced transcriptional activation.

β catenin is known to act as a transcriptional activator in in gonadotrope cell lines leading to

transcriptional activation of gonadotropin subunit genes [15]. We hypothesized that GnRH-

induced nuclear translocation of β catenin would lead to transcriptional responses at other gene

targets in gonadotrope cells. To test this, we examined a gene promoter known to be activated

upon stimulation of gonadotrope cells with GnRHa; the orphan nuclear receptor, Nur77 [17].

Moreover, the Nur77 gene promoter has very recently been demonstrated to be β catenin-

responsive in a colon cancer cell line [18]. Combined, these observations support the possibility

that the Nur77 gene promoter is an attractive candidate gene to examine further. **Figure 6A**

depicts the organization of AP-1 sites within the Nur77 gene promoter that were identified as

regulatory elements associated with β catenin action in colon cancer cells. In our experiment,

plasmids containing a series of promoter deletions (750, 400, 205 and 95 nucleotides upstream of

the ATG) of the mouse Nur77 gene cloned upstream of a luciferase reporter were transiently

transfected into αT3-1 cells. Either a control plasmid or a β catenin expression plasmid was

cotransfected with the luciferase reporter. Following transfection, cells were lysed and luciferase

activity was determined and standardized to protein levels **(Figure 6B)**. Overexpression of β

Figure 6. Identification of promoter sequences within the 5' flanking sequence of the Nur77 gene that are required for β catenin-induced transcriptional activation. A. αT3-1 cells were transiently transfected with luciferase reporter plasmids containing nucleotides from -750, -400, -205, or -95 from the transcription start site of the mouse Nur77 gene. Cells were co-transfected with 1 μg of a control vector (open bars) or and an expression plasmid encoding wild type β catenin (black bar). Twenty four hours following transfection, cells were lysed and lysates were assayed for luciferase activity. The luciferase data was standardized for protein levels and presented as mean ± standard error of the mean for fold induction. **B.** A similar transfection study was carried out as described above except the luciferase reporter contained nucleotides from -600 to the transcription start site of the mouse GnRH R gene promoter.

Figure 6

A.

= AP1 site

Nur77

-750   -205   -200 -180   -37 -8

B.

Relative Luciferase
(Fold)

0   1.25   2.50   3.75

Nur77

-750

-400

-205

-95

Control

β-Catenin

C.

Relative Luciferase
(Fold)

0   1.25   2.50   3.75

GnRH R

-600

Control

β-Catenin

catenin consistently increased Nur77 luciferase activity when sequences from -750 to -205 were present; however, the effects of β catenin overexpression were lost when sequences from -205 to -95 were deleted. The effects of β catenin on promoter activity appear to be specific since similar overexpression studies using a 600 nucleotide fragment of the GnRHR gene promoter linked to luciferase (also GnRHa responsive) was not affect by β catenin overexpression **(Figure 6C)**. These studies support the conclusion that sequences between -205 and -95 of the Nur77 promoter are required for β catenin action. This leads to speculation that the AP-1 sites located at positions -200 and -180 may play critical roles to facilitate β catenin action on Nur77.

Since we had previously identified Nur77 as an immediate early response gene within the GnRH signaling network [17] and found that Nur77 was indeed responsive to β catenin overexpression in a sequence-specific manner, we sought to determine if GnRH signaling and β catenin might function combinatorially to regulate the Nur77 gene promoter. To address this, αT3-1 cells were transfected with the -750, -205 or -95 Nur77 promoter-luciferase reporters. The -205 and -95 promoter fragments were selected based on our previous observations that the -205 Nur77 reporter was clearly the most responsive to β catenin, while the -95 promoter fragment lacked this responsiveness.   For each reporter, some cells were co-transfected with the β catenin expression plasmid and/or administered Buserelin (GnRHa) in a 2x2 factorial experiment. Following transfection and GnRHa treatment, cells were lysed and luciferase activity determined and standardized to protein content of the cell lysates. The preliminary results of these studies are reported in **Figure 7**. In the context of the -750 Nur77 reporter, GnRH induced a robust ~4 fold activation consistent with our previous observations [17]. β catenin action was more modest

Figure 7. Combined actions of GnRHa and β -catenin on the Nur77 gene promoter. αT3-1 cells were transiently transfected with luciferase reporter plasmids containing nucleotides from -750, -205, or -95 from the transcription start site of the mouse Nur77 gene. Some cells received either control solution (open bars) or GnRHa (10nM; grey bars) as indicated. Some cells were co-transfected with 1 μg of a control vector (open bars) or and an expression plasmid encoding wild type β catenin (black bar) or the combination of β -catenin and GnRHa (black/grey bars). Twenty four hours following transfection, cells were lysed and lysates were assayed for luciferase activity. The luciferase data was standardized for protein levels and presented as mean $\pm$ standard error of the mean for fold induction.

Figure 7
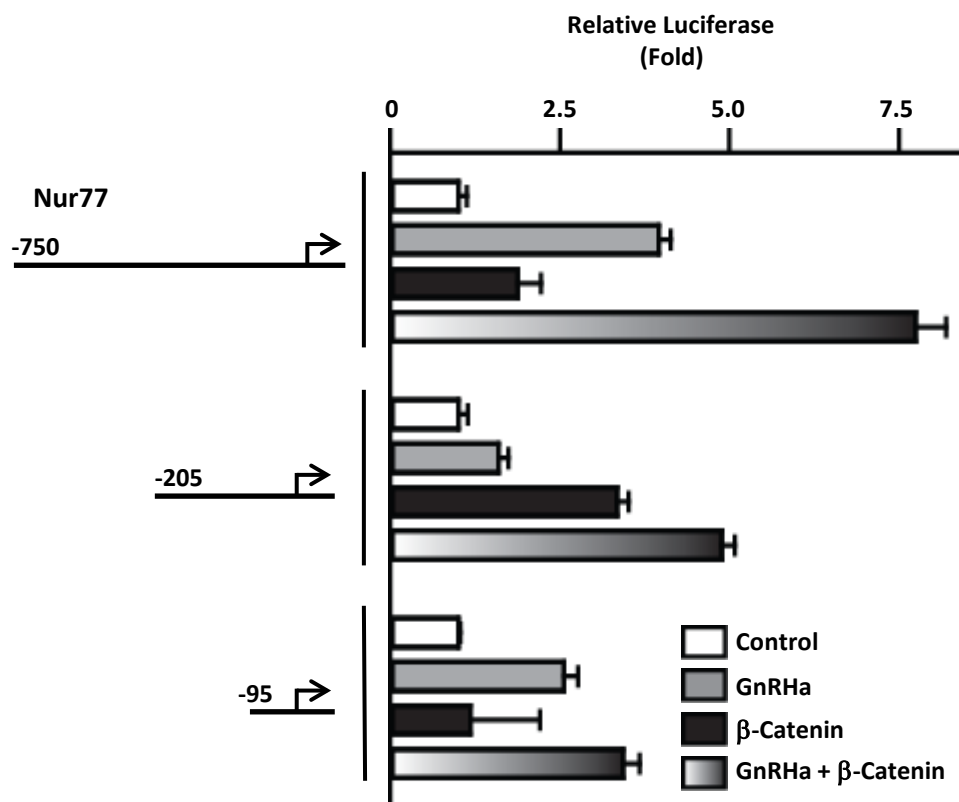
(~2 fold); however, the combined actions of GnRHa and β catenin resulted in a synergistic transcriptional response. A similar trend was evident using the -205 Nur77 reporter; however, GnRH action was less robust (~70% increase) and the response to β catenin was more robust as observed in earlier studies. When β catenin action was lost with the deletion of the Nur77 promoter from -205 to -95, the synergistic response was lost. These preliminary studies suggest that signaling activities induced by GnRH interact with β catenin to promote Nur77 gene transcription in a synergistic manner. Additional studies in this area are necessary to fully elucidate the molecular mechanisms associated with these compelling observations.

DISCUSSION

β catenin emerged from our data sets as a likely candidate for further study in part because it was represented in all of our different approaches to determine the cohort of peptides that populate the raft compartment, and because it has been implicated in signaling processes involving the GnRHR and its associated signaling networks [[15, 19, 20]. β catenin is a ubiquitously expressed protein originally identified, along with α and γ (junctional plakoglobulin) catenin, in complex with the calcium-dependent transmembrane glycoproteins, cadherins [21, 22]. Although β catenin is capable of binding to cadherins at the cell membrane, studies in Madin-Darby Canine Kidney cells have demonstrated that perhaps 50% of catenins within a cell are not associated with cadherins [23]. Further, Stewart and coworkers identified four distinct pools of catenins of which only one includes association with cadherins [24]. These findings suggest that the roles for β catenin within cells are likely many and complex. Consistent with this, β catenin is also known to participate in Wnt/Frizzled signaling where it acts as a transcriptional activator

129

of Wnt-responsive genes [14]. Recently, β catenin has been shown to participate in a variety of GPCR-mediated signaling pathways, including those of the GnRHR [20].

In the present studies, we have shown that β catenin is not only capable of participating in downstream signaling processes, but that it also associates with the GnRHR either directly or as part of a larger complex of proteins within membrane rafts. Interestingly, GnRH treatment has been shown to induce changes in cell morphology and rearrangement of the actin cytoskeleton [13, 25, 26]. In light of this information, our finding that β catenin is found in association with the GnRHR within the membrane raft compartment may be suggestive of a mechanistic connection(s) between GnRHR occupancy and changes in the actin cytoskeletal network. This notion is substantiated from data described within Chapter 2 of this dissertation. β catenin appears to be a likely candidate for communication to the actin cytoskeleton since it is known to bind to at least two proteins which are also capable of binding actin: α catenin and adenomatous polyposis coli (APC). Additionally, our analysis of proteins identified by MS that were immunoprecipitated with the GnRHR included a subset of proteins predicted to be involved in the "Regulation of Actin-based motility by Rho" canonical pathway which may also point toward mechanistic means by which the GnRH signal is "perceived" by the cytoskeleton. Finally, the cell-to-cell signaling network in which we identified β catenin includes integrins, Rap (a small GTPase), and MAPK family members. This further suggests that β catenin may be participating in a network which is capable of relaying the GnRH signal to the actin cytoskeletal network resulting in the activation of MAPK family members possibly including ERK.

β catenin is capable of responding to GnRH treatment by translocating to the nucleus in gonadotrope-derived αT3-1 cells, consistent with the finding of Gardner et. al. in LβT2 cells, a related cell line [27]. Once in the nucleus, β catenin appears to act as a transcriptional activator in αT3-1 cells regulating the expression the orphan nuclear receptor, Nur77. This is consistent with the findings of Salisbury and colleagues who have shown that β catenin is capable of binding to SF-1and acting as a co-activator in association with Egr-1 to promote maximal expression of the LHβ subunit gene promoter in HEK293 and LβT2 cells [15]. Importantly, we show that the effects of β catenin were relatively specific since β catenin overexpression was not sufficient to regulate the GnRHR gene promoter, another GnRH-responsive gene in aT3-1 cells [28-30]. These studies have also identified the region from -205 to -95bp from the ATG of the Nur77 gene as important for β catenin-dependent transcriptional aactivation. Inclusion of the more distal segment from -750 to -205 appears to reduce β catenin's ability to induce transcription of the luciferase reporter element relative to the profound increase seen when sequences distal to -205bp are removed. This suggests that a repressive element may be present distal to 205 bp upstream of ATG which then requires the binding of another factor for maximal promoter activity. Curiously, loss of this distal segment also blunts the synergistic effect seen with both overexpression of β catenin and GnRH stimulation. This suggests that a GnRH-response element (GnRH-RE) or other hormone-responsive enhancer element might be present within the distal segment of the promoter to mediate a putative synergistic effect. Since overexpression of β catenin alone was sufficient to induce activation of these promoter elements in our studies, it seems that endogenous levels of β catenin may be limiting in the absence of hormone treatment. However, transcriptional activity of these promoter fragments could be regulated even higher once cells were stimulated with GnRH suggests that GnRH-induced

signaling events may augment β catenin-induced transcriptional activity. In particular, the activation of MAP kinases and resultant activation of the transcription factors they regulate are likely to be important in determining the maximal activity of this promoter. Interestingly, Salisbury also discovered that β catenin is capable of interacting with TCF/LEF family members in response to GnRH to regulate expression of the transcription factor, cJun [19], while another group identified cJun itself as an interacting partner of β catenin, augmenting transactivation of the Nur77 promoter in SW480 cells [18]. These findings support our conclusion that β catenin overexpression is sufficient to activate the Nur77 promoter, but that response to GnRH treatment likely requires the participation of activated transcription factors possibly including cMyc, cJun, cFos or other factors previously shown to participate in the transactivation of immediate early genes in response to GnRH treatment.

In our studies, we were unable to show phosphorylation of GSK3β, the canonical negative regulator of β catenin in Wnt signaling, on serine 9. In Wnt/Frizzled signaling, phospho-inactivation of GSK3β is prerequisite for rescue of β catenin from the destruction complex and subsequent translocation to the nucleus where β catenin activates the transcription of Wnt-responsive genes. That we were unable to see enhanced phosphorylation of Ser 9 on GSK3β does not necessarily provide supporting evidence that this kinase is not involved in our system. Diverse mechanisms for regulation of GSK3β exist within cells and phosphorylation of Ser 9 represents a single potential mechanism. GSK3β is capable of associating with other proteins in a complex which then augments its kinase activity. For example, GSK3β catalytic activity is enhanced when it is associated with APC and Axin in the so-called destruction complex [31-33]. Meanwhile GSK3β association with one of the GSK3β-binding proteins, Frat 1 or Frat 2 results

in inhibition of its catalytic activity [33-35]. Further, GSK3β has recently been shown to be directly phosphorylated by ERKs in hepatocellular carcinoma cells on its threonine 43 residue [36] indicating that there may be multiple phosphorylation sites capable of activating and inactivating GSK3β and those at play may vary with cell type and signaling pathway.

Finally, we have demonstrated that β catenin is constitutively but not exclusively localized to detergent resistant membrane raft microdomains along with the GnRHR. We propose that membrane raft domains may play a role in β catenin activation in response to GnRH stimulation. In a manner similar to that proposed for ERK activation [Bliss 2007], we speculate that membrane rafts serve as a platform for the association of β catenin with key signaling intermediates. As we have previously demonstrated that kinases including c-Raf and ERK [7-9] are present within membrane raft microdomains, it seems reasonable to predict that β catenin phosphorylation could be facilitated within this membrane compartment where receptor and signaling intermediates are predicted by be in close proximity. It is possible that membrane raft-associated β catenin might become phosphorylated on a number of residues triggering its activation and nuclear translocation. For example, phosphorylation of β catenin on Thr 393 has previously been shown to promote β catenin stability and transcriptional activity [37]. Similarly phosphorylation on Tyr residues (Y86, Y654, and Y670) is associated with β catenin translocation to the nucleus and nuclear stabilization [38, 39]. Thus, it seems reasonable to postulate that β catenin cycles through the membrane raft compartment as a necessary step to becoming activated and translocating to the nucleus, similar to what occurs GnRH-induced ERK activation [8, 9, 40]. There is also the intriguing possibility that β catenin activation might be associated with its dissociation from proteins such as α catenin or APC that then mediate effects

133

on the actin cytoskeleton linking GnRH stimulation to changes in cell morphology. While such speculation is exciting, it is clear that more work needs to be done in this area to sort out the relationship between β catenin, the GnRHR, membrane raft microdomains, and downstream signaling events.

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Chapter 4: Conclusions and Future Directions

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is the central hormone of reproduction in vertebrates. Neurons in the hypothalamus integrate environmental cues, steroid hormone feedback, and other stimuli into the pulsatile secretion of GnRH into the median eminence [1-4]. The hypophyseal portal vasculature then delivers GnRH to the anterior pituitary where it binds to its receptor on the surface of gonadotrope cells. The agonist-bound GnRH Receptor (GnRHR) stimulates a variety of intracellular signaling events culminating in the differential release of the gonadotropin hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH) [5, 6]. The gonadotropin hormones are glycoprotein heterodimers composed of the common α subunit with the hormone-specific β subunits. In addition to secretion of gonadotropin hormones, GnRH also stimulates the transcription of the gonadotropin subunit genes as well as stimulating the transcription of its own receptor [7-11]. The activation of mitogen-activated protein kinase (MAPK) cascades is an essential component of hormone-induced transcription of target genes.

The GnRHR has been shown in recent years to be a constitutive occupant of membrane raft microdomains within the plasma membrane [12-14]. Membrane raft microdomains are defined as small (10-200 nm), dynamic microdomains within the plasma membrane that are enriched in cholesterol and sphingolipids [15]. Additionally, a number of studies have suggested actin to be intimately involved in the organization or maintenance of membrane raft microdomains in cells. Interestingly, the ability of the GnRHR to initiate some downstream signaling processes

140

including activation of the MAPK, extracellular signal regulated kinase (ERK) has been shown to require an unperturbed membrane raft microdomain as well as a dynamic and responsive actin cytoskeleton [12-14]. GnRHR-induced ERK activation is absolutely required for gonadotropin subunit gene expression and optimal fertility in mice [16]. Therefore elucidating the means by which the GnRHR is able to partition into membrane raft microdomains and productively couple to ERK activation is essential to our understanding of fertility.

THE GnRHR AND FLOTILLINS

Flotillin-1 and flotillin-2 (also known as reggie-2/reggie-1, respectively) are widely expressed, highly conserved membrane raft microdomain-associated proteins [17, 18] capable of forming stable homo- and hetero-tetramers [19]. Flotillin proteins are constitutively associated with membrane raft domains in several cell types [18, 20, 21], are capable of forming preassembled platforms in living cells under physiologic conditions [22], and serve as useful markers of membrane raft compartments in some situations. Although their precise function is not yet known, flotillins are thought to participate in membrane receptor-mediated signaling, membrane raft-associated endocytosis, phagocytosis and regulation of neurite outgrowth, cytoskeletal organization, and scaffolding of molecular processes [23]. Recent identification of flotillin-1 as a binding partner for the G-protein subunit, $G\alpha_{q/11}$ [24], the same G-protein subunit which participates in GnRHR-associated signaling in pituitary gonadotropes, led us to hypothesize that flotillins interact with the GnRHR or other members of its' signaling network.

In this dissertation we demonstrate that the flotillin/reggie proteins are found in association with the GnRHR in membrane raft microdomains using biochemical, immunoprecipitation and imaging approaches in mouse pituitary and clonal αT3-1 cells. GnRH administration to αT3-1 cells appears to reorganize the spatial distribution of flotillins 1 and 2 in a manner consistent with

141

actin reorganization. At this point the mechanistic relevance for flotillin association with the GnRHR is unclear. Future studies are needed to determine whether flotillins are required for GnRHR participation in the membrane raft compartment or for coupling to downstream signaling events. Experimental approaches utilizing siRNA silencing or knock down of each of the flotillins followed by assays to determine whether the GnRHR is still capable of partitioning into membrane rafts and activating downstream signaling targets such as ERK will be instrumental to defining the requirement for flotillins. Generation of a dominant negative flotillin may also provide an important tool for the study of flotillin's involvement in the organization of a GnRHR-associated membrane raft compartment. Another intriguing possibility is that the flotillins take the place of caveolins in cells, such as the αT3-1 cell line, which lack caveolae. To determine whether this may be the case future studies may include establishing a clonal line of αT3-1 cells in which flotillin expression has been silenced. In such cells exogenous caveolin can then be expressed in order to determine whether caveolae can take the place of flotillin-associated membrane microdomains and whether GnRHR-mediated signaling still progresses in a similar fashion.

THE GnRHR-ASSOCIATED MEMBRANE RAFT PROTEOME

We have previously demonstrated that the GnRHR constitutively and exclusively localizes to low density membrane raft microdomains [12-14]. Accompanying the GnRHR in membrane raft compartments are several components of its signaling network including $G\alpha_{q/11}$, c-Raf kinase, and ERK [12, 13, 25]. As mentioned above, ERK activation in response to GnRH to be dependent upon an intact membrane raft microdomain and productive engagement of the actin

cytoskeleton [13, 14, 26]. Curiously activation of ERK could still be achieved with phorbol ester treatment despite the perturbation of either membrane raft microdomains or stabilization of actin polymerization [12, 13, 26]. These studies revealed that our understanding of how the GnRH signal is relayed between receptor binding and PKC/ERK activation is incomplete. It appears that some factor (or factors) is missing from our current model of GnRH-induced intracellular signaling including ERK activation. We hypothesized that such a factor would be found in association with the GnRHR within membrane raft microdomains. In order to identify a list of candidate proteins we employed a combination of sub-cellular fractionation and immunoprecipitation to generate samples for Mass Spectrometry analysis. This approach allowed us to examine the low density membrane raft compartment while taking advantage of more selective antibody-antigen interactions to add specificity and relevance to our list of proteins.

Mass spectroscopy (MS) is a powerful tool, when applied to biology it allows for the systematic and unbiased identification of a multitude of targets including those not previously considered. The test is exquisitely sensitive, but the results might not be very specific. Sample contamination can be a problem, whether from keratin dust in the environment, from imperfect sample preparation, or incomplete purification, the detection limits of MS machines are so low that even miniscule amounts of contamination will likely be detected and reported [27]. Further complicating matters, MS may not be able to detect highly hydrophobic proteins which might precipitate out during the process, or proteins which lack trypsin cleavage sites, or which are simply not abundant in the sample [28]. The large data sets it is possible to produce with the use of Mass Spectrometry are both a blessing and a curse. At present MS is unable to tell us what role (if any) identified proteins might be playing or whether that role is crucial or redundant.

There are currently tools being produced to help with this including such things as pathway analysis software and interactome libraries which show great promise, but are still ultimately dependent upon experimental methods to fill out their data sets and increase the predictive power of these computer programs. For the time being, there is still a need for proteins identified by MS to be validated by traditional biochemical and molecular biology techniques.

In this dissertation we have used MS to generate both a large, potentially less accurate list of 2,032 proteins that might be involved in the membrane raft microdomain in αT3-1 cells, and a shorter list of 129 proteins that immunoprecipitated along with both the GnRHR and flotillin out of membrane raft fractions. We further went on to use complimentary techniques to validate that a selection of the proteins we identified here represent true participants within the GnRHR-associated membrane raft proteome and potentially within the GnRHR-induced transcriptional network. Many proteins still remain to be validated from these MS experiments and future studies will be needed to determine whether all of the proteins we identified hold biological relevance within the context of GnRHR signaling in pituitary gonadotropes. It is also important to note that all of our MS studies were performed from cells at rest. As we expect both membrane microdomains and cellular signaling processes to be highly dynamic, these studies might have missed several components of the signaling network which are recruited into the membrane raft compartment only following stimulation. Future studies comparing cells at rest to cells stimulated for various lengths of time will be needed to fully characterize the GnRHR-associated signaling network within the membrane raft compartment.

GnRHR SIGNALING AND β CATENIN IN GONADOTROPE CELLS

Pathway analysis of the 129 proteins identified by MS as noted above provides evidence that a number of these peptides are linked to known signaling pathways and cellular processes. Unique

among the peptides identified by MS within the membrane raft compartment was β catenin. β catenin is a protein known for its ability to bind E-cadherin and as a signaling molecule in the Wnt/Frizzled signaling pathway. In canonical Wnt signaling, β catenin translocates to the nucleus where it acts as a transcriptional coactivator on Wnt-responsive genes [29]. Recently, β catenin has been shown to participate in transcriptional coactivation of gonadotropin subunit genes in pituitary-derived LβT2 cells in response to GnRH stimulation [30]. Thus, β catenin may be playing a key role in pituitary gonadotrope function.

In this dissertation we provide evidence that β catenin is present constitutively but not exclusively in membrane rafts from IP studies linked to the GnRHR and flotillin 1. In αT3-1 cells, GnRH administration resulted in a rapid translocation of β catenin to the nuclear compartment putatively independent of phosphorylation of GSK3β, a known modulator of β catenin activity. We therefore hypothesized that β catenin likely regulates key gene transcriptional events linked to GnRH stimulation of gonadotropes. Indeed overexpression of β catenin in αT3-1 cells induced activation of the promoter for Nur77; an orphan nuclear receptor known to be regulated by GnRH as an immediate early gene in gonadotropes.   Further this activation proved to be sequence- and promoter-specific. The combined actions of β catenin and GnRH resulted in a synergistic activation of the Nur77 promoter suggesting important combinatorial action of GnRH signaling with β catenin. These studies support the conclusion that membrane raft-localized β catenin may play a critical role in the expression of immediate early gene transcription induced by GnRH in gonadotropes.

Missing from our current understanding is how GnRH stimulation is capable of activating β catenin. Future studies to determine whether β catenin is phosphorylated in response to GnRH

treatment in αT3-1 cells will help to elucidate the mechanism by which raft-associated β catenin becomes activated and translocates to the nucleus. As we have seen that overexpression of β catenin is sufficient to drive expression of a GnRH-inducible gene the obvious counterpart would be to determine whether β catenin is required for the activation of Nur77 transcription. Experiments utilizing siRNA knock down of β catenin followed by luciferase assays to determine whether basal or GnRH-induced activity of the Nur77 promoter is disrupted following loss of β catenin will be vital to understanding the role of β catenin in this system. Similarly future studies to specifically identify and mutate the binding sites for β catenin on the Nur77 promoter will help us to define how β catenin is capable of transactivating this promoter. Our studies also revealed an apparently synergistic response to overexpression of β catenin and GnRH stimulation on the activity of the Nur77 promoter. This result points to the potential involvement of other transcription factors activated by GnRH treatment in modulating the activity of β catenin on the Nur77 promoter. Several GnRH-responsive transcription factors are known including cJun and Fos. Future experiments to overexpress or pharmacologically block activation of these transcription factors will help to determine the extent of their involvement in β catenin-associated activation of the Nur77 promoter. Complementary studies including chromatin immunoprecipitation (ChIP) or mobility shift assays can also be utilized to determine what other factors may be involved in β catenin's ability to recognize and activate specific promoter elements.

SUMMARY

The GnRHR-associated signaling network in pituitary gonadotropes is an amazingly complex system which is tightly regulated at all levels. Despite decades of study there is much we still do not know about how the GnRHR is capable of coupling to downstream signaling targets

including ERK. In this dissertation we provide evidence for the association of the GnRHR with flotillins and β catenin in membrane raft microdomains in gonadotrope cells. We have further generated a list of proteins by Mass Spectroscopy that may be involved in mediating the GnRHR's ability to stimulate actin cytoskeletal reorganization and induce downstream signaling events. Future studies will be needed to better define which proteins from this list are involved in the GnRHR-associated signaling network and to characterize the requirement for these proteins in the coordination and control of fertility in animals.

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