DISSERTATION

MEMBRANE ORGANIZATION OF THE GONADOTROPIN RELEASING HORMONE RECEPTOR

Submitted by

Amy M. Navratil

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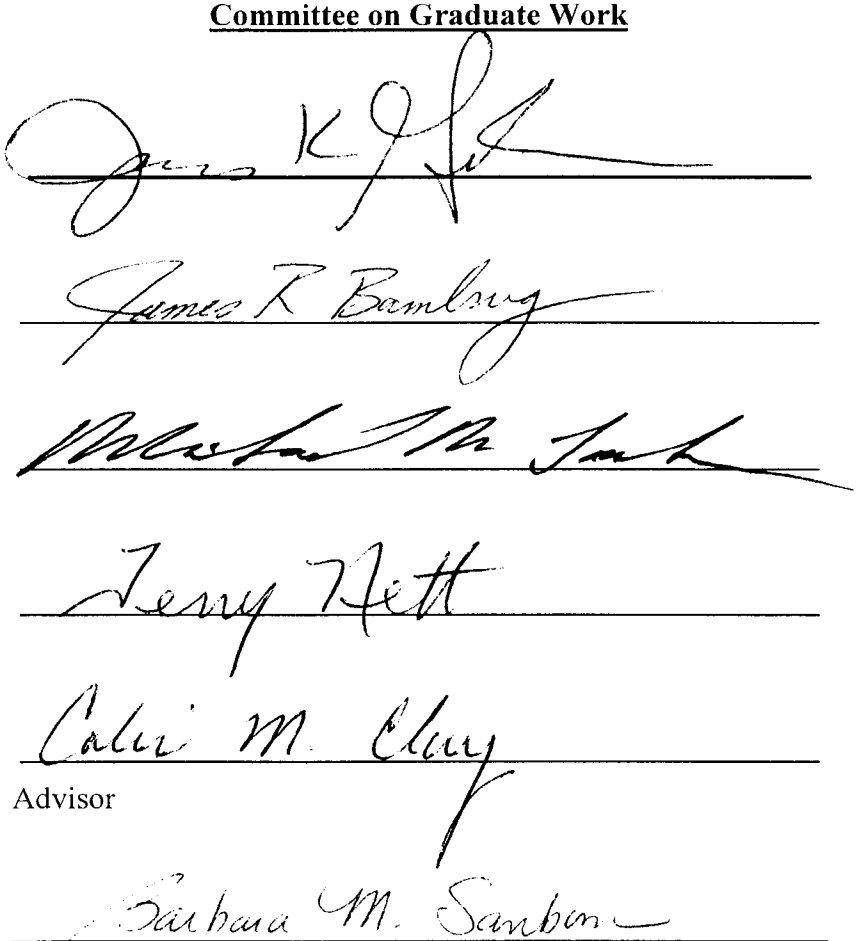
We hereby recommend that the dissertation prepared under our supervision by Amy Navratil entitled "Membrane Organization of the Gonadotropin Releasing Hormone Receptor" be accepted as fulfilling in part requirements for the Degree of Doctor of Philosophy.

Committee on Graduate Work



Advisor

Department Head

ABSTRACT OF DISSERTATION

MEMBRANE ORGANIZATION OF THE GONADOTROPIN RELEASING HORMONE RECEPTOR

Sometimes referred to as the "first hormone of reproduction", GnRH is the key hypothalamic input that stimulates and maintains the functional integrity of gonadotrope cells in the anterior pituitary gland. Thus, the interaction of GnRH with its cognate pituitary receptor is central to the normal reproductive function of mammals. Accordingly, enormous effort has been expended toward understanding physiological, cellular and molecular regulation of GnRH and the GnRH receptor (GnRHR). We have found that the GnRHR and down stream signaling intermediates, including c-raf kinase are constitutive residents of discrete membrane domains termed lipid rafts. These raft domains are thought to consist of tightly packed sphingolipid and cholesterol. From a signaling standpoint, lipid rafts have generated much interest as membrane scaffolds for spatial and temporal organization of cell-surface receptors and their downstream effectors. Towards this end, we have found that disruption of lipid rafts by cholesterol depletion in αT3-1 cells using methyl-β-cyclodextrin disrupted GnRHR but not c-raf kinase association with rafts and shifted the receptor into higher density fractions. Cholesterol depletion also significantly attenuated GnRH but not phorbol ester-mediated

activation of extracellular signal-related kinase (ERK) and c-fos gene induction. We were able to rescue raft localization and GnRHR signaling to ERK upon repletion of membrane cholesterol. Thus, the organization of the GnRHR into low-density membrane microdomains appears to be critical for the ability of GnRH to propagate an intracellular signal to the level of MAP kinase activation.

Given the central role lipid rafts play in the activation of the GnRHR, another key issue is the identity of structural motifs that direct lipid raft localization of the GnRHR. Because the absence of an intracellular carboxyl terminal domain is one of the more conspicuous and unique features of the GnRHR, we were intrigued with the possibility that the association of the GnRHR with lipid rafts may reflect both a loss (C-terminus) and gain (raft association address) of structural characteristics. To address this, we fused either the full length C-terminus from the non-raft associated LH Receptor (LHR) (GnRHR-LF) or a truncated (t631) LHR C-terminus, (GnRHR-LT) to the GnRHR. These chimeric receptors are trafficked to the plasma membrane, bind ligand and display increased agonist induced receptor internalization but do not partition into lipid rafts. Thus, a heterologous C-terminus from a non-raft associated GPCR redirects localization of the GnRHR to non-raft domains. In contrast to the murine GnRHR, the catfish GnRHR (cfGnRHR) possesses an intracellular C-terminus. We find that the cfGnRHR is localized to lipid rafts and that the cfGnRHR C-terminus does not alter raft localization of the mammalian receptor. Consistent with placement in different lipid microenvironments within the plasma membrane, fluorescence recovery after photobleaching (FRAP) reveals different lateral diffusion phenotypes of the raft associated GnRHR and cfGnRHR vs. the non-raft associated GnRHR-LF fusion protein. We conclude that while an intracellular

C-terminus is capable of redirecting the GnRHR to non-raft compartments, this is not a generalized feature of GPCR C-terminal tails. In summary, while the research presented in this dissertation confirms the constitutive presence of GnRHR to lipid rafts, the localization is not simply due to the loss of an intracellular C-terminus.

Amy Navratil
Department of Biomedical Sciences
Colorado State University
Fort Collins, CO 80523
Fall 2005

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A special thank you goes to Dr. Mark Roberson, our collaborator at Cornell University. Thanks for all the advice and guidance throughout graduate school. I'll never look at c-raf kinase the same!

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To Margie

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Since the initial characterization of GnRH in 1971 (Matsuo et al., 1971) a clear picture of the key role of this molecule in controlling reproductive function has emerged. The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but is obligatory for synthesis and secretion of LH and, to a lesser extent, FSH from gonadotrope cells of the anterior pituitary gland (Brinkley, 1981; Clarke et al., 1983; Clayton and Catt, 1981; Desjardins, 1981). In addition, expression of genes encoding for both the common α and unique β subunits of LH is absolutely dependent on GnRH input (Gharib et al., 1990). Given the central role of GnRH in reproduction much effort has been devoted toward understanding the physiological consequences of regulation of GnRH and its pituitary receptor. In regard to the latter, a significant breakthrough was the initial cloning of a cDNA encoding the murine GnRHR (Reinhart et al., 1992; Tsutsumi et al., 1992). Hydrophobicity analysis predicted a polypeptide containing 7 hydrophobic amino acid domains consistent with membership of the GnRHR in the superfamily of heptahelical G-protein coupled receptors (GPCR). Although classified as a member of the rhodopsin class of GPCR, the GnRHR has an extremely short carboxyl-terminal cytoplasmic domain of only 1-2 amino acids (Stojilkovic et al., 1994). The lack of an intracellular C-terminus makes the mammalian GnRHR a unique member of the GPCR

family not only structurally but also functionally (Gether, 2000). For example, the mammalian GnRHR is not phosphorylated and internalization is not mediated via a β-arrestin dependent mechanism like almost all other GPCRs (McArdle et al., 2002).

At the time I began in the laboratory, the lipid raft model was beginning to emerge. Lipid rafts are domains that are resistant to solubilization using non-ionic detergent that "float" when separated through a non-linear sucrose gradient due to their high level of cholesterol and sphingolipid (Brown and London, 1998). Lipid rafts are thought to play a crucial role in signal transduction by organizing and concentrating signaling molecules (Simons and Toomre, 2000). Given the unique structural and functional attributes of the GnRHR, the central focus of my initial work was to determine if membrane microdomains play a role in GnRHR mediated early membrane events.

Herein, I describe that mammalian Type I GnRHR, atypical member of the rhodopsin-like family of G-protein coupled receptors (GPCR's), and c-raf kinase are constitutively localized to low-density fractions independent of hormone treatment in various cell types. Furthermore, the organization of the GnRHR in lipid rafts appears to be critical for the ability of GnRH to propagate an intracellular signal to the level of MAP kinase activation (Navratil et al., 2003).

Next, I wanted to explore the molecular determinants that direct raft localization of the GnRHR. I hypothesized that the absence of an intracellular carboxyl terminal domain in the GnRHR may reflect a raft association address. Essentially, does the lack of C-terminus from the GnRHR cause it to "default" to lipid rafts. To address this, I fused the intracellular C-terminus from either the non raft associated rat luteinizing hormone receptor (LHR) or a raft associated non-mammalian (catfish) GnRHR (cfGnRHR) to the

C-terminus of the murine GnRHR and examined these chimeric proteins for raft distribution after transient expression in CHO cells. My results show that while an intracellular C-terminus is capable of redirecting the GnRHR to non-raft compartments, this is not a generalized feature of GPCR C-terminal tails. Thus, constitutive raft localization of the GnRHR is not simply due to the loss of an intracellular C-terminus.

CHAPTER TWO

LITERATURE REVIEW

I. THE HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS

A. Overview

Proper reproductive function in mammals is dependent upon critical input from the hypothalamus, pituitary, and the gonads. One of the key molecules underlying regulation of the HPG axis is gonadotropin releasing hormone (GnRH). Since the characterization of GnRH, (Matsuo et al., 1971) The role this molecule plays in controlling reproductive function has become evident. The pulsatile discharge of GnRH from hypothalamic neurons is necessary for synthesis and secretion of lutenizing hormone (LH) and, to a lesser extent, follicle-stimulating hormone (FSH) from gonadotrope cells of the anterior pituitary gland (Clayton and Catt, 1981). These gonadotropins are released into systemic circulation where they affect the gonads (Brinkley, 1981; Clarke et al., 1983; Clayton and Catt, 1981; Desjardins, 1981). LH stimulates ovulation and corpus luteum formation in females and androgen secretion in males (Arimura et al., 1967). FSH stimulates follicle growth and maturation in females while stimulating spermatogenesis in males (Kalra and Prasad, 1967). The gonadal steroid hormones then act in a negative feedback mechanism to prevent further gonadotropin production. It should be noted that in females, estrogen can cause an

increase in gonadotropin release at different reproductive cycle stages (Pierce and Parsons, 1981).

B. The Anterior Pituitary

Central to the HPG axis is the anterior pituitary gland. There is no direct neural connection between the anterior pituitary and the hypothalamus, but there is a vascular connection, specifically through the hypophyseal portal system (Reichlin, 1989). Via this portal system, hormones secreted by the hypothalamus circulate directly to the anterior pituitary where they regulate the release of a number of hormones through a variety of different cell types. The anterior pituitary is composed of five known endocrine cell types: the lactotrope, somatotrope, thyrotope, corticotrope, and the gonadotrope (Reichlin, 1989)(**Figure 1**).

Somatotropes release Growth Hormone (GH) which stimulates long bone growth, protein synthesis, and increased muscle mass. GH release is regulated by two hypothalamic hormones with antagonistic effects. Growth hormone releasing hormone stimulates GH release while somatostatin inhibits it. Lactotropes release prolactin. In females, prolactin stimulates mammary duct development during pregnancy and milk production following childbirth. Prolactin release is stimulated by vasoactive inhibiting protein while the neurotransmitter dopamine inhibits secretion. Clearly the most abundant secretory cells are the lactotropes and the somatotropes, together they make up approximately 70% of the anterior pituitary (Guillemin, 2005).

Thyrotropes, under the influence of thyrotropin releasing hormone from the hypothalamus, secrete the glycoprotein hormone thyroid stimulating hormone (TSH).

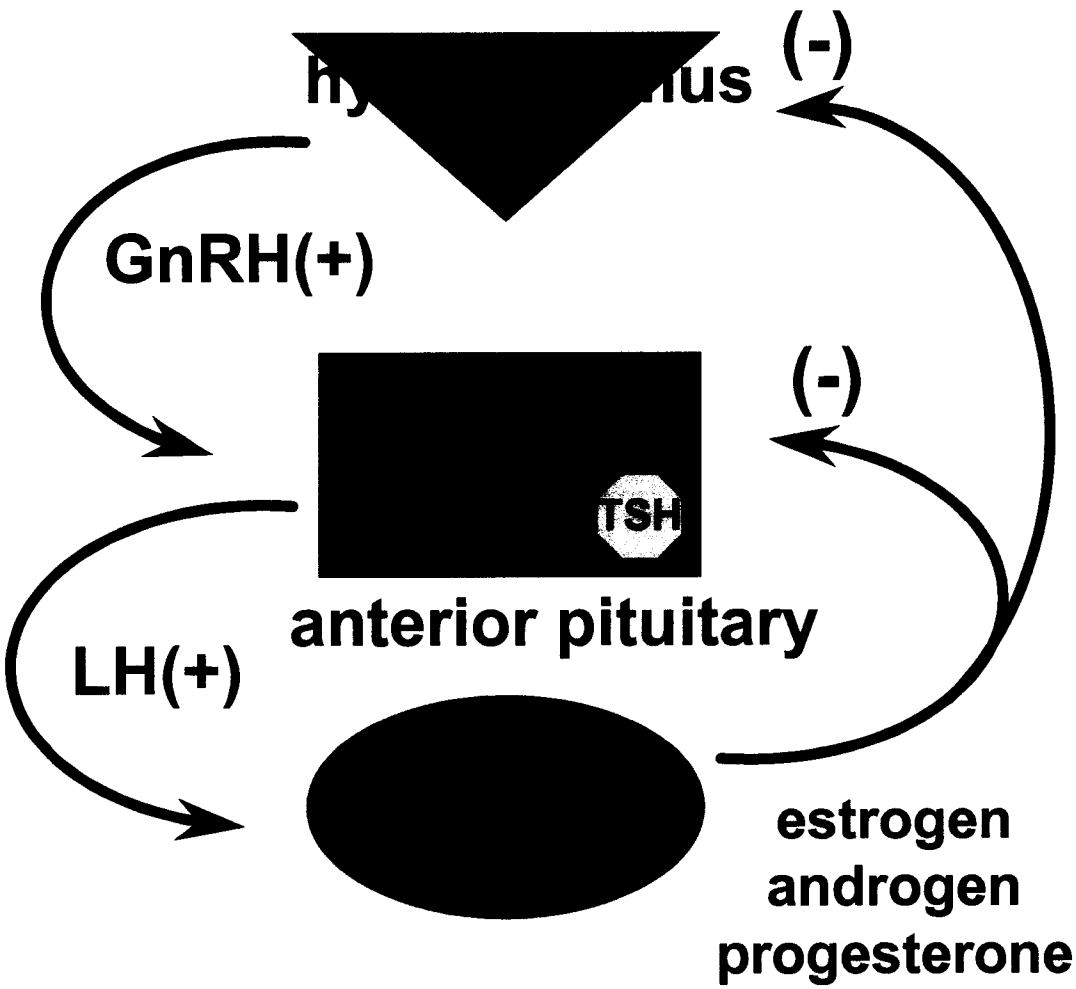


Figure 1. The Hypothalamic-Pituitary-Gonadal-Axis. GnRH from the hypothalamus stimulates the release of LH and FSH from the gonadotopes within the anterior pituitary. Gonadal steroid hormones (esterogen, adrogen, and progesterone) then act in a negative feedback to suppress the release of the gonadotropins (LH and FSH). In addition to the gonadotropes, the anterior pituitary has four other secretory cell types: the corticotropes (ACTH), the lactotropes (PRL), somatotropes (GH), and thyrotropes (TSH).

TSH stimulates the release of thyroid hormone (thyroxine and triiodothyronine). These hormones are critical for the proper development of the skeletal and nervous systems, maintaining the body's basal metabolic rate, and maintaining blood pressure. Corticotropes release adrenocorticotropic hormone (ACTH). ACTH is produced through the proteolytic processing of proopiomelanocortin (POMC). ACTH regulates metabolic function through stimulation of glucocorticoid synthesis in the adrenal cortex. ACTH release is stimulated by corticotrophin-releasing hormone (CRH) from the hypothalamus (Reichlin, 1989).

Gonadotropes, which make up 8-12% of the anterior pituitary in adult mammals (Ibrahim et al., 1986), bind the decapeptide GnRH. GnRH is synthesized in the pre-optic area of the vertebrate brain and is released in a pulsatile fashion from nerve terminals in the median eminance into portal vein circulation (Clayton and Catt, 1981). GnRH then binds the GnRH receptor (GnRHR) on gonadotropes in the anterior pituitary which causes the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Clayton and Catt, 1981; Counis et al., 2005). While both LH and FSH are present in the gonadotropes, they are packaged and released in separate granules (Jablonka-Shariff and Boime, 2004). FSH, in both sexes, stimulates gamete (egg and sperm) production (Gharib et al., 1990). FSH in females binds granulosa cells within the ovary, while FSH in males binds sertoli cells within the testes. LH in females aids in maturation of a follicle, ovulation of an egg, and production of estrogen and progesterone by specifically binding thecal, granulosal, and luteal cells in the ovary. LH in males stimulates Leydig cells within the testes to produce testosterone (Gharib et al., 1990).

In the absence of GnRH communication to the anterior pituitary, whether through mutation of the GnRH gene (Mason et al., 1986a), ablation of gonadotropes (Kendall et al., 1991), immunoneutralization of GnRH (Turzillo and Nett, 1997), or hypothalamic-pituitary disconnection (Hamernik et al., 1986), proper reproductive function in mammals ceases. Thus, binding of GnRH to the gonadotrope represents the first link in activating the HPG axis.

C. The Gonadotrope

Gonadotrope cells are defined by a unique phenotype that ultimately must include expression of at least 4 gene products, the common glycoprotein hormone α subunit, the unique LHβ and FSHβ subunits and the GnRH receptor (Marian and Conn, 1983). Furthermore, expression of each of these genes is tightly regulated by a variety of different stimulatory and inhibitory endocrine inputs. Of the different hormones that directly mediate gonadotrope function, none are more fundamental than GnRH. Binding of GnRH increases expression of the genes coding for LHβ, FSHβ and GnRHR (Pierce and Parsons, 1981). This in turn stimulates the synthesis and release of LH and to a lesser extent FSH as well as an increase in GnRH receptor expression (**Figure 2**) (Pierce and Parsons, 1981).

Although the release of LH and FSH from the gonadotrope overlaps during the pre-ovulatory surge, their release diverges under various physiological conditions. Work done in the ovine pituitary suggests that 20-30% of LH containing granules polarize themselve to the gonadotrope cell membrane abutting the vascular sinusoid. During the

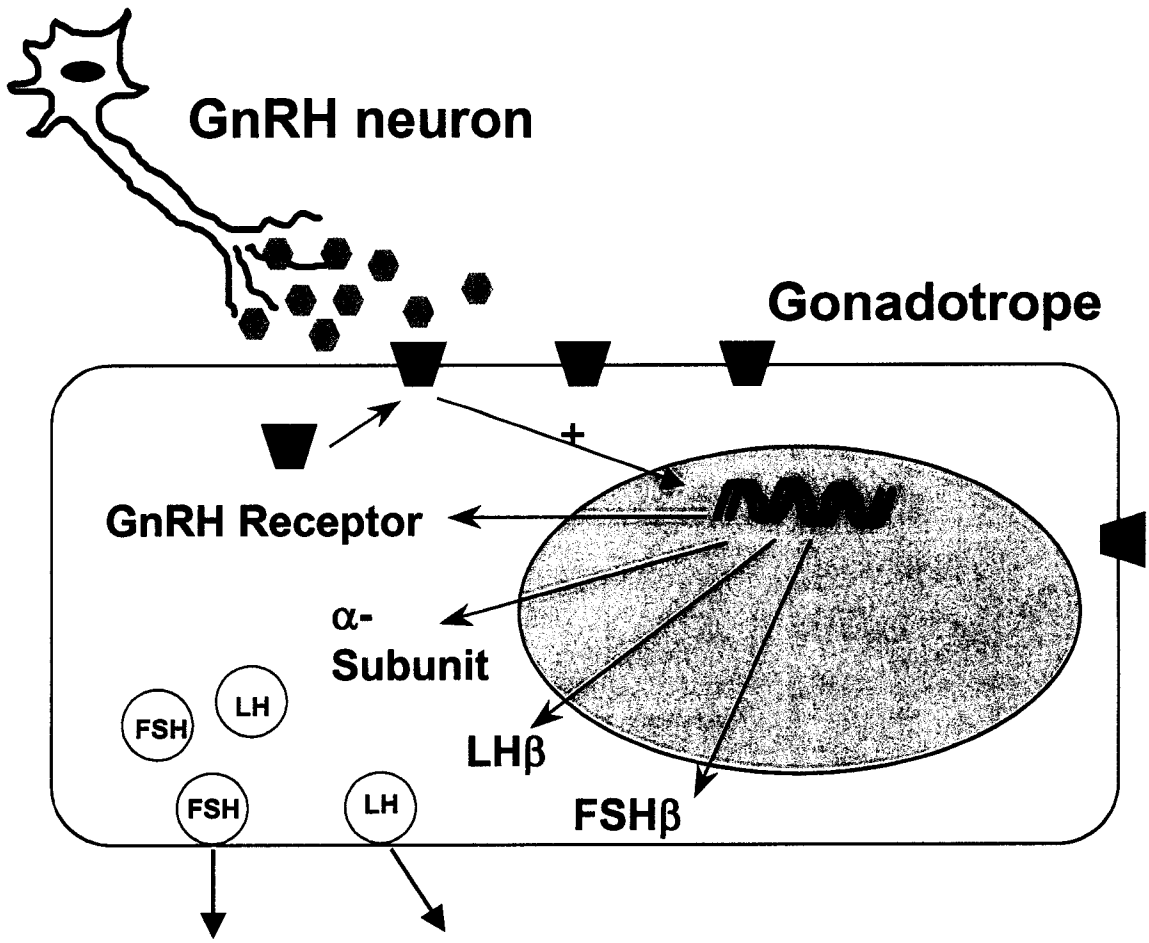


Figure 2. Model of the Gonadotrope. Hypothalamic GnRH binds to specific high-affinity GnRH receptors located on gonadotropes. This stimulates the espression of genes encoding the GnRH receptor, the common α-subunit, LHβsubunit, and the FSHβsubunit. GnRH also causes the release of LH and FSH stores as well as the synthesis of GnRH receptors.

preovulatory surge, this number increases to about 80% (Currie and McNeilly, 1995). Information contained within the carboxy terminus of the LHβ subunit is believed to contribute to the differential sorting (Jablonka-Shariff and Boime, 2004). In constrast, FSH granules appear to be more dispersed within the gonadotrope and show no preferential sorting (Thomas and Clarke, 1997). Recent work in polarized Madin-Darby Canine Kidney (MDCK) cells shows different secretion patterns of LH and FSH. Granules containing LH are preferentially secreted from the basolateral surface while FSH displays equal distribution in apical and basolateral compartments (Jablonka-Shariff et al., 2002). Thus, it appears that LH and FSH exit the gonadotrope via different secretion patterns.

Studying the biology of gonadotropes has often proven difficult because of the small percentage of the pituitary they occupy (only 8-12%) (Ibrahim et al., 1986). Also, gonadotropes only have a limited life span in primary pituitary cultures. Thus, an important development in studying gonadotrope function was the generation of the gonadotrope derived αT3-1 cell line (Windle et al., 1990). Like gonadotropes, αT3-1 cells express the α-subunit protein and GnRH receptors but lack the unique LH/FSH β subunit (Windle et al., 1990). Although these cells do not completely mimic the characteristics of "true" gonadotropes, they have proven to be an important model for studying the GnRH receptor and its signal transduction pathways.

D. Glycoprotein Hormones

The glycoprotein hormones include TSH, LH, FSH as well as placental chorionic gonadotropin (Pierce and Parsons, 1981). These glycoprotein hormones consist of an α

subunit and a β subunit that are encoded by separate genes on different chromosomes. Upon association of the α and β subunits, a conformational change occurs that results in an active non-covalently linked heterodimer (Bokar et al., 1989; Ingham et al., 1976). Within a given species, the α chain, common to all glycoprotein hormones, contains an identical amino acid sequence and has two N-linked carbohydrate residues (Fiddes and Talmadge, 1984).

The β subunits are unique for each hormone and are important for determining binding specificity (Combarnous, 1988). Unlike the α subunit, β subunits vary in size. All the β subunits contain 12 conserved cysteine residues that form 6 disulfide bridges. LHβ and TSHβ contain one N-linked carbohydrate residue while CGβ and and FSHβ contain two. In addition, CGβ also contains 4 O-linked carbohydrate moieties (Boime and Ben Menahem, 1999). In the human, LHβ and CGβ are extremely homologous, they both bind LH receptor but hCG differs from LH in that it has a 25 amino acid extension on its C-terminus (Pierce and Parsons, 1981). This C-terminal extension allows for a longer circulating half-life of hCG due to the higher degree of glycosylation (Jameson and Hollenberg, 1993).

As their name suggests, approximately 18-45% of the total glycoprotein hormone weight is due to carbohydrates (Baenziger, 1990). These oligosaccarides undergo hormone specific posttranslational modifications that prove to be critical in receptor binding, secretion, intracellular stability, and half-life in circulation (Petaja-Repo et al., 1993). Deglycosylation of either LH or hCG through Asp mutantion, causes an increase in LH receptor affinity, however, the deglycosylated hormones do not exhibit hormonal

activity (Matzuk et al., 1989). Thus, the proper glycosylation of glycoprotein hormones appears to be critical for their biological activity.

II. THE GONADOTROPIN RELEASING HORMONE RECEPTOR

A. Overview

Given the central role of GnRH in reproduction, much effort has been devoted toward understanding the physiological consequences of regulation of GnRH and its pituitary receptor. In regard to the latter, a significant breakthrough was the initial cloning of a cDNA encoding the murine GnRHR (Reinhart et al., 1992; Tsutsumi et al., 1992). The cDNA for the murine GnRHR encodes a 327 amino acid protein that has an extracellular ligand binding domain, 7 hydrophobic transmembrane domains that are connected by alternating extracellular and intracellular loops, and an extremely short intracellular C-terminal tail (Stojilkovic et al., 1994). The characteristics of the GnRHR are consistent with membership in the superfamily of heptahelical G-protein coupled receptors (GPCR). The lack of an extensive C-terminus found in other GPCRs, makes the GnRHR a unique member of the GPCR superfamily (Sealfon and Millar, 1995). Due to the lack of an intracellular C-terminus the GnRHR has been described as a naturally occurring desensitization and internalization mutant (McArdle et al., 2002b). Although traditionally thought of as a product of gonadotropes, the GnRHR has been found in various tissues in humans, including the gonads (Latouche et al., 1989), the placenta (Bramley et al., 1992), the adrenal glands (Eidne et al., 1985) and the central nervous system (Jennes et al., 1988). Interestingly, GnRHR expression is also found in multiple

reproductive neoplasias including, breast, prostate, and ovarian cancers (Harrison et al., 2004). For this reason, there has been significant interest in the pharmacology of GnRH behavior and signaling (Chengalvala et al., 2003; Limonta et al., 2003).

B. Regulation of the number of GnRH receptors in the anterior pituitary

Relative changes in GnRH secretion from the hypothalamus are clearly an important determinant of gonadotropin secretion (Clayton and Catt, 1981; Crowder and Nett, 1984; Nett et al., 1988; Savoy-Moore et al., 1980; Wise et al., 1984). Similarly, changes in the number of pituitary receptors for GnRH have also been implicated as an important mechanism underlying the regulation of gonadotropin secretion (Bauer-Dantoin et al., 1995). Thus, changes in pituitary content and secretion of LH are not only dependent on changes in GnRH availability but also the number of GnRH receptors available for binding and, consequently, the responsiveness of the pituitary to a given dose of GnRH (Adams et al., 1981; Clayton and Catt, 1981; Kaiser et al., 1993; Smith MS, 1981; Turzillo et al., 1998; Wise et al., 1984). Since the availability of cDNA's encoding the GnRHR, a number of groups have demonstrated coordinate changes in GnRHR numbers and pituitary concentrations of GnRHR mRNA (Bauer-Dantoin et al., 1993; Brooks et al., 1993; Hamernik et al., 1995; Kaiser et al., 1993; Sealfon et al., 1990; Turzillo et al., 1994). Receiving particular attention is an increase in GnRHR gene expression during the pre-ovulatory period that leads to heightened responsiveness of the pituitary gland to GnRH (Brooks et al., 1993; Crowder and Nett, 1984; Kaiser et al., 1993; Koves et al., 1989). Of the multiple endocrine inputs that have been implicated in affecting changes in GnRHR numbers, perhaps the most dramatic are those associated

with estradiol-17β, activin, and GnRH itself (Frager et al., 1981; Katt et al., 1985). The research involved in elucidating the molecular mechanisms of GnRH receptor gene regulation has been an ongoing effort of many investigators for many years, as recently reviewed by Hapgood et al (Hapgood et al., 2005).

C. The GnRHR is an atypical member of the rhodopsin-like family of G-protein coupled receptors

Although classified as a member of the rhodopsin class of GPCR, the GnRHR displays several unique structural features. Perhaps the most striking of these is an extremely short carboxyl-terminal cytoplasmic domain of only 1-2 amino acids (Sealfon et al., 1997; Stojilkovic et al., 1994). In more prototypical GPCRs this domain is quite extensive and contains phosphorylation sites for GPCR kinases (GRK), second-messenger regulated kinases such as protein kinase A (PKA) and casein kinases (Pierce et al., 2002). In many GPCR, phosphorylation of the C-terminus appears to be requisite for subsequent interaction with β-arrestin, which hinders further G-protein activation and targets the deactivated receptor for internalization (Ferguson, 2001; Zhang et al., 1997). Due to the absence of an intracellular C-terminus, the mammalian GnRHR is not phosphorylated and internalization is not mediated via a β-arrestin dependent mechanism (Heding et al., 2000; Hislop et al., 2001; Willars et al., 2000). Thus, the life cycle of the GnRHR is quite unique compared to other GPCRs. Therefore, we must exercise caution with applying other GPCR paradigms, like the extensively studied β-Adrengeric receptor (Zhang et al., 1997), to the GnRHR.

While not as immediately striking as the absence of a C-terminus, the GnRHR is also distinguished by sequence divergence at several key amino acid residues. The first of these relates to highly conserved D and N residues located in transmembrane domain (TMD)2 and TMD7, respectively. While this arrangement is 98% conserved in other GPCR, these residues are effectively reversed in the GnRHR such that N replaces D at position 87 in TMD2 and D rather than N is found at position 318 of TMD7. Thus, the GnRHR represents a naturally occurring reciprocal mutation of these 2 highly conserved residues. Directed mutational studies have revealed a critical role for both N^{87} and D^{318} in ligand binding and signal transduction. Specifically, mutation of N^{87} to D impairs ligand binding and signaling. In contrast, conversion of D^{318} to N had no effect on binding but uncoupled inositol 1,4,5-trisphosphate (IP_3) production in response to agonist. Thus, the functional role of N^{87} in TMD2 in other GPCR appears to be transferred to D^{318} in TMD7 of the GnRHR (Awara et al., 1996; Flanagan et al., 1999; Zhou et al., 1994). Finally, another unusual feature of the GnRHR is a modified DRY motif at the junction of TMD3 and the second intracellular loop. The DRY motif is characteristic of most GPCR and is critical for proper initiation of intracellular signaling. In the GnRHR, S^{140} replaces the highly conserved Y residue. Conversion of the GnRHR DRS motif to the more prototypical DRY sequence increased the affinity of ligand binding and increased the rate of internalization, however, G-protein coupling did not appear to be compromised (Arora et al., 1997) (**Figure 3**).

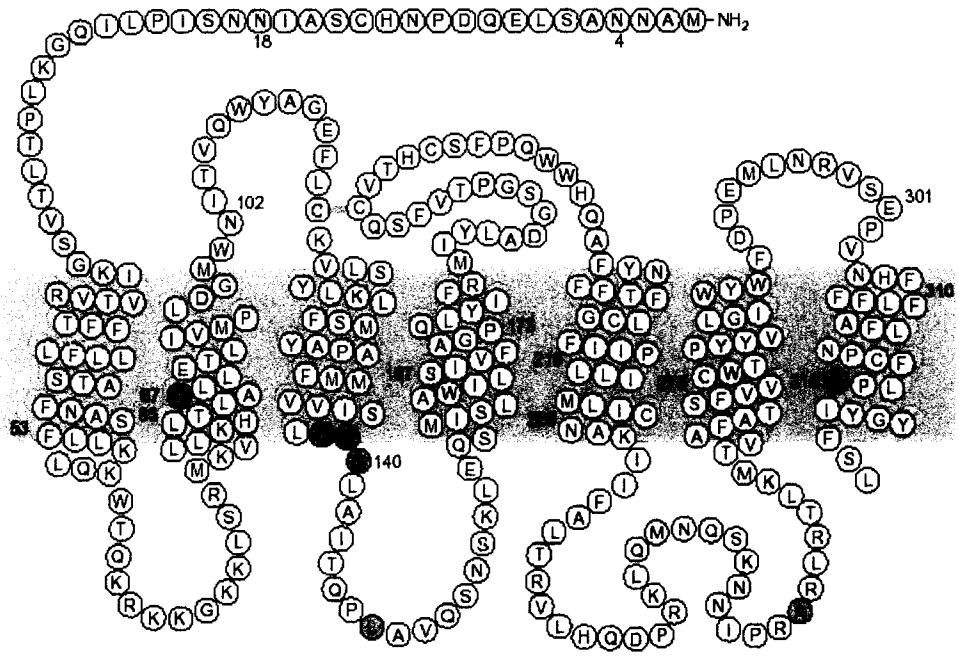


Figure 3. Primary amino acid sequence of the murine GnRHR. Highlighted amino acids represent unique divergence from prototypical G-protein coupled receptors. Modified from Flanagan et al. 1997.

D. GnRHR Signaling

It is well established that, upon ligand binding, agonist occupied GnRHR undergoes a conformational change that promotes the activation of heterotrimeric G proteins, specifically, $G\alpha_{q/11}$. Upon dissociation, $G\alpha_{q/11}$ activates phospholipase C β which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce two signaling intermediates, inositol 1,4,5, bisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes an elevation of intracellular free calcium via ER stores. The rise of intracellular calcium enhances PKC activation and also activates calmodulin which leads to LH release. DAG leads to an activation of one or more isoforms of protein kinase C (PKC) (Stanislaus et al., 1997). These early events underlie GnRH activation of multiple mitogen activated protein kinase (MAPK) signaling cascades including p38 MAPK (Bonfil et al., 2004; Naor et al., 2000), c-Jun N-terminal kinase (JNK) (Levi et al., 1998) and extracellular signal regulated kinase (ERK) (Roberson et al., 1995; Weck et al., 1998).

Phosphorylation of ERK1 and ERK2 in response to GnRH depends mainly on the phosphorylation of Raf1 by PKC. After activation, Raf1 is believed to be recruited to the plasma membrane and in turn activate MEK (1/2), which is the immediate upstream activator of ERK1 and ERK2 (Kraus et al., 2001). Calcium influx via L-type voltage gated calcium channels (VGCC) also appears to be PKC dependent and contribute to ERK activation (Mulvaney et al., 1999). In heterologous cells, the GnRHR has also been shown to couple to $G\alpha_s$ (Krsmanovic et al., 2003; Liu et al., 2002b; Standaert et al., 1992) and more recently, DAG kinase (Davidson et al., 2004a). GnRHR signaling in heterologous systems should be interpreted cautiously, as these pathways are often not

recapitulated in homologous cells raising issues about the physiological relevance of the data.

In addition to the ERK cascade, GnRHR has also been shown to activate both of the stress related MAPK pathways, JNK, and p38. JNK (1/2) activation proceeds through a Rac-CDC42/MLK/MEK pathway and does not appear to be PKC dependent (Ellsworth et al., 2003). Phosphoylated JNK can then activate such transcription factors as JunD and FosB. These transcription factors, which bind to the AP-1 element within the GnRHR gene, have been shown to be important for the GnRH responsiveness of the GnRHR gene promoter (Ellsworth et al., 2003). Unlike JNK, activation of the p38 kinase pathway is PKC dependent (Roberson et al., 1999). Activation of p38 proceeds through a Rac-Cdc42/MEKK/MEK pathway (Kraus et al., 2001). Although the precise role of GnRH induced p38 activation is not known, there is evidence to suggest that p38 contributes to GnRH integration of c-*fos* promoter activity (Roberson et al., 1999) (**Figure 4**).

The GnRHR has also been shown to communicate to the actin cytoskeleton in HEK 293 cells. It is believed that upon GnRH binding, profound changes in cellular architecture occur through the activation of Rac, a Rho family GTPase. Rac activation causes enhanced association of intergrin dimers (α and β) with the extracellular matrix proteins. The dimerization of integrins activates focal adhesion kinase (FAK) which autophosphorylates itself after binding β integrin. It is believed that FAK serves as a scaffold for c-src which is involved in the activation of ERK (Davidson et al., 2004b). However, GnRH induced activation of c-src, Rac and FAK has not been shown in all experimental systems. Thus, the significance of these findings in a homologous cell line remains unclear. In fact, neither Dr. Mark Roberson (Cornell University, personal

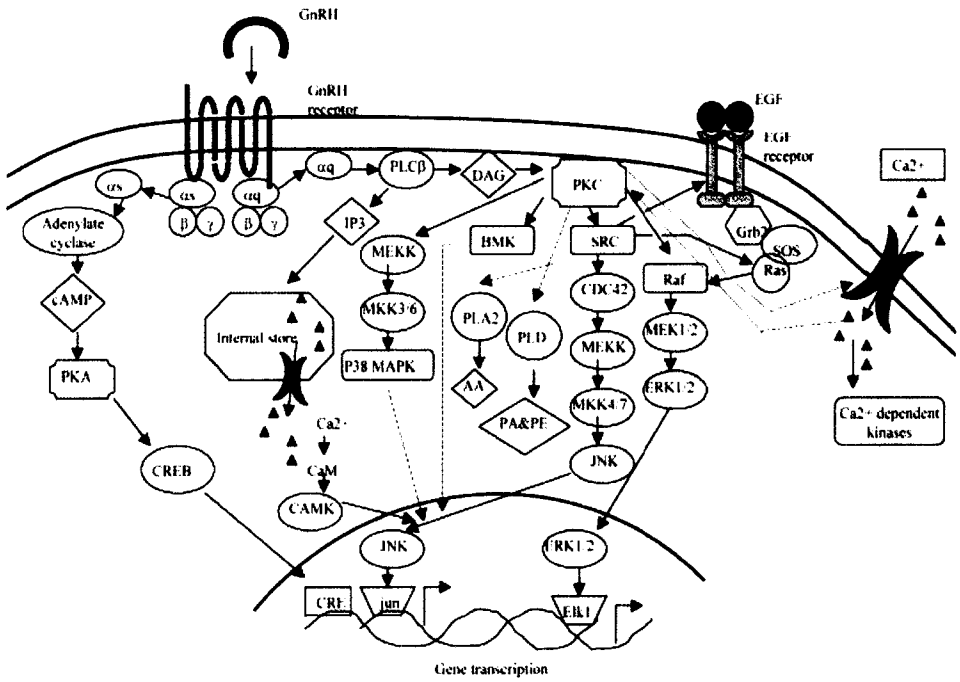


Figure 4. Signaling events evoked by GnRH binding to the mammalian Type I GnRHR. Coupling of GnRHR to Gαq is well established. This event leads to activation of Phospholipase C (PLC) and formation of inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). Elevation of intracellular [Ca++] is mediumted by IP3 binding to Endoplasmic Reticulum and, perhaps, activation of L-type VGCC. Activation of ERK and potentially JNK and p38 are mediumted by PKC. These paths lead to activation of GnRH dependent gene program in gonadotropes. In heterologous cell systems, GnRH has been shown to activate EGF receptor and Gαs, but the physiological significance in homologous cells is unknown. Figure from Ruf et al. 2004.

communication) or our laboratory has been able to demonstrate src activation, nor can we find any effect on cellular function utilizing the src inhibitor PP2 in homologous αT3-1 cells (unpublished data).

E. GnRHR membrane dynamics

Studying the lateral mobility of proteins within the plasma membrane can help determine the extent of protein-protein interactions as well as their association the membrane environment (Lippincott-Schwartz and Patterson, 2003). Utilizing fluorescence recovery after photobleaching (FRAP) techniques with a green fluorescent protein (GFP) tagged GnRHR, it has been established that the binding of agonist slows the rate of lateral movement of the GnRHR in the plasma membrane and leads to a significant reduction in the percentage of receptors that are laterally mobile. The binding of an antagonist (antide) also leads to a reduction in the rate of lateral diffusion but, in contrast to agonist, does not affect the fraction of mobile receptors (Nelson et al., 1999). The reduction in the rate of lateral diffusion of the GnRHR-GFP fusion protein was similar with GnRH and antide. In contrast, the reduction in the mobile fraction observed only with GnRH appeared to reflect additional interactions unique to the agonist occupied receptor, most likely receptor dimerization.

Dimerization of the GnRHR has long been thought to an obligatory event in signal transduction. In early studies, it was established that crosslinking an antagonist bound GnRHR with a 150 Å distance, converted the antagonist to an agonist and the GnRHR was capable of signal transduction (Conn et al., 1982). Although this work

suggested that self-association of the GnRHR was necessary to propagate a signal, there was no direct evidence that the GnRHR dimerized without crosslinking. Recently, our lab, as well as others, has found that agonist but not antagonist leads to a dose dependent increase in fluorescence resonance energy transfer (FRET) between GnRH receptors fused to spectral variants of GFP (Horvat et al., 2001b). Similar results have been reported using both imaging FRET (Cornea et al., 2001) and bioluminescence resonance energy transfer (BRET) (Kroeger et al., 2001).

F. GnRHR displays unique early membrane characteristics

Following agonist activation, signaling by GPCR's is carefully regulated by multiple processes that include, desensitization, internalization and down-regulation (Ferguson, 2001). According to this paradigm, the earliest event, desensitization, occurs very rapidly and often involves G protein receptor kinase (GRK) mediated phosphorylation of S and T residues located in the intracellular C-terminus. This event promotes interaction of the GPCR with a β-arrestin family member and subsequent targeting to clathrin coated pits leading to internalization. Following internalization, GPCR's typically enter early endosomes that can be sorted into a recycling pathway (resensitization) or degradation. Down-regulation then is a more latent event that partially reflects a loss of cell-surface receptor due to internalization and receptor degradation (Ferguson, 2001).

Interestingly, the mammalian GnRHR does not fit with the paradigm developed for GPCR endocytosis. The GnRHR is unique in that it does not contain a C-terminal tail, couple to β-arrestin, or undergo agonist induced phosphorylation (McArdle et al., 1999).

The GnRHR is therefore resistant to rapid agonist induced desensitization found in other GPCRs. GnRH induced IP3 production in αT3-1 cells show that IP3 accumulation is sustained for over 90 minutes while "prototypical" GPCR's show reduction in IP3 levels after 1 min (Willars et al., 1998). This phenomenon is a consequence of receptor structure rather than cell type because the GnRHR has subsequently shown a lack of desensitization in numerous cell systems (McArdle et al., 2002b). It is hypothesized that the lack of receptor desensitization is advantageous for mammals for the generation of the pre-ovulatory surge of LH (McArdle et al., 2002a).

GnRHR internalization is also unique among other GPCRS. At present, the cellular mechanisms that mediate internalization of the GnRHR are not firmly established; however, several key observations have been made. First, the rate of internalization of the Type I GnRHR is slower than GPCR's that possess a C-terminal tail (McArdle et al., 2002; Willars et al., 2000). For example the murine GnRHR has a t1/2 of 60 min while other GPCRs, like the adenosine receptor, show a t1/2=20 min (McArdle et al., 2002a). Second, the internalization process is not dependent on receptor phosphorylation or β-arrestin (Heding et al., 2000; Hislop et al., 2001). Third, clathrin mediated endocytosis is typically thought to be dynamin dependent (Sever et al., 2000). Dynamin is a mechano-GTPase that acts to sever endosomes from the membrane (Hinshaw, 2000). Intersestingly, the GnRHR appears to display a unique internalization phenotype that is clathrin dependent but relatively insensitive to overexpression of dominant-negative dynamin (Heding et al., 2000; Hislop et al., 2001).

G. The non-mammalian GnRH Receptor

The work described above is focused on the mammalian type I GnRHR – the receptor that mediates the essential hypothalamic input to gonadotrope cells of the anterior pituitary gland. This mammalian type I receptor does, however, differ from GnRH receptors found in non-mammalian vertebrates (e.g. catfish (Bogerd et al., 2002), goldfish (Illing et al., 1999), chicken (Sun et al., 2001), Xenopus (Troskie et al., 2000)). In contrast to the mammalian type I GnRHR, the non-mammalian GnRH receptors possess C-terminal tails, are phosphorylated by GRK's and show rapid desensitization (Blomenrohr et al., 1999; Heding et al., 1998). Also, non-mammalian GnRHR's undergo rapid agonist-induced internalization whereas the mammalian type I GnRHR internalizes at much slower rates (McArdle et al., 2002b; Vrecl et al., 2000; Willars et al., 2000). When the C-terminal tail from the non-mammalian catfish GnRHR is added to the mammalian GnRHR, this chimeric receptor shows agonist induced phosphorylation, rapid desensitization, and accelerated internalization kinentics. Thus, the natural resistance of mammalian GnRHR's to desensitization and internalization has been attributed to the lack of C-terminal phosphorylation sites that bind β-arrestin (McArdle et al., 1999).

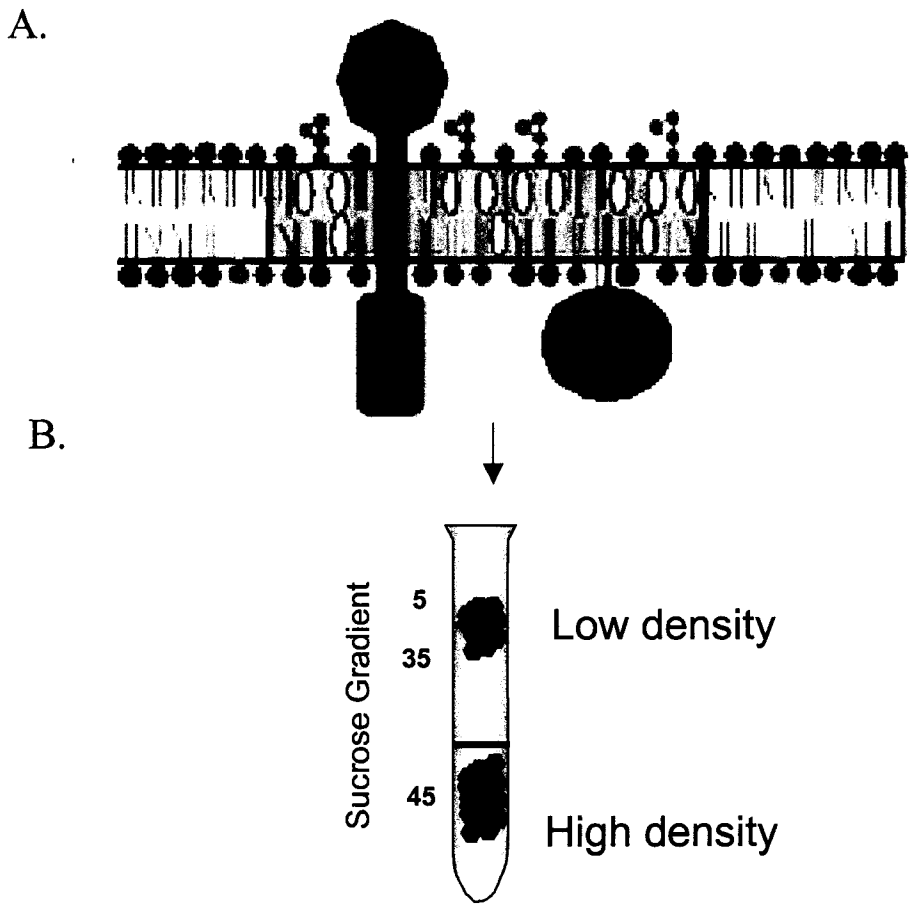
III. LIPID RAFTS

A. Overview

The plasma membrane for many years has been described as a fluid mosaic of lipids and proteins (Singer and Nicolson, 1972). Over the past decade, evidence suggests

that the plasma membrane is not a random sea of lipids but rather contains distinct microdomains, typically less than 100 nm in diameter, that have elevated levels of cholesterol and sphingolipid. Due to the tight packing of the saturated acyl chains of the sphingolipids and the presence of cholesterol, these domains are less fluid than the surrounding plasma membrane (Simons and Ikonen, 1997). The first definition of these microdomains was described by Brown and Rose in 1992 who found that sphingolipids and glycosyl-phosphatidyl-inositol (GPI) anchored proteins are insoluble in cold detergent extraction (Triton X-100). These insoluble domains would float, like a raft, to the top of a density gradient (Brown and Rose, 1992). The term lipid raft was then introduced to refer to a population of cellular membranes that were insoluble in non-ionic detergents and had a light buoyant density in sucrose gradients (**Figure 5**).

Although related in lipid composition, two distinct subsets of membrane microdomains have been described based on morphological and biochemical characteristics. The first of these, caveolae, are flask shaped invaginations of the plasma membrane that are defined by the presence of the marker protein caveolin (Anderson, 1998; Razani and Lisanti, 2001). In contrast, non-caveolar lipid rafts lack caveolin. While not topologically distinct within the plasma membrane, these non-caveolar domains have been visualized with fluorescent lipid probes (Schutz et al., 2000) and, more recently, 2-photon microscopy (Gaus et al., 2003). In the latter, these liquid-ordered domains (rafts) cover 35% of the cell surface and are particularly enriched on membrane protrusions and adhesion points. It should be noted that utilizing sucrose gradient centrifugation as a method for isolating lipid rafts does not distinguish between



Adapted from: Edidin, M. Sci STKE. 2001

Figure 5. Model of a Lipid Raft. A. Lipid rafts are localized regions of elevated cholesterol (yellow) and glycosphingolipid content (pink) within cell membranes. Caveolae, small plasma membrane invaginations that are coated with the cholesterol-binding protein caveolin, are a subset of lipid rafts. The acyl groups of the phospholipids present in lipid rafts and caveolae are more highly saturated than those in the surrounding membrane. This allows close packing of these phospholipid side chains with the saturated acyl chains of sphingolipids and probably leads to phase separation. Due to the presence of cholesterol, a liquid ordered domain is formed that exhibits less fluidity than the surrounding plasma membrane. Lipid rafts are also enriched in gangliosides (orange). The ability of cholera toxin B subunit to associate with the lipid raft enriched ganglioside GM_1 is widely used for analyzing raft distribution and localization in the plasma membrane. **B.** Due to the their unique biochemical characteristics, lipid rafts have a light buoyant density in sucrose gradients compared to bulk plasma membrane.

different microdomain populations (ie. caveolae and lipid rafts co-purify together) (Lagerholm et al., 2005).

B. Lipid Raft Marker Proteins

As the study of lipid rafts evolves, it is becoming clear that the population of microdomains within a cell is heterogenous. For example, some lipid rafts are permanently enriched with particular structural proteins (Razani and Lisanti, 2001; Stuermer and Plattner, 2005). These proteins are thought to affect the morphology, stability, and function of the lipid raft.

The caveolin family of proteins (caveolins 1,2, and 3) are enriched in low-density membrane domains referred to as caveolae. Caveolin 1 and caveolin 2 are usually coexpressed in a number of cell types including endothelial, epithelial, fibroblast, and adipose, while caveolin 3 is largely muscle cell specific. Interestingly, caveolae are rare or absent in lymphocytes and neuronal cells. Caveolins are cholesterol binding proteins that form an unusual hairpin structure within the plasma membrane. These flasked shaped invaginations are approximately 50-100 nm in size. Caveolins act as a scaffold to regulate a number of signaling molecules targeted to caveolae such as: src-family tyrosine kinases, HA-Ras. G protein α subunit, PKC, among others. The scaffolding domain of caveolin interacts with several signaling molecules that contain a ΦXΦXXXXΦ or ΦX XXXΦXXΦ sequence motif, where Φ represents an aromatic amino acid (Trp, Tyr, or Phe). To date, caveolae are the only raft domains that can be recognized morphologically (Okamoto et al., 1998; Razani and Lisanti, 2001; Schlegel and Lisanti, 2001).

Flotillins (Flotillin 1 and Flotillin 2), also known as reggies, are another known ubiquitous marker protein for membrane microdomains. Evidence suggests that flotillin occupies domains that are distinct from caveolae. Flotillin does not possess a transmembrane domain but is rather anchored to the cytoplasmic leaflet of the plasma membrane via lipid modification (acylation). Despite ubiquitous expression, the function of flotillin is still unclear. Recent studies suggest that flotillin is a scaffolding protein involved in the control of cytoskeletal dynamincs (Stuermer et al., 2005; Stuermer and Plattner, 2005).

In addition to caveolin and flotillin, there are a number of other marker proteins that appear to reside constitutively in lipid rafts. Some of these proteins include: stomatin, which is in the same protein family as flotillin, VIP36, which is involved in intracellular transport of glycoproteins, and prominin which interacts with cholesterol in lipid rafts (Lucero and Robbins, 2004).

C. Lipid rafts in Signaling and Intracelluar Trafficking.

Over the past few years, the potential role of lipid rafts as platforms for efficiently and specifically organizing the initiation of intracellular signaling has emerged (Hoessli et al., 2000; Okamoto et al., 1998; Pike, 2003; Simons and Toomre, 2000). It is evident that many raft-associated or caveolar proteins represent cell-surface receptors or components of established intracellular signaling cascades such as G-proteins, Ras, Raf-kinase and Src-family kinases (Simons and Toomre, 2000). Thus, lipid rafts may serve to co-localize membrane receptors and their cognate downstream signaling components in

either pre-assembled complexes or in separate complexes that, upon ligand activation, co-segregate to form a transient signaling platform (Hoessli et al., 2000).

When lipid rafts were first identified, the original interest was not so much in signaling as it was in membrane sorting. For example, in polarized Madin-Darby Canine Kidney (MDCK) cells, it has been found that sphingolipids, cholesterol and glycosyl-phosphatidly-inositol (GPI) anchored proteins are sorted preferentially to the apical side of the cell (Benting et al., 1999; Brown and Rose, 1992). Additionally, Kai Simons recent work suggests that lipid rafts constitute segregation and sorting devices into which proteins specifically associate. For example, in budding yeast, Saccharomyces cerevisiae, lipid rafts serve as sorting platforms for proteins destined to the cell surface. Simons suggests that the sorting is based on O-glycosylation (Proszynski et al., 2004). The segregation capacity of rafts also provides the basis for the polarization of proteins at the cell surface during mating (Bagnat and Simons, 2002).

D. Lipid rafts can include or exclude plasma membrane proteins.

One of the unique features of lipid microdomains is their ability to include or exclude plasma membrane-associated proteins. For example, caveolin is constitutively associated with the unique sub-population of microdomains termed caveolae, while dynamic partitioning from the bulk plasma membrane to lipid rafts characterizes a number of cell-surface receptors (including several GPCRs) following ligand activation (de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997; Sabourin et al., 2002).

Fundamental to cell biology is the theory that proteins contain intrinsic molecular addresses that direct sub-cellular localization or secretion. As such, protein-targeting

theory would predict that the segregation of raft-associated proteins into lipid microdomains is dependent on unique structural determinants found within the protein itself. For example, caveolin appears to possess intrinsic cholesterol binding properties that contribute to its concentration in cholesterol rich caveolae (Schlegel and Lisanti, 2001). In contrast, lipid or glycolipid modifications such as GPI-anchors appear to be the essential targeting motif in a number of raft-associated proteins (Brown and London, 1998). In this scenario, the lipid modification (e.g. myristolation, palmitoylation) may represent the anchor that directly interacts with membrane microdomains (Zacharias et al., 2002). Also implicated in protein targeting to lipid rafts are the lengths of membrane spanning domains and the presence of specific extracellular glycosylations (Anderson and Jacobson, 2002). For transmembrane proteins, such as cell-surface receptors, critical amino acid residues have been identified in membrane-proximal extracellular domains and even the transmembrane domain itself. For example, it has been proposed that lipid binding properties of specific amino acid motifs located in membrane spanning regions of raft-associated transmembrane proteins results in a "lipid shell" that directs the segregation of these proteins into lipid microdomains. Finally, for some transmembrane proteins, critical amino acid residues present in cytoplasmic domains function as molecular addresses (Anderson and Jacobson, 2002). For GPCR's, this could represent motifs present in 1 of 3 intracellular loops or the cytoplasmic C-terminus. The latter is particularly intriguing as cysteine residues located in the intracellular C-terminus are targets for palmitoylation in several GPCR's (Munshi et al., 2001; O'Dowd et al., 1989; Tanaka et al., 1998). While it appears that multiple mechanisms account for segregation

of membrane proteins into lipid microdomains, our understanding of this process is minimal.

E. The GnRHR and downstream signaling intermediates are localized to lipid rafts.

Specialized membrane microdomains are also thought to contribute to GnRHR signaling by organizing the GnRHR and its cognate signaling molecules into discrete membrane domains. Recently, our lab has found that the GnRHR is constitutively localized into lipid rafts in homogenates of αT3-1 cells expressing endogenous GnRHR or Chinese hamster ovary cells expressing an epitope-tagged GnRHR. It appears that downstream GnRHR signaling intermediates are also localized to low density microdomains. For example, c-raf kinase and Gaq are also constitutively localized to raft fractions independent of hormone treatment. If these microdomains are disrupted in αT3-1 cells by cholesterol depletion using methyl-β-cyclodextrin, there is significant attenuation in GnRH but not phorbol ester-mediated activation of extracellular signal-related kinase (ERK) and c-*fos* gene induction. Raft localization and GnRHR signaling to ERK and c-Fos can be rescued upon repletion of membrane cholesterol. Thus, the organization of the GnRHR into low-density membrane microdomains appears critical in mediating GnRH induced intracellular signaling (Navratil et al., 2003).

F. Lipid rafts organize membrane cytoskeletal communication.

Many of the molecular components regulating the actin cytoskeleton have been shown to associate with lipid rafts (Golub et al., 2004). Among these are members of the Rho

family of GTPases. Represented by 3 primary forms (Rho, Rac, cdc42), the Rho GTPases regulate a diverse array of cellular activities ranging from actin cytoskeletal dynamics, cellular adhesion, endocytosis and downstream activation of multiple MAP kinase family members including ERK and JNK (Bishop and Hall, 2000;Ridley, 2001). In the past several years it has become apparent that Rho family members can be targeted to lipid raft domains in response to extracellular ligands or chemotactic gradients. An emerging concept then is that lipid rafts serve as centers for organizing ligand mediated communication between the plasma membrane and the actin cytoskeleton and that Rho GTPases are part of larger protein networks that control nucleation of actin from the plasma membrane (Golub et al., 2004; Orth and McNiven, 2003 ;Schlunck et al., 2004). Consistent with this notion is the enrichment of phosphatidylinositol 4,5-bisphosphate (PIP_2) in cholesterol rich membrane microdomains. This phosphatidyl inositol derivative serves not only as the precursor for IP_3 production but also as a structural scaffold for phosphoinositide (PI) binding proteins via conserved phospholipid binding domains. Among these are the pleckstrin homology (PH) domains of phospholipase C, dynamin and multiple actin modifying proteins (Yin and Janmey, 2003).

G. The Lipid Raft Debate

Despite increasing evidence, there has been debate as to whether lipid rafts actually exist on the cell surface (Lai, 2003). Much of the controversy surrounding lipid rafts relates to their definition as membranes resistant to extraction in 1% Triton X-100 (TX-100) that float in low-density sucrose fractions (5-30%). In fact, during their initial characterization, lipid rafts were often referred to as detergent resistant membrane

(DRMs) (Pike, 2003). Unfortunately, studies have revealed that if the detergent extraction is not done accurately, artificial formation of membrane microdomains can occur, sometimes up to several microns in diameter (Heerklotz, 2002). Thus, the field has slowly moved away from detergent extraction with sucrose gradient centrifugation as a means for raft separation and is advancing to more precise biophysical and visual approaches for the identification of lipid microdomains. Some of these approaches include, single particle tracking, co-localization of proteins with fluorescent raft markers, and FRET/FRAP studies (Lagerholm et al., 2005).

Another method that is often used to verify the existence of lipid rafts is the removal of cellular cholesterol (Edidin, 2003). Due to the unique biochemical composition of lipid rafts, removal of cellular cholesterol can disrupt the microenvironment of these membrane microdomains, altering their buoyant density and function (Edidin, 2003). This is achieved by utilizing cholesterol sequestering agents such as methyl-β-cyclodextrin and fillipin. Less commonly, disruption of cholesterol biosynthesis is also utilized. The caveat of these approaches is their global effects on cellular function and architecture that may not be related to lipid rafts (Kwik et al., 2003). Unfortunately, at the present, the methods for accomplishing lipid raft disruption are largely limited to altering cholesterol content.

IV. SUMMARY

Binding of GnRH to the GnRHR initiates a cascade of events that culminate in synthesis and secretion of the gonadotropin hormones LH and FSH (Pierce and Parsons,

1981). These gonadotropins are essential for steroidogenesis and gametogenesis. In the absence of GnRH communication to the anterior pituitary, proper reproductive function is lost (Mason et al., 1986a). Given the central role of GnRH in reproduction, much effort has been devoted toward understanding the GnRHR at both the genetic and protein level.

In regard to the latter, it appears that the mammalian GnRHR is an atypical member of the GPCR superfamily in that it lacks an intracellular C-terminus, does not undergo agonist induced phosphorylation, or couple to β-arrestin. Due to the lack of a C-terminus, the GnRHR also displays resistance to desensitization and slow internalize kinetics (McArdle et al., 2002a). In contrast, non-mammalian GnRH receptors do contain a C-terminal tail and behave like more "prototypical" GPCRs . Given the unique features of the mammalian GnRHR, my focus has been to expand our understanding of the functional organization of the GnRHR in the plasma membrane.

More recently, our view of the plasma membrane has been shifted from a random sea of membrane lipids to membrane regions that display elevated levels of glycosphingolipid and cholesterol as compared to the "bulk" plasma membrane (Simons and Ikonen, 1997). These localized membrane microdomains, termed lipid rafts, are thought to serve as platforms for efficiently and specifically organizing the initiation of intracellular signaling because of their enrichment of signaling molecules, like G-proteins and GPCRs (Simons and Toomre, 2000).

In the following chapters, I will address the localization of the GnRHR within lipid microdomains and how these domains are critical for GnRHR signaling to MAPK. Lastly, I will discuss GnRHR targeting to lipid rafts. Specifically, how the addition of different intracellular C-termini to the murine GnRHR alters lipid raft localization.

CHAPTER THREE

CONSTITUTIVE LOCALIZATION OF THE GONADOTROPIN-RELEASING HORMONE (GNRH) RECEPTOR TO LOW-DENSITY MEMBRANE MICRODOMAINS IS NECESSARY FOR GNRH SIGNALING TO ERK

INTRODUCTION

Over the past several years it has become evident that the specificity and fidelity of intracellular signaling is partially achieved through compartmentalization of interacting proteins into discrete subcellular domains. In this regard, the plasma membrane is no exception. Of particular interest are discrete microdomains consisting of tightly packed sphingolipids and cholesterol in the exoplasmic leaflet (Simons and Ikonen, 1997). Due to this unique lipid character, these membrane microdomains are characterized by resistance to solubilization in low-ionic strength detergents and a low-buoyant density in sucrose gradients as compared to the "bulk" plasma membrane (Brown and Rose, 1992). Although related in regard to lipid composition, two distinct subsets of membrane microdomains have been described based on different morphological and biochemical characteristics. The first of these, caveolae, are flask shaped invaginations of the plasma membrane that are defined by the presence of the marker protein caveolin (Anderson, 1998; Razani and Lisanti, 2001). In contrast, lipid

rafts lack caveolin and are not topologically distinct from the plasma membrane (Simons and Ikonen, 1997; Simons and Toomre, 2000). Although the molecular determinants that dictate inclusion or exclusion of proteins from lipid rafts or caveolae are not fully understood, it is evident that many of these raft-associated or caveolar proteins represent components of established intracellular signaling cascades such as G-proteins, Ras, and Src-family kinases (Razani and Lisanti, 2001;Shaul and Anderson, 1998). Thus, membrane microdomains may represent a form of signaling platform that organizes efficient and specific signal transduction by facilitating interactions among co-segregated proteins such as membrane receptors and their cognate signaling proteins. Within these domains, further specificity of signaling is likely mediated by scaffolding proteins, such as caveolins and 14-3-3 proteins, which bring sequential members of a signaling cascade into direct proximity (Aitken et al., 2002; Okamoto et al., 1998).

Consistent with the presence of G-proteins in lipid microdomains, several members of the superfamily of G-protein coupled receptors (GPCR) have been shown to partition into lipid rafts and caveolae (Chun et al., 1994; de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997; Rybin et al., 2000; Sabourin et al., 2002). Often, the translocation of GPCR into membrane microdomains appears to require ligand activation; however, β-adrenergic receptor subtypes have been found distributed between both caveolin containing and bulk plasma membrane fractions (Rybin et al., 2000; Schwencke et al., 1999). Herein we have sought to determine the membrane localization of the mammalian type I gonadotropin releasing hormone receptor (GnRHR). An atypical member of the rhodopsin-like family of GPCR, the GnRHR is located on the cell surface of pituitary gonadotropes. The binding of the hypothalamic decapeptide GnRH to the

pituitary GnRHR not only stimulates but is obligatory for the synthesis and secretion of luteinizing hormone (LH) (Gharib et al., 1990; Hamernik et al., 1986) . In the absence of GnRH input to the pituitary gland, LH production and, consequently, gonadal function in mammals ceases (Mason et al., 1986a; Mason et al., 1986b). Thus, the GnRHR represents the site that mediates the primary stimulatory input to gonadotrope cells. Upon ligand activation, it is well established that agonist-occupied GnRHR couples to $G_{\alpha q/11}$ leading to stimulation of phospholipase C, formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), elevation of intracellular free calcium and activation of one or more isoforms of protein kinase C (PKC) (Kaiser et al., 1997; Stanislaus et al., 1997). These early events underlie GnRH activation of multiple mitogen activated protein kinase (MAPK) signaling cascades including p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) (Naor et al., 2000). ERK activation by GnRH is dependent on both PKC and calcium influx via L-type voltage-gated calcium channels and proceeds through a c-raf kinase dependent mechanism (Mulvaney and Roberson, 2000; Naor et al., 2000).

Although classified as a member of the rhodopsin class of GPCR, the GnRHR displays several unique structural characteristics of which perhaps the most striking is an extremely short carboxyl-terminal cytoplasmic domain of only 1-2 amino acids (Sealfon et al., 1997; Stojilkovic et al., 1994). In more prototypical GPCRs, this domain is quite extensive and contains phosphorylation sites for G-protein coupled receptor kinases, second-messenger-regulated kinases (such as protein kinase A) and casein kinases. In some GPCRs, these phosphorylation events allow for interaction with β-arrestins and subsequent receptor deactivation and internalization (Ferguson, 2001; Zhang et al., 1997).

Consistent with the lack of an extensive C-terminal tail, the GnRHR does not appear to undergo arrestin-dependent internalization or desensitization (Heding et al., 2000; Hislop et al., 2001; McArdle et al., 1999; McArdle et al., 2002b; Willars et al., 2000). In fact, the GnRHR has been considered as a naturally occurring internalization resistant mutant (McArdle et al., 1999). Thus, the GnRHR is both structurally and functionally unusual.

Given its unique structural and functional attributes we sought to assess the membrane distribution of the mammalian GnRHR. Herein, we find that unlike other GPCRs, such as the muscarinic acetylcholine receptor and B2 bradykinin receptor that are uniformly distributed throughout the plasma membrane and localize to caveolae only in the presence of ligand (de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997), the GnRHR constitutively resides in a low-density membrane microdomain in both the gonadotrope-derived αT3-1 cell line that expresses the endogenous GnRHR gene (Windle et al., 1990) and Chinese hamster ovary (CHO) cells. We also find that c-raf kinase, a target of GnRHR signaling, is constitutively, but not exclusively, localized to low-density membrane fractions. Furthermore, disruption of raft organization by cholesterol depletion attenuates GnRHR activation of ERK and induction of c-fos gene expression suggesting that the sub-localization of GnRHR and c-raf kinase to membrane microdomains is functionally significant.

MATERIALS AND METHODS

Materials

The C-terminal anti-GnRHR, anti-caveolin-1, anti-c-fos, anti-$G_{\alpha q/11}$, anti-c-raf kinase, anti-b-raf kinase, anti-p-ERK, and anti-ERK-1 antibodies were purchased from Santa

Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against H+ ATPase was a generous gift from Dr. Klaus Bayenbach (Cornell University). The anti-flotillin-1 and pan-caveolin antibodies were purchased from BD Transduction Laboratories (San Jose, CA). The anti-tubulin antibody and methyl-β-cyclodextrin (CD) were obtained from Sigma (St. Louis, MO). The anti-HA antibody was purchased from Roche (Indianapolis, IN). Anti-goat, anti-rabbit, and anti-mouse secondary antibodies were from Pierce (Rockford, IL), Santa Cruz Biotechnology or Bio-Rad, (Hercules, CA). [^{3}H] inositol (14 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). D-Ala6-GnRH purchased from Sigma.

Cell Culture

αT3-1 cells were maintained in high glucose DMEM from Mediatech (Herndon, VA) containing 2 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 5% FBS, and 5% horse serum. CHO cells were maintained in high glucose DMEM containing 2 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 10% FBS and 1 X nonessential amino acids from Life Technologies (Grand Island, NY). All cells were grown in 5% CO_2 at 37°C in a humidified environment.

Construction of Hemagglutinin tagged GnRH receptor attached to Green Fluorescent Protein (GFP)

The construction of the GnRHR-GFP fusion cDNA has been described (Nelson et al., 1999). A 2-step PCR procedure was used to attach the HA sequence (YDYDVPDYA) immediately adjacent to the initiation codon of the GnRHR-GFP fusion protein. Appropriate placement of the HA tag was confirmed by sequencing.

Preparation of cholesterol loaded methyl-β-cylcodextrin (CLCD)

Cholesterol (200 mg) was dissolved in 1 ml of chloroform. In a separate beaker, 1 g of methyl-β-cylcodextrin (CD) was dissolved in 2 ml of methanol. A 0.45 ml aliquot of the cholesterol solution was added to the CD solution, stirred and then placed under a stream of nitrogen gas until the chloroform and methanol evaporated. The resulting crystals were allowed to dry for 24 hours and stored in glass at room temperature. A CLCD working solution was prepared by adding 50 mg of the CLCD crystals to 1 ml of serum free DMEM, warming to 37°C and vortexing briefly.

Detergent-Free Preparation of Lipid Rafts

αT3-1 cells, a generous gift from Dr. Pam Mellon, or CHO cells were grown to confluence in 150 mm tissue-culture plates. Cells were harvested in phosphate buffered saline (PBS) and centrifuged for 3 minutes at 300 x g. Cells were resuspended in PBS to a final volume of 1 ml and administered either vehicle or 100 nM GnRH for 15 minutes at 37°C followed by centrifugation for 3 minutes at 300 x g. Detergent-free lipid raft preparations were conducted according to Song et al. (Song et al., 1996). The cell pellet was resuspended in 2 ml of 500 mM sodium carbonate buffer (pH 11). Cells were then homogenized using a Wheaton loose fitting glass dounce homogenizer (10 strokes) followed by sonication (three 20 second bursts) on ice using a Branson 250 sonifier. Two ml of 90% sucrose prepared in MES buffer (20% glycerol, 150 mM NaCl, 2mM EDTA, 25mM MES, pH 6.5) was added to the homogenized samples yielding a final concentration of 45% sucrose in a total volume of 4 ml. A discontinuous sucrose

gradient was then layered on the surface of the 45% fraction (4 ml 35% sucrose, 4 ml 5% sucrose in MES containing 250 mM sodium carbonate). Isopycnic ultracentrifugation was then carried out at 38,000 rpm using a SW 41 rotor for 16-20 hours at 4°C. Following ultracentrifugation, 645 µl samples were collected representing a total of 18 fractions. Proteins that migrated to the interface of the 5% and 35% gradients (approximately fractions 6 and 7) were considered to be raft-associated (Song et al., 1996).

CD treated samples were prepared as above with the exception that αT3-1 cells in monolayer were incubated in 12 ml of serum free medium containing 2% CD for 1 hour at 37°C followed by 2 hours of serum free medium alone. Control cells were incubated in serum free medium without CD over the same time period. For the cholesterol repletion studies, αT3-1 cells were treated with CD as described above. Following CD treatment, cells were washed with PBS and incubated in 12 ml of serum free medium containing 1 mg/ml of CLCD for 2 hours. Raft samples were then prepared as described above.

Tx-100 Preparation of Lipid Rafts

αT3-1 or CHO cells were grown to confluence in monolayer cultures. Cells were harvested and treated with hormone as in the detergent-free raft method. Following centrifugation, the cell pellet was resuspended to a final volume of 500 µl in PBS. 500 µl of 2X TX-100 lysis buffer (0.2% TX-100 prepared in MES buffer) was then added to yield a final 1X lysis buffer concentration (TX-100 concentration = 0.1%). Cells were then homogenized either by 3 passes through a 30 gauge needle or douncing. PBS was added to adjust the final volume to 1 ml. One ml of 80% sucrose (in MES buffer) was

added to the samples to yield a 40% sucrose fraction in a final volume of 2 ml. A discontinuous sucrose gradient was then prepared by layering 2 ml of each sucrose fraction (80%, 60%, 40%-containing sample, 30%, 20% and 10%) in a 12 ml ultracentrifuge tube (Field et al., 1995) (**Figures 6D, 7B**) or layering 4 ml of 45, 35 and 5% sucrose in a 12 ml ultracentrifuge tube (Figure 11). Samples were subjected to isopycnic ultracentrifugation in an SW 41 rotor for 20 hours at 37,000 rpm. Following ultracentrifugation, 645µl samples were collected representing a total of 18 fractions and Western analyses were conducted. In **Figure 12**, whole mouse pituitaries were obtained following euthanasia, minced, rinsed free of blood in PBS and lysed in a 0.1% Triton lysis buffer (in MES) by douncing. Whole pituitary lysates were adjusted to 45% sucrose and layered within a sucrose gradient (45 (sample), 35 and 5% sucrose) in a total volume of approximately 3.5 ml to reduce a potential dilution effect of the larger gradients described for αT3-1 and CHO cells. These samples were centrifuged as described above in an SW50.1 rotor. Following centrifugation, gradients were harvested in 10 equal fractions.

Silver staining

Sucrose gradient fractions from αT3-1 cells prepared with carbonate lysis were resolved by SDS-PAGE. The resolved proteins within the gel were visualized by silver staining using methods as described by Blum et al. (Blum et al., 1987). Identical results were obtained using gradient fractions from αT3-1 lysates generated from 0.1% Triton-X 100 lysis buffer (data not shown).

Western blots

Samples representing individual fractions were subjected to SDS polyacrylamide gel electrophoresis (acrylamide:bis-acrylamide ratio of 29:1) and electro-blotted to nitrocellulose (Bio-Rad Hercules, CA) or PVDF membranes (Perkin Elmer, Boston MA). In **Figure 10B**, SDS-PAGE was carried out using a 37.5:1 acrylamide:bis-acrylamide ratio to favor the separation of phosphorylated and non-phosphorylated c-raf kinase. Membranes were blocked in 5% non-fat dried milk in Tris buffered saline (TBS) or TBS containing 0.1% Tween-20 (TBST). Anti-GnRHR antibody (1:500 dilution in 5% milk) was incubated for 8 hours at 4°C on an orbital shaker. Blots were washed for 30 minutes (3 washes x 10 minutes) with TBS and then incubated with anti-goat HRP (1:5,000) for 2 hours. Anti-$G_{\alpha q/11}$ antibody was used at a 1:500 dilution for 2 hours at room temperature followed by washing with TBS and incubation with anti-rabbit HRP (1:5,000) for 2 hours. Anti-HA and anti-flotillin antibodies were used at a 1:1,000 dilution with an incubation time of 1 hour. Blots were washed and then incubated with a 1:10,000 dilution of anti-mouse HRP for 1 hour at room temperature. Anti-c-raf, b-raf and H+ ATPase antibodies were used at a dilution of 1:1,000 and incubations were overnight at 4°C. Blots were washed in TBST and then incubated with a 1:5,000 dilution of anti-rabbit HRP for 2 hours at room temperature. Anti-caveolin-1, anti-tubulin, and anti-pan caveolin were used at a 1:2,000 dilution with a 1:10,000 dilution of the appropriate secondary antibody for 1 hour. All blots were washed for 60 minutes (6 x 10 minutes) with TBS or TBST after secondary antibody and then visualized by chemiluminescence using either Pierce SuperSignal or Perkin Elmer Western Lightening reagents on Pierce CL-XPosure film.

ERK and c-fos Activation Assays

Monolayers of αT3-1 cells in 6-well tissue culture plates were subjected to 1 of 3 treatments. First, control cells were washed with PBS and then incubated with 1 ml of serum free medium for 3 hours. Second, for cholesterol depletion, cells were washed with PBS and incubated with 1 ml of serum free medium containing 2% CD for 1 hour. Cells were then washed with PBS and incubated for 2 hours in serum free medium. Third, for cholesterol repletion, cells were washed with PBS and administered 1 ml of 2% CD in serum free medium for 1 hour. Cells were then washed with PBS and incubated for 2 hours in serum free medium containing 1 mg/ml CLCD. Following the 3 hour treatment protocols, cells were washed with PBS and serum free medium was replaced containing either 0 or 100 nM GnRH. After a 30 or 60 minute incubation, cells were washed in ice cold PBS and lysed in RIPA buffer containing 20mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholate and 0.2 mM PMSF. 6X sample buffer (300 mM Tris-HCl, pH 6.8, 60% glycerol, 30 mM DTT, 6% SDS) was added to yield a final concentration of 1X. Aliquots (15 μl) of each lysate were heated to 95°C for 5 minutes and subjected to SDS-PAGE and Western analysis. Nitrocellulose membranes were incubated for 2 hours with either a phospho-ERK or c-fos antibody (both at 1:1,000 dilutions) followed by a 2 hour incubation with a 1:2,000 dilution of HRP conjugated secondary antibody. Phospho-ERK blots were then stripped at room temperature with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) heated to 50°C for 30 minutes. After stripping, membranes were washed twice for 15 minutes with TBS and blocked with 5% milk for 1 hour. Blots were then re-probed with a 1:10,000 dilution of

an anti-ERK antibody that recognizes ERK-1 and ERK-2 independent of phosphorylation state. Following washing in TBS, blots were incubated with a 1:2,000 dilution of anti-rabbit HRP and immunoreactive bands were visualized by chemiluminescence.

Cholesterol Assays

αT3-1 cells were either incubated in serum free medium alone for 3 hours, cholesterol depleted or cholesterol depleted followed by incubation with CLCD as described in ERK and c-fos Activation Assays above. At the end of the 3-hour treatment period, all cells were washed and harvested in PBS. Cells were pelleted by centrifugation and resuspended to a final volume of 500 μl in PBS. An equal volume of 2X lysis buffer containing 0.2% TX-100 in MES buffer cooled to 4°C was then added to solubilize the plasma membrane. Cholesterol content was determined using Infinity cholesterol reagent (Sigma). Briefly, lysates were diluted 1:5 with cholesterol reagent and samples were incubated for 10 minutes at 37°C followed by spectrophotometric analysis (absorption at 500 nm). Cholesterol concentration was determined using a 6 point standard curve generated with known concentrations of cholesterol. To standardize cholesterol content, protein concentrations were determined using a BCA protein assay (Pierce). Data are expressed as mg of cholesterol per mg of protein.

Transmission Electron Microscopy (TEM)

CHO and αT3-1 cells were cultured to approximately 60-70% confluence then scraped from dishes in ice-cold Hepes buffered saline (HBS; pH 7.4) and washed once in HBS. The cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH

7.4 for 30 minutes at room temperature followed by 1.5 hours at 4° C. The cells were then washed 3 times 10 minutes each in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were post-fixed in 2% osmium tetroxide for 1 hour at room temperature, followed by washes in sodium cacodylate buffer as described above. Cells were then dehydrated through a standard graded ethanol series followed by an acetone rinse. Gradual infiltration of epon araldite resin followed, after which the cells were placed into Beem capsules and cured in a 60° C oven. The resin blocks were cut on a Reichert OmU2 ultramicrotome and ~70 nm sections were stained with uranyl acetate and lead citrate. The grids were viewed in a Tecnai 12 Biotwin transmission electron microscope (FEI Corp). For quantitation of each cell type, 100 cells were visualized and the number of putative caveolae was obtained. Digital images were captured with a Gatan Model 791 Multiscan Camera.

[^{125}I] D-Ala6-GnRH Binding Assay

D-Ala^{6}-desGly^{10}-GnRH-Pro^{9}-ethylamine ([D-Ala^{6}] GnRH) was radioiodinated using a glucose-oxidase procedure and purified by chromatography in QAE Sephadex as described by Wagner et al (Wagner et al., 1979). Following a 1 hour incubation in either serum free medium alone or serum free medium containing 2% CD, αT3-1 cells were washed with PBS and incubated for 2 hours in serum free medium alone. Cells were harvested in ice-cold PBS. Following centrifugation, cell pellets were resuspended in assay buffer (10 mM Tris-HCl, .1% BSA, .01 mM $CaCl_2$) to a final concentration of 1 x 10^7 cells/50 µl. Triplicate 12 x 75 mm assay tubes were prepared containing 50 µl aliquots of cell suspension and 5 x 10^4 cpm of [^{125}I] D-Ala^{6}-GnRH (61.4 pM) in 50 µl

assay buffer in the presence or absence of 50 μl of non-radioactive D-Ala^6 GnRH (340 nM). The total volume for each tube was adjusted to 250 μl by addition of ice-cold assay buffer. Following a 2 hour incubation at 4°C, 3 ml of ice-cold assay buffer was added and samples were immediately centrifuged for 15 minutes at 16,000 x g. The supernatents were decanted and radioactivity in the cell pellets was quantified using an Apex automatic gamma counter (Micromedic systems, Horsham, PA). Specific binding was determined by subtracting the cpm in samples containing [^{125}I] D-Ala^6-GnRH in the presence of unlabeled D-Ala^6-GnRH from the cpm in samples containing only [^{125}I] D-Ala^6-GnRH samples. To standardize binding for protein concentration, a BCA protein assay (Pierce) was performed. The binding activity presented in **Figure 8A** (inset) was adjusted for protein concentration.

[^{3}H] inositol assays

Phospholipase C activity was assessed by quantifying cellular accumulation of phosphorylated inositol using previously described methods (Evans et al., 1997). Briefly, αT3-1 cells were plated overnight in 24-well culture plates. The DMEM culture medium was washed from the cells with serum-free M199 culture medium (Mediatech; Herndon, VA) supplemented to 0.37 % (w/v) sodium bicarbonate, 20 mM HEPES buffer, 100 U penicillin/ml, and 100 μg streptomycin/ml. After washing, cells were incubated at 37°C for 5 hours in 0.3 ml of serum-free M199 containing 2 μCi of myo-[2-^{3}H]inositol. The labeled cells were then washed with serum-free DMEM containing 5 mM LiCl and incubated for an additional hour at 37 °C in either 1 ml serum-free DMEM with 5 mM LiCl or 1 ml serum-free DMEM containing 5 mM LiCl and 2% CD. After 1 hour of

cholesterol depletion, this medium was removed and then control and cholesterol depleted cells remained untreated or were challenged with 100 nM GnRH in 1 ml serum-free DMEM containing 5 mM LiCl. These treatment conditions were maintained at 37 °C for 1 hour, after which the medium was removed and cells were immediately lysed by addition of 0.05 ml RIPA buffer (described above) and 1 ml water heated to 95 °C. The cells were then frozen overnight and thawed at room temperature. Cell lysates from each individual well were collected and loaded separately onto Dowex 1-X8, 200-400 mesh, formate-form columns with an approximate bed volume of 0.4 ml. Free, unphosphorylated inositol was eluted from the lysate by the addition of 10 bed volumes of water. After collection of the eluent containing the free inositol, total inositol phosphates were collected by the addition of 10 bed volumes of 1 M ammonium formate in 0.1 M formic acid. The amounts of radioactivity in both the free and phosphorylated inositol eluents were quantified using a Beckman LS-5000CE liquid scintillation counter. Data are presented as phosphorylated inositol expressed as a percentage of the total [^{3}H]-inositol.

RESULTS

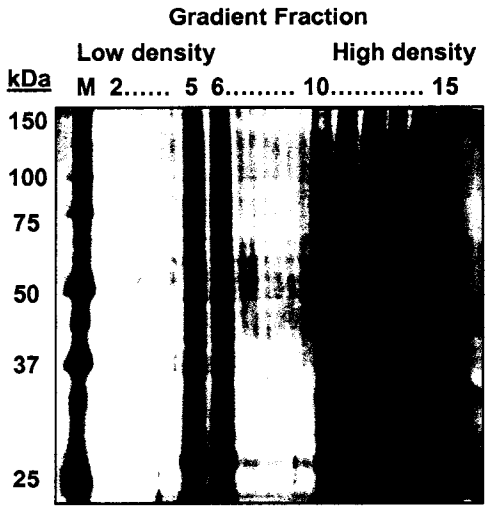
The GnRHR is a resident protein in low-density membrane microdomains.

Silver staining of non-detergent fractions from αT3-1 cells was conducted to assess the efficacy of protein separation using sucrose gradients (**Figure 6A**). This analysis revealed enrichment of distinct populations of proteins associated with either low density (fractions 5 and 6) or high-density fractions (fractions >10) suggestive of effective separation of raft-associated proteins. To assess membrane sub-localization of

the GnRHR, αT3-1 cells were administered either vehicle or 100 nM GnRH for 15 minutes. Cells were then sonicated in a detergent-free sodium carbonate buffer and fractions separated in a discontinuous sucrose gradient. Fractions were isolated and subjected to SDS PAGE followed by Western blotting using an anti-GnRHR, anti-tubulin, or anti-flotillin antibody. Immunodetectable GnRHR localized to low-density fractions within the gradient independent of GnRH treatment (**Figure 6B**, lanes 6 and 7). Lanes 6 and 7 represent the interface of the 5% and 35% sucrose fractions where raft-associated proteins would migrate (Song et al., 1996). The absence of caveolin expression in αT3-1 cells (**Figure 6C**) precluded the utility of this protein as a marker protein for low-density membrane microdomains, thus, we used an antibody directed against flotillin – another raft associated protein (Kokubo et al., 2003; Morrow et al., 2002). Using the same detergent free raft isolation method in αT3-1 cells, flotillin appropriately localized to low-density fractions within the sucrose gradient (**Figure 6B**). Not considered a raft-associated protein (Martens et al., 2000), tubulin did not partition into membrane rafts in these conditions. To confirm GnRHR localization to membrane microdomains, an independent approach based on resistance to detergent solubilization was utilized to prepare samples prior to isopycnic centrifugation. This technique takes advantage of the relative insolubility of lipid rafts in non-ionic detergents at 4°C. As with the detergent-free raft preparations, the GnRHR localized to the low-density fractions regardless of hormone treatment. Tubulin was again localized to high-density fractions (**Figure 6D**).

To assess whether GnRHR localization to low-density membrane microdomains was specific to a gonadotrope derived, non-caveolin expressing cell line, we next studied

A



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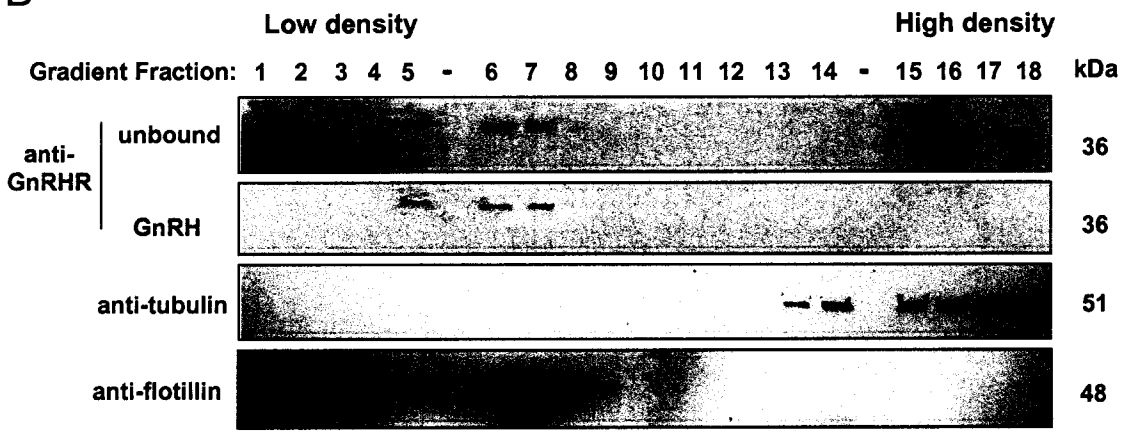

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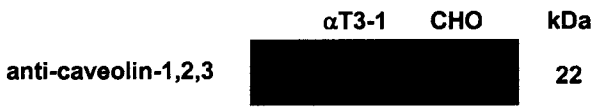

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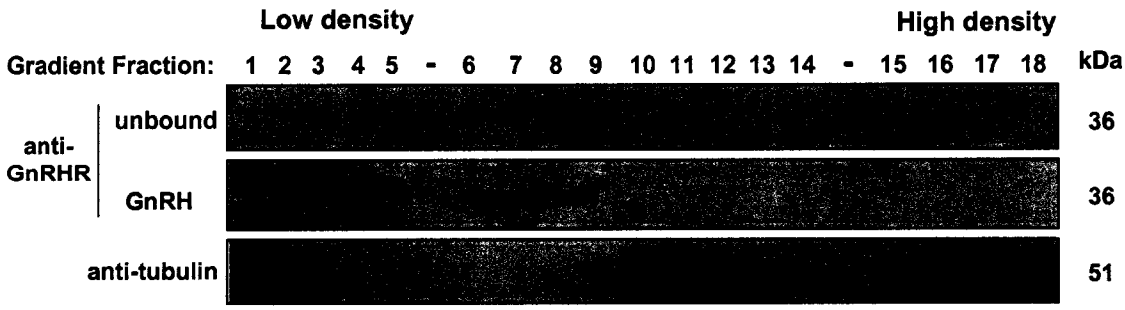


Figure 6. Endogenous GnRHR in αT3-1 cells localizes to low-density membrane microdomains. A. Raft samples were prepared using a detergent free carbonate buffer and separated by isopycnic ultracentrifugation through a non-linear sucrose gradient (45%, 35%, 5%). Sucrose fractions were electrophoresed in PAGE and then silver stained to determine the efficiency of membrane separation. **B.** αT3-1 cells were incubated in the presence or absence of 100 nM GnRH for 15 min at 37°C. Raft samples were then prepared using a detergent free carbonate buffer and separated by isopycnic ultracentrifugation through a non-linear sucrose gradient (45%, 35%, 5%). Fractions were collected from the top and separated by SDS-PAGE. Western blots were conducted using an antibody directed against the C-terminus of the GnRHR, tubulin or flotillin-1. **C.** Whole cell RIPA lysates were prepared from either αT3-1 or CHO cells. Western blots were conducted using a pan-caveolin antibody that detects caveolin-1, caveolin-2 and caveolin-3. **D.** αT3-1 cells were incubated in the absence or presence of 100 nM GnRH for 15 min at 37°C. Samples were then prepared using a 0.1% TX-100 based raft preparation and separated by isopycnic ultracentrifugation through a non-linear sucrose gradient (80%, 60%, 40%, 30%, 20%, 10%). Fractions were collected and electrophoresis and immunoblotting were performed as above.

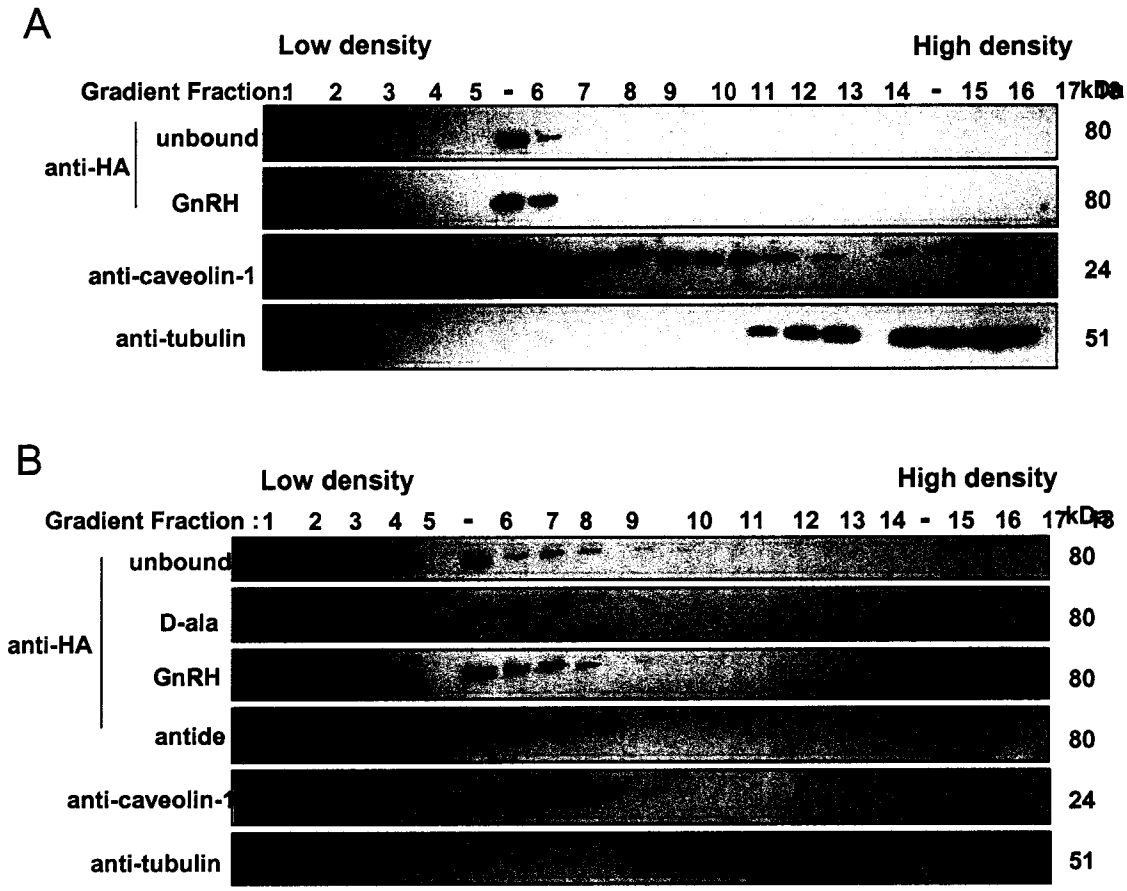


Figure 7. Stably transfected GnRHR localizes to low-density membrane microdomains in CHO cells. A. CHO cells stably transfected with HA-GnRHR-GFP were administered either control vehicle or 100 nM GnRH for 15 min at 37°C. Detergent free raft samples were prepared as in Figure 1A. Fractions were collected and separated by SDS-PAGE. Western blots were conducted using antibodies directed against an hemagglutinin (HA) epitope tag on the GnRHR, tubulin, or caveolin-1 **B.** Using a 0.1% TX-100 raft preparation as in Fig 1C, CHO cells were incubated either in the absence of hormone or in the presence of 100 nM GnRH, 10 nM super-agonist (D-Ala6-GnRH), or 10 nM antagonist (antide). Western blots were conducted using an antibody against an hemagglutinin (HA) tag on the GnRHR, tubulin, and caveolin-1.

an HA tagged GnRHR-GFP fusion protein stably expressed in CHO cells (Nelson et al., 1999). Using the detergent-free raft preparation, we found that the GnRHR migrated to low-density fractions 6 and 7 (the 5/35% interface). As in αT3-1 cells, the migration of the GnRHR in the sucrose gradient was unaffected by hormone treatment (**Figure 7A**). Similar results were evident when cells were prepared using non-ionic detergent (**Figure 7B**). Specifically, the GnRHR localized to the low-density fractions, and the migration of the GnRHR in the sucrose gradient was unaffected by treatment with agonist (GnRH), super-agonist (D-Ala^6-GnRH) or antagonist (antide). With both the detergent and non-detergent preparations, tubulin failed to migrate out of the high-density fractions of the gradient. Finally, unlike αT3-1 cells, CHO cells express the caveolar raft marker protein caveolin-1 that appropriately localized to low-density fractions. Thus, GnRHR localization to membrane microdomains or lipid rafts appears to be independent of cell-type or the presence of caveolin-1.

Transmission electron microscopy (TEM) reveals topological differences in the plasma membrane of αT3-1 and CHO cells.

The absence of caveolin expression in αT3-1 cells suggests that the GnRHR localizes to non-caveolar rafts in homologous cells, consistent with expression of flotillin-1. We wanted to examine the potential topological differences that could possibly exist in the plasma membrane of αT3-1 and CHO cells. The topological differences should be expressed as the presence of flask shaped invaginations with the plasma membrane of CHO cells but not αT3-1 cells. For quantitation, 100 cells were visualized and the number of flask-shaped invaginations in the plasma membrane of each cell type

was determined (**Figure 8A**). Consistent with non-detectable levels of caveolin-1, αT3-1 cells contain 20-fold fewer morphologically distinct membrane invaginations relative to CHO cells (**Figure 8B**). These ultrastructural studies support the notion that GnRHR most likely partitions to non-caveolar lipid rafts.

GnRH activation of ERK and c-fos expression is lost with cholesterol depletion.

The microenvironment necessary for raft formation is sensitive to cholesterol depletion (Brown and London, 2000). Thus, to assess the functional relevance of GnRHR localization in lipid rafts we sought to disrupt raft organization utilizing the cholesterol-sequestering agent, methyl- β -cyclodextrin (CD) (Simons and Toomre, 2000). αT3-1 cells were incubated for 1 hour in serum free medium with or without 2.0 % CD. One hour exposure to 2.0% CD effectively reduced cholesterol content by approximately 55% (**Figure 9A**). To address the functional consequence of cholesterol depletion and, presumably, raft disruption we next examined the effects of CD treatment on GnRH activation of ERK. Consistent with our previous studies (Mulvaney and Roberson, 2000; White et al., 1999), 30 minute exposure to GnRH increased the dual phosphorylated forms of ERK (ERK1 and ERK2) (**Figure 9B**). In contrast, phosphorylated ERK was not detectable in cells that had been exposed to 2.0 % CD. GnRH activation of c-fos gene expression proceeds through an ERK-dependent mechanism (Liu et al., 2002a; Mulvaney et al., 1999). Consistent with this notion, the effects of cholesterol depletion were also evident as a loss of GnRH activation of c-fos expression (**Figure 9C**). Finally, it seemed possible that the loss of GnRH activation of ERK and c-fos gene expression in CD treated cells might simply reflect a significant attenuation in the number of cell-surface

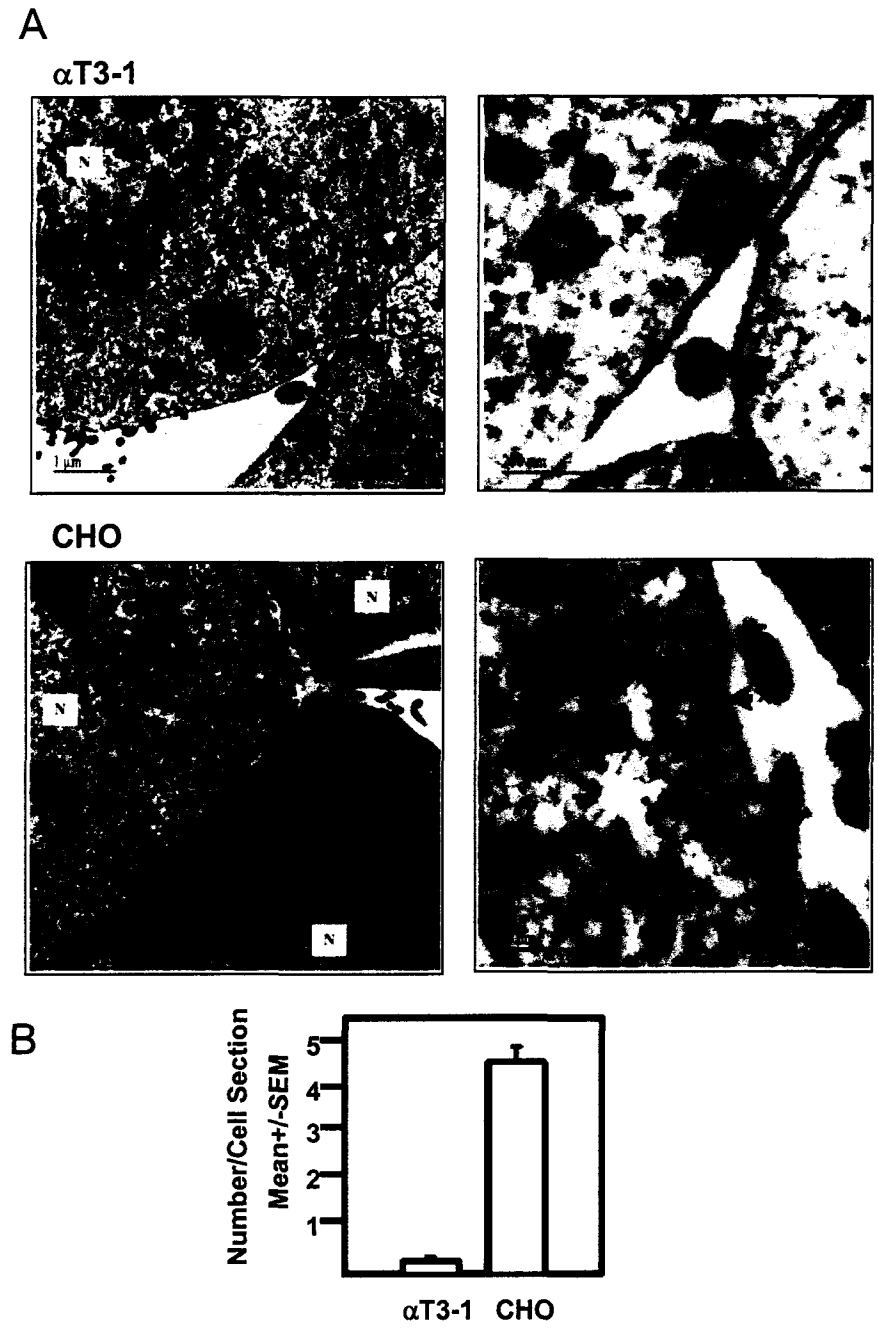


Figure 8. Plasma membrane topologies differ between αT3-1 and CHO cells using transmission electron microscopy (TEM) A. 70 nm sections of CHO and αT3-1 cells were fixed, stained with uranyl acetate and lead citrate, and viewed by transmission electron microscopy. **B.** To quantify the relative amounts of putative caveolae, the number of membrane invaginations per cell section was counted and the mean was calculated for 100 cell sections

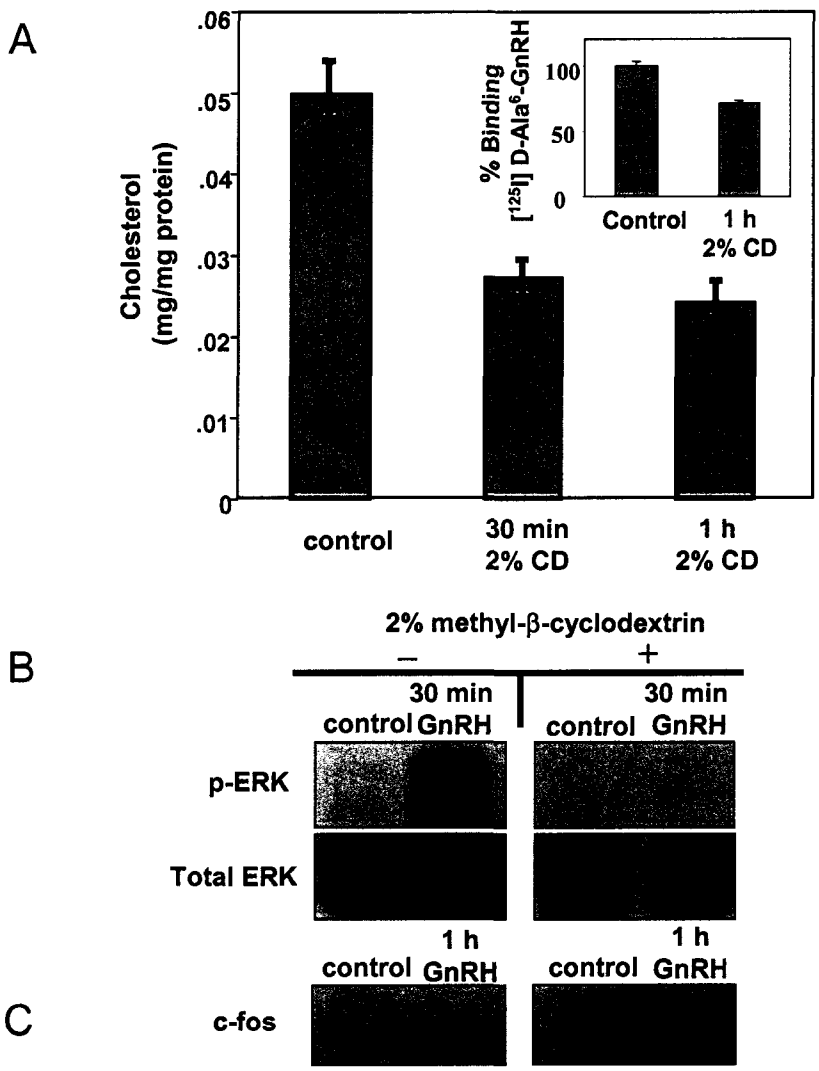


Figure 9. Cholesterol depletion leads to a loss of ERK and c-fos activation by GnRH in αT3-1 cells. **A.** αT3-1 cells were incubated in serum free medium containing 2.0% CD for 1 hour while control cells received serum free medium alone. Samples were lysed in MES buffer containing 0.1% TX-100. Cholesterol content was assayed as described in *Materials and Methods* and adjusted for protein concentration. **(Inset).** Binding of [125I] D-Ala6-GnRH (61.4 pM) to αT3-1 cells +/- CD treatment was determined as counts bound in the absence of unlabeled D-Ala^6-GnRH subtracted from counts bound in the presence of unlabeled D-Ala^6-GnRH (340 nM). Binding was then adjusted for protein concentration. **B.** αT3-1 cells were treated for 1 hour with serum free medium containing 2% CD while control cells received serum free medium alone. Cells were then washed with PBS and incubated with serum free medium containing 0 or 100 nM GnRH for 30 minutes at 37°C. Samples were then lysed in RIPA buffer and lysates analyzed by Western blotting using antibodies specific for phosphorylated ERK. After probing with anti-phospho-ERK, blots were stripped and reprobed with an antibody that detects ERK 1 and 2 independent of phosphorylation. **C.** Treatment of αT3-1 cells was identical to **B** except that RIPA lysates were prepared following 1 hour of GnRH treatment and analyzed in Western blots using a c-fos specific antibody.

GnRH receptors available for binding. This would not, however, appear to be the case as cholesterol depleted αT3-1 cells retained the ability to bind [^{125}I] D-Ala^{6}-GnRH at levels approximately 72% that of control cells (**Figure 9A inset**).

c-raf kinase localizes to low-density membrane microdomains in αT3-1 cells.

GnRH receptor activation of the ERK cascade and c-fos gene expression is thought to proceed through a c-raf kinase dependent mechanism (Mulvaney and Roberson, 2000). Thus, we were intrigued with the possibility that the loss of ERK and c-fos activation associated with cholesterol depletion may reflect disruption of membrane microdomains containing both the GnRHR and c-raf kinase. Consistent with this possibility, we find that c-raf kinase is also localized to low-density microdomains in αT3-1 cells prepared using the non-detergent based approach (**Figure 10A**). Partitioning of c-raf kinase to the low-density sucrose fractions did not appear to be affected by hormone treatment. Thus, c-raf kinase was constitutively but not exclusively present in lipid rafts. In contrast to c-raf kinase, b-raf-kinase, a closely related member of the raf kinase family, was only evident in high-density fractions. Thus, partitioning into lipid rafts appeared to be specific to the c-raf isoform of raf-kinase. Also supportive of the specificity of fractionation, an integral membrane protein H^{+}ATPase migrated to high-density sucrose fractions. Finally, to test if raft-associated c-raf kinase is a target for GnRH-mediated phosphorylation, fractions 6 and 7 were isolated from the sucrose gradient and subjected to electrophoresis in a low-crosslinking gel. Consistent with GnRH-induced phosphorylation, the electrophoretic mobility of raft-associated c-raf kinase was reduced with GnRH treatment (**Figure 10B**). Previous studies have

demonstrated that the retarded electrophoretic mobility of c-raf kinase correlates with GnRH-induced catalytic activation of this enzyme (Mulvaney and Roberson, 2000).

GnRH binding enhances the association of c-raf kinase with lipid microdomains.

We next sought to confirm raft localization of c-raf kinase using the detergent based preparation. As with the non-detergent approach, immunoblots of lysates prepared using a 0.1% TX-100 lysis buffer revealed constitutive but not exclusive localization of c-raf kinase low-density fractions independent of hormone treatment (**Figure 11A**). It is interesting to note, however, that increasing the concentration of Triton X-100 from 0.1 to 1.0 % resulted in a distinctly different pattern of segregation such that c-raf kinase was evident in the low-density fractions only under conditions of GnRH binding (**Figure 11B**). Thus, in the unbound state, the association of c-raf kinase in lipid rafts was more susceptible to disruption by non-ionic detergent suggesting that GnRH treatment may act to stabilize or enhance the association of c-raf kinase in lipid microdomains.

c-raf kinase is present in low-density membrane microdomains in mouse pituitary cells.

To address whether c-raf kinase localization to lipid rafts was a unique property of the αT3-1 cell line, detergent-free raft preparations were prepared from CHO, JEG3 (choriocarcinoma) and NIH 3T3 cells and subjected to sucrose-density gradient centrifugation. As with αT3-1 cells, a raft-associated population of c-raf kinase was detectable in all 3 cell lines (data not shown). Thus, c-raf kinase is present in low-density membrane microdomains independent of cell type and expression of caveolin-1. Most importantly, c-raf kinase was also evident in low-density fractions prepared from whole

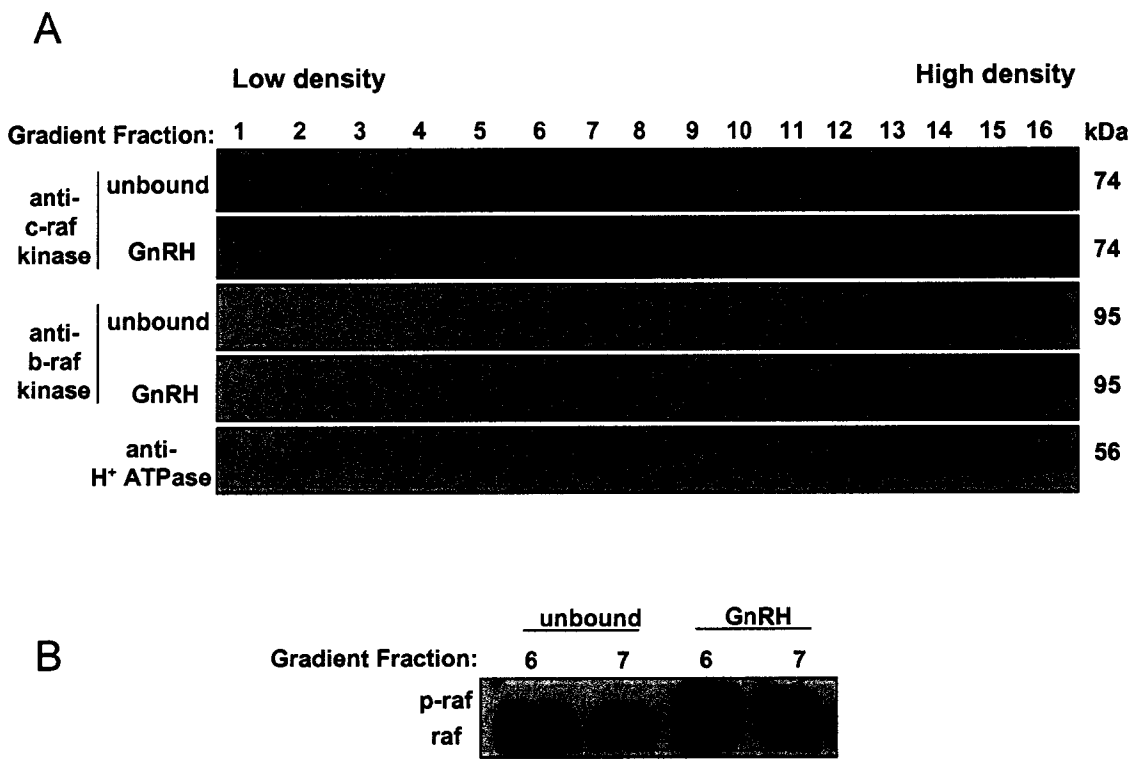
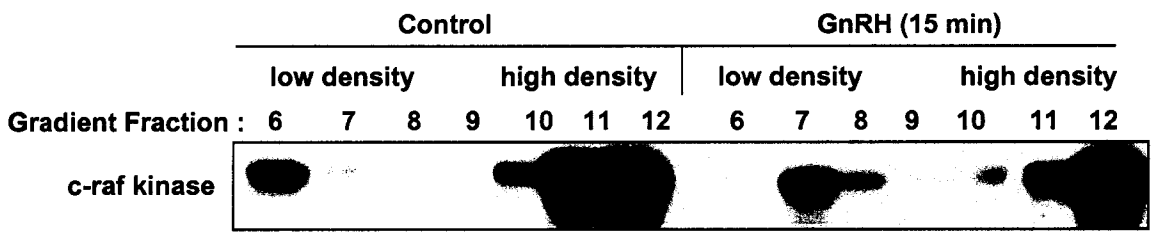


Figure 10. c-raf kinase localizes to lipid rafts in αT3-1 cells. A. αT3-1cells were treated with PBS or 100 nM GnRH for 15 minutes at 37°C. Following detergent free raft preparation and density gradient centrifugation, Western blots were probed for antibodies specific for c-raf kinase, b-raf-kinase and anti-H+–ATPase. **B.** Low-density fractions 6 and 7 were isolated from the sucrose gradient and subjected to electrophoresis in a low-crosslinking gel to determine the effects of GnRH treatment on c-raf kinase.

A

Prepared with 0.1% Triton X-100



B

Prepared with 1.0% Triton X-100

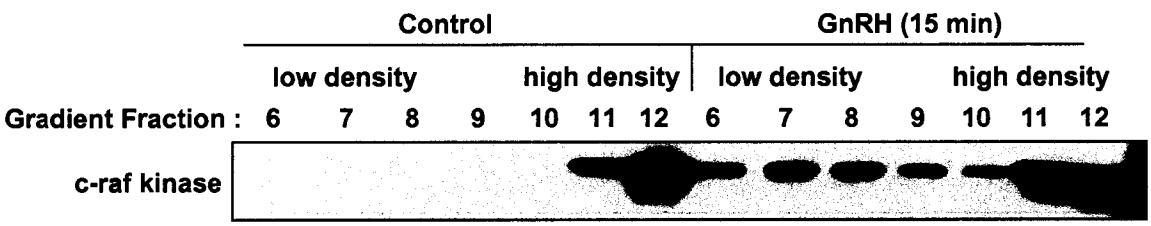


Figure 11. GnRH stabilizes the association of c-raf kinase with low-density membrane microdomains. A. αT3-1 cells were incubated in the presence or absence of 100 nM GnRH for 15 minutes at 37°C and then solubilized using a 0.1% TX-100 detergent raft preparation. Western blots were probed with an antibody specific for c-raf kinase. **B.** αT3-1 cells were incubated with 100 nM GnRH for 15 minutes and solubilized using a 1.0% TX-100 raft preparation. Western blots were probed for an antibody specific for c-raf kinase.

pituitary lysates from adult female mice (**Figure 12**). Pituitary expression of caveolin-1 allowed us to use this protein as a marker for low-density microdomains. Clearly, we do not suggest that these results reflect a unique contribution of GnRHR expressing cells (gonadotropes) as there are multiple cell lineages present in the adult pituitary. These data do, however, demonstrate that raft localization of c-raf kinase is evident in non-transformed, GnRHR expressing tissues and is not simply an aberrant feature of the αT3-1 cell line. Unfortunately, we were unable to detect the GnRHR in mouse pituitaries due to the low number of gonadotropes and insufficient protein concentrations.

Disruption of raft localization of GnRHR by CD treatment of αT3-1 cells is reconstituted by cholesterol repletion.

In **Figure 9**, we demonstrate that GnRH activation of ERK and c-fos expression is lost in cholesterol depleted αT3-1 cells. Presumably, the loss of cholesterol is associated with raft disruption. If correct, then the effects of CD should be revealed as a loss of both GnRHR and potentially c-raf kinase in low-density sucrose fractions. Furthermore, if the effects of CD are specific to cholesterol depletion then reconstitution of cholesterol content should accordingly reconstitute raft microdomains. To directly test these possibilities, αT3-1 cells were incubated in the presence or absence of 2.0% CD for 1 hour. Cholesterol repletion was accomplished by incubation of cholesterol depleted cells with 1 mg/ml CLCD for 2 hours. Cellular lysates were then prepared in sodium carbonate buffer and subjected to sucrose-density gradient centrifugation. As in the previous study (**Figure 9**), 1 hour exposure to CD resulted in an approximate 55% reduction in cholesterol in CD treated cells as compared to control cells (**Figure 13A**).

Subsequent incubation of cholesterol-depleted cells with CLCD was effective in restoring cholesterol content to approximately 85 % of control levels (**Figure 13A**). Consistent with raft disruption, cholesterol depletion was associated with a loss of GnRHR from the low-density fractions 6 and 7 (**Figure 13B**). Importantly, however, cholesterol repletion was effective in reconstituting the localization of GnRHR to low-density fractions in the sucrose gradient (**Figure 13B**). In contrast, partitioning of c-raf kinase into lipid rafts was not remarkably affected by cholesterol depletion (**Figure 13C**) suggesting that fundamental differences exist in the mechanism(s) underlying the partitioning of c-raf kinase and GnRHR into low-density compartments.

Cholesterol repletion reconstitutes GnRH activation of ERK and c-fos expression in CD treated αT3-1 cells.

Based on the data in the previous section, incubation of cholesterol depleted αT3-1 cells with CLCD effectively restored cholesterol content and raft localization of GnRHR. Next, we sought to determine if this is sufficient to reconstitute GnRH signaling to ERK and c-fos. Accordingly, GnRH activation of ERK and c-fos expression was assessed utilizing the same cholesterol depletion/repletion paradigm. As in **Figure 9**, GnRH-induced phosphorylation of ERK and c-fos expression was lost upon incubation of αT3-1 cells for 1 hour in 2.0 % CD (**Figure 14**). Partial reconstitution of both parameters was, however, evident after incubation of cholesterol-depleted cells with CLCD (**Figure 14**).

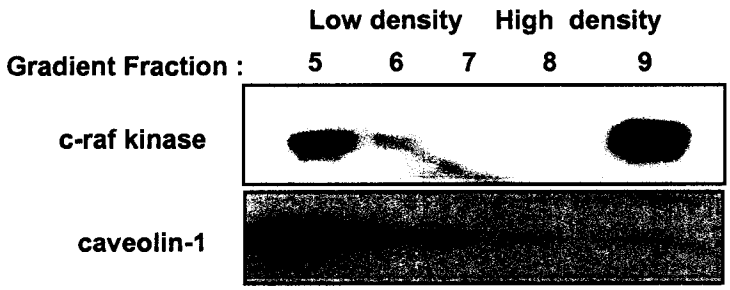


Figure 12. c-raf kinase localizes to low-density membrane microdomains in mouse pituitary lysates. A pooled sample representing 10 whole mouse pituitaries was prepared using a 0.1% TX-100 raft procedure and then separated by sucrose gradient centrifugation. Ten fractions were collected and Western blotting was carried out using antibodies specific for c-raf kinase and caveolin-1.

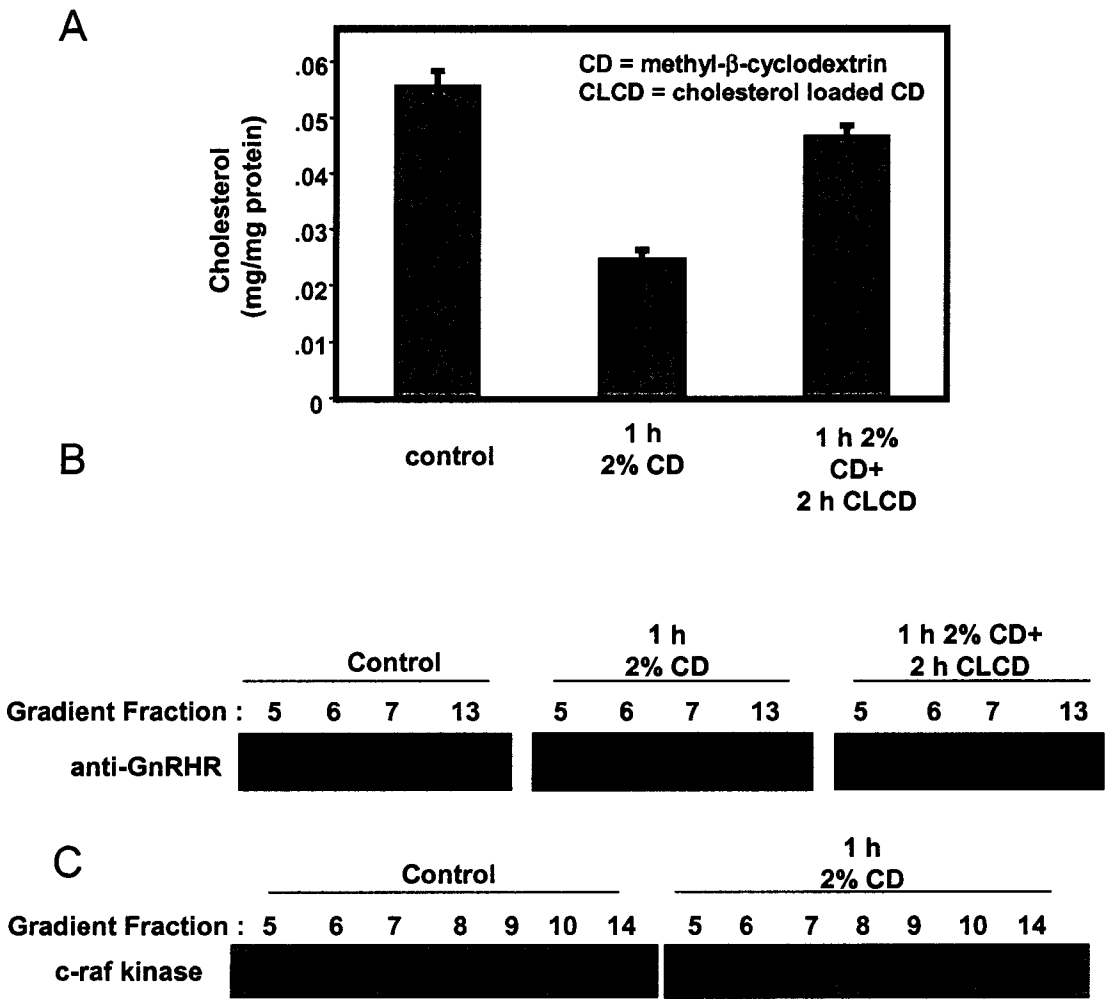


Figure 13. Disruption of GnRHR raft localization can be rescued by cholesterol repletion. A. αT3-1 cells were incubated for 1 hour in serum free medium containing 2.0% CD at 37°C. Cells were then incubated with either 1 mg/ml of CLCD or serum free medium alone for 2 hours. Control cells received 3 hours of serum free medium alone. Cholesterol content was assayed as described in the *Materials and Methods* and then adjusted for protein concentrations. **B.** αT3-1 cells were incubated for 1 hour in serum free medium containing 2.0% CD at 37°C followed by treatment with 1 mg/ml of CLCD or serum free medium alone for 2 hours. Western blots were probed with an antibody that detects GnRHR. **C.** αT3-1 cells were incubated for 1 hour in serum free medium containing 2.0% CD at 37°C. Cells were then incubated with serum free medium alone for 2 hours. Control cells received 3 hours of serum free medium alone. Western blots were probed with an antibody that detects c-raf kinase.

Cholesterol depletion uncouples GnRHR but not phorbol ester mediated activation of ERK.

To begin to localize the lesion in GnRH signaling to ERK we next asked if ERK activation in response to phorbol ester (PMA) treatment was retained in cholesterol depleted cells. The use of PMA in these studies would, presumably, directly activate PKC isozymes thus effectively bypassing the GnRHR, $G\alpha_{q/11}$ and phospholipase C. Consistent with earlier studies (Liu et al., 2002a; Mulvaney et al., 1999; Reiss et al., 1997; White et al., 1999), both GnRH and PMA induced ERK phosphorylation in control cells (**Figure 15A**). As expected, cholesterol depletion resulted in a loss of GnRH-induced ERK phosphorylation. Importantly, however, CD treatment did not visibly compromise ERK activation in response to PMA. Thus, cholesterol depletion and the resulting raft disruption appears to uncouple the GnRHR from signaling intermediates that lie upstream of PKC isozymes and may, in fact, reflect uncoupling of the GnRHR from its cognate heterotrimeric G-protein complex. Consistent with this possibility, we find that, like the GnRHR, cholesterol depletion of αT3-1 cells leads to an attenuation in the amounts of immunodetectable $G_{\alpha q/11}$ localized to low-density fractions (**Figure 15B**) suggesting that raft organization may be critical for GnRH coupling to $G_{\alpha q}$. If correct, disruption of raft organization should be revealed as a loss of GnRH signaling to phospholipase C and, as a consequence, attenuation in the ability of GnRH to liberate IP_3 from membrane phospholipid. In accordance with this we find that CD treatment virtually eliminates the GnRH induced IP_3 response in αT3-1 cells (**Figure 15C**).

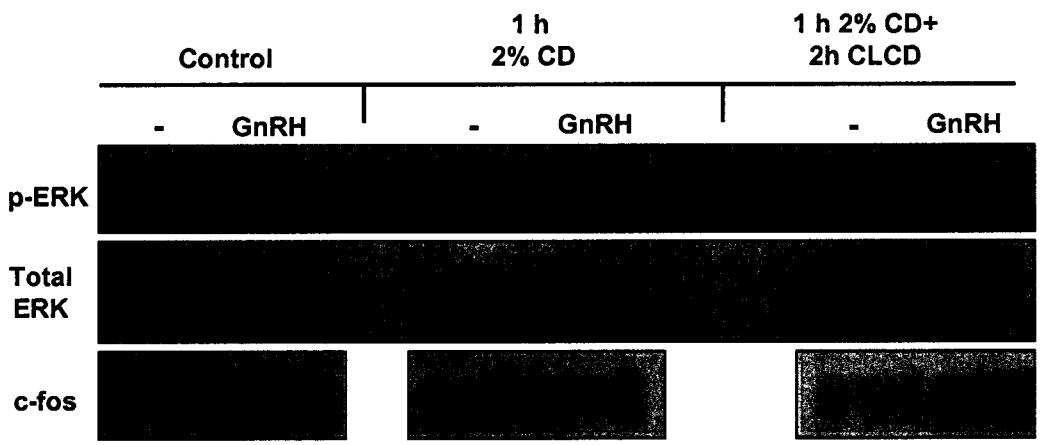
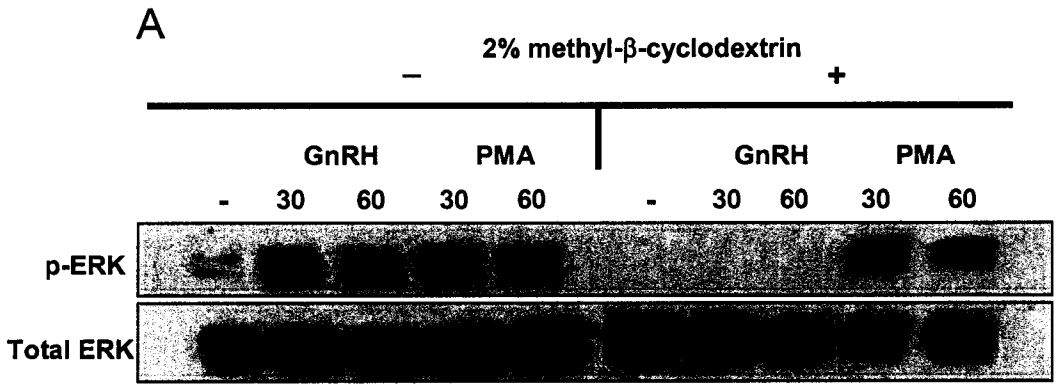
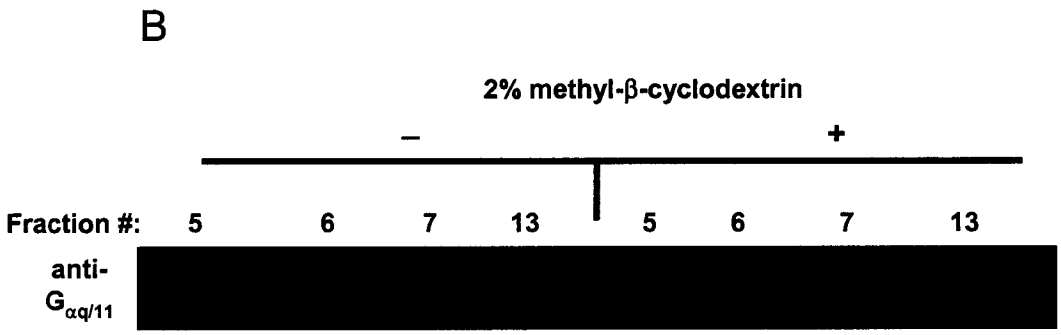


Figure 14. Repletion of membrane cholesterol in aT3-1 cells restores the ability of GnRHR to signal to ERK and c-Fos. αT3-1 cells were incubated for 1 hour in serum free medium containing 2.0% CD at 37°C. Following the CD treatment, cells were incubated with either 1 mg/ml of CLCD or serum free medium alone for 2 hours. Control cells received serum free medium alone for 3 hours. After treatments, medium was removed and replaced with serum free medium containing 100 nM GnRH for 1 hour. Separate Western blots were then probed with antibodies specific for the phosphorylated forms of ERK and c-fos. Phospho-ERK blots were subsequently stripped and reprobed with an antibody that detects ERK independent of phosphorylation.





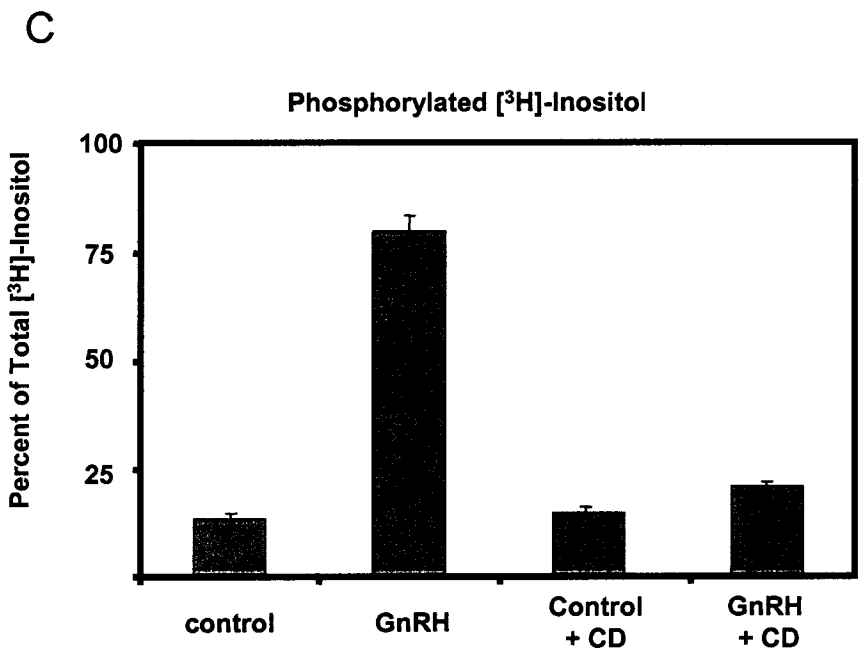


Figure 15. Cholesterol depletion uncouples GnRH but not phorbol ester mediumted activation of ERK. A. αT3-1 cells were incubated for 1 hour in serum free medium containing 2.0% CD at 37°C. CD medium was removed and replaced with serum free medium for 2 hours. Control cells received serum free medium alone for three hours. After 2 hours of serum free medium, samples were incubated with 100 nM GnRH or 100 nM PMA for either 30 or 60 minutes. Western blots were then probed with antibodies specific for the phosphorylated forms of ERK. Phospho-ERK blots were subsequently stripped and reprobed with an antibody that detects ERK independent of phosphorylation. **B.** αT3-1 cells were incubated in the presence or absence of 2.0% CD for 1 hour at 37°C followed by detergent free raft preparation and density gradient centrifugation as described in Materials and Methods. Western blots were probed using an antibody specific for Gαq/11. **C.** αT3-1 cells were preloaded with [3H]-inositol for 5 hours and then incubated with or without 2.0% CD for 1 hour followed by treatment with either 0 or 100 nM GnRH for an additional hour. Lysates were prepared and subjected to ion exchange chromatography to separate free and phosphorylated inositol. Phosphorylated inositol is expressed as a percentage of total [3H]-inositol.

DISCUSSION

The organization of cell-surface receptors and their cognate signaling proteins into pre-formed signaling platforms contributes to maintaining the specificity and integrity of ligand-induced intracellular signaling cascades (Hoessli et al., 2000). Furthermore, it appears that these signaling platforms are not uniformly distributed throughout the plasma membrane but rather are often localized to discrete microdomains characterized by an enriched population of sphingolipids and cholesterol (Simons and Ikonen, 1997). The relative buoyancy of these microdomains in sucrose gradients accounts for the evolution of the term "lipid raft" as a general descriptor of these regions of the plasma membrane (Simons and Ikonen, 1997). Over the past 3-5 years, a wide array of signaling proteins, scaffolding proteins and receptors has been shown to associate with lipid rafts (Shaul and Anderson, 1998). Typically, this association is characterized as either static or dynamic. For example, caveolin is constitutively associated with and, in fact, defines a unique sub-population of microdomains termed caveolae (Anderson, 1998). Dynamic partitioning from the bulk plasma membrane to lipid rafts characterizes ligand activation of a number of cell-surface receptors including members of the GPCR superfamily such as the muscarinic acetylcholine receptor and B2 bradykinin receptor (de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997). In contrast to this dynamic behavior, we find that, independent of ligand activation, the mammalian GnRHR is constitutively localized to non-caveolar lipid rafts in the gonadotrope-derived αT3-1 cell line. Raft localization of the GnRHR was equally evident in heterologous cells (CHO) suggesting that this constitutive segregation into lipid rafts is not unique to αT3-1 cells but rather is intrinsic to the GnRHR itself.

As yet we do not know what structural features of the GnRHR account for raft localization. However, perhaps the most unique structural feature of this GPCR is the virtual absence of an intracellular carboxyl terminal tail (Stojilkovic et al., 1994). In more prototypical GPCRs, this region is quite extensive and important for coupling to G-proteins, agonist induced receptor internalization, and phosphorylation-mediated desensitization (Dohlman et al., 1991). Phosphorylation of the c-terminus is typically thought of as requisite for subsequent interaction with β-arrestin which hinders further G-protein activation and targets the deactivated receptors for internalization (Ferguson, 2001; Goodman et al., 1996). Due to the absence of an intracellular c-terminus extenstion, the mammalian GnRHR is neither phosphorylated nor internalized via a β-arrestin dependent mechanism (Heding et al., 2000; McArdle et al., 2002b; Vrecl et al., 1998). Finally, in several GPCRs, cysteine residues located in the intracellular c-terminal domain are targets for reversible palmitoylation (Munshi et al., 2001; O'Dowd et al., 1989; Tanaka et al., 1998) – a particularly intriguing observation as palmitoylation has been implicated in increasing a protein's affinity with lipid rafts and caveolae (Melkonian et al., 1999; Zacharias et al., 2002). In short, given this fundamental difference in the structural and functional properties of the GnRHR vis a vis prototypical GPCRs, it is tempting to speculate that the unusual behavior of the GnRHR in the plasma membrane may partially reflect the absence of an intracellular carboxyl terminal tail. In this regard, it should be of particular interest to examine the in-membrane behavior of non-mammalian and Type II GnRH receptors which possess intracellular c-terminal tails (Millar et al., 2001; Neill et al., 2001; Tensen et al., 1997).

Regardless of the structural features of the GnRHR that direct raft association it appears that localization of the GnRHR to lipid rafts is functionally significant. In support of this notion, raft disruption resulting from the removal of cholesterol leads to a loss of GnRH activation of ERK and c-fos gene expression. Importantly, this loss of signaling was not due to a loss of cell-surface binding and was reversible by cholesterol replenishment and reconstitution of lipid rafts. It is also important to underscore that the lesion in GnRH signaling resulting from cholesterol depletion was not reflected as a generalized loss of signaling. Specifically, while GnRH activation of ERK was lost in CD treated cells, this same paradigm had little effect on the efficacy of PMA induced ERK phosphorylation. Thus, the ERK signaling cascade in cholesterol depleted αT3-1 cells is sufficiently intact to transduce a PMA signal but not a GnRH signal. As such, the primary lesion in the GnRH response likely lies upstream of PKC. Based on several lines of evidence we suggest that this lesion may partially reflect uncoupling of the GnRHR from its cognate heterotrimeric G-protein complex. First, consistent with others, we find that the presence of $G_{\alpha q}$ in low-density membrane fractions is susceptible to disruption by cholesterol depletion. Second, cholesterol depletion significantly attenuates the ability of GnRH to increase intracellular levels of IP_3.

GnRH activation of ERK proceeds through c-raf kinase (Benard et al., 2001; Mulvaney and Roberson, 2000). Consistent with this, we find that, like the GnRHR, c-raf kinase also segregates into low-density sucrose fractions prepared from αT3-1 cells, whole mouse pituitaries and several clonal cell lines. Thus, the localization of at least a portion of c-raf kinase to low-density membrane compartments is not cell-type specific. It is also clear, however, that the localization of c-raf to low-density microdomains is not

exclusive. The limited presence of c-raf kinase in lipid rafts relative to the larger pool of c-raf kinase associated with cytosol or other membrane compartments (higher density fractions) may reflect the rate-limiting presence of a putative platform or scaffolding protein(s) reminiscent of the yeast protein Ste5p that is known to bind multiple members of the ERK signaling cascade (Elion, 2001). Similarly, several mammalian scaffold proteins have also been characterized including 14-3-3 proteins and multiple isoforms of JNK inhibitory protein (JIP) (Aitken et al., 2002; Okamoto et al., 1998; Yasuda et al., 1999). If correct, then compartmentalization of such a scaffold or platform into lipid rafts associated with GnRHR would reflect a key mechanism for the initiation and organization of GnRH signaling in gonadotropes. Finally, we should point out that caveolin itself has been implicated as a scaffolding protein (Okamoto et al., 1998); however, the absence of expression of caveolin in αT3-1 cells would suggest that this protein is not requisite for organizing the GnRHR and c-raf kinase into a signaling platform or scaffold.

The constitutive presence of c-raf kinase within lipid rafts is consistent with studies of the insulin signaling cascade and, more recently, the use of artificial rafts to examine the interaction of c-raf kinase with lipid microdomains. In regard to the latter, Ulf Rapp and colleagues (Hekman et al., 2002) demonstrated c-raf kinase preferentially associated with artificial lipid rafts with high affinity. In the absence of conditions that promote lipid raft formation, raf kinase displayed moderate binding affinity directly with cholesterol; however, the binding affinity increased markedly in the context of lipid rafts. Similar observations with c-raf kinase were demonstrated for binding of ceramides within lipid rafts. In bulk plasma membrane not associated with rafts, c-raf kinase associated

with phospholipids such as phosphotidylserine and phosphatidic acid, a product of phospholipase D catalytic activity. In addition to these studies, c-raf kinase has been shown to associate with lipid rafts in the context of insulin signaling via recruitment into internalized endosomes (Rizzo et al., 2000; Rizzo et al., 2001). In this system, c-raf kinase appears to be recruited to membrane rafts by an interaction with raft-associated phosphatidic acid generated by insulin activation of phospholipase D. This interaction appears to be necessary for activation of the ERK cascade in a ras-dependent manner. This mechanism stands in contrast to the present studies in which c-raf kinase is constitutively present in low-density fractions independent of any recruitment by signaling intermediates downstream of GnRHR activation.

In the insulin signaling system, cholesterol depletion and, presumably, raft disruption blocks the recruitment of ras to lipid rafts and ERK activation but does not appear to affect c-raf kinase membrane association or activation (Rizzo et al., 2001). In the case of αT3-1 cells, cholesterol depletion to levels approximately 50% of control cells was sufficient to disrupt GnRHR association with lipid rafts. These same conditions, however, did not affect the association of c-raf kinase with lipid rafts. Thus, it is possible that the association of c-raf kinase with lipid rafts in αT3-1 cells may be independent of cholesterol content within the membrane. Alternatively, if raft association of c-raf kinase in αT3-1 cells is cholesterol dependent then it is possible that the cholesterol depletion was not sufficient to disrupt this association. Unfortunately, a marked reduction in αT3-1 cell viability precludes more extensive cholesterol depletion. Finally, it is interesting to note that the association of c-raf kinase within membrane rafts was sensitive to increasing levels of non-ionic detergent. In relatively low detergent

conditions, c-raf kinase was constitutively localized to membrane rafts independent of GnRH administration. However, while increasing detergent concentrations effectively reduced c-raf localization to membrane rafts in unstimulated αT3-1 cells, GnRH receptor activation restored c-raf kinase to this low-density membrane compartment. This increased resistance to detergent solubilization raises the possibility that GnRH action resulting in modification of c-raf kinase (such as phosphorylation) may increase the stability of the association of c-raf kinase with lipid microdomains.

Since the isolation of the first cDNA encoding the mammalian GnRHR much progress has been made in identifying structure-function relationships in this unique GPCR (Sealfon et al., 1997; Stojilkovic et al., 1994). Additionally, the use of epitope or fluorophore tagged GnRH receptors has allowed direct observations of the "in-membrane" behavior of unoccupied, agonist occupied and antagonist occupied receptors. For example, we and others (Cornea et al., 2001; Horvat et al., 2001b; Kroeger et al., 2001) have utilized resonance energy transfer methods to demonstrate that agonist but not antagonist leads to self-association of GnRH receptors in the plasma membrane. Based on the present studies, we suggest that this self-association occurs in the context of discrete lipid microdomains that contain not only the GnRHR but also downstream signaling components, such as $G_{\alpha q}$ and c-raf kinase, that serve to transduce a GnRH signal to the level of ERK activation. As such, disrupting these microdomains effectively lesions the ability of GnRH to activate ERK and increase c-fos gene expression. In this model then, the GnRHR exists as a resident protein in a pre-formed signaling platform that is poised to both receive and efficiently transmit the GnRH signal. Confirmation of this model awaits definition of the complement of proteins organized within this platform

including identification of potential scaffolding proteins and definition of the structural features of the GnRHR that direct its association with lipid microdomains.

CHAPTER FOUR

DIFFERENTIAL IMPACT OF INTRACELLULAR CARBOXYL TERMINAL DOMAINS ON LIPID RAFT LOCALIZATION OF THE MURINE GONADOTROPIN RELEASING HORMONE RECEPTOR

INTRODUCTION

Over the past decade it has become evident that the plasma membrane is not a random sea of lipids but rather displays regions, typically less than 100 nm in length, that contain elevated levels of glycosphingolipid and cholesterol compared to the "bulk" plasma membrane (Brown and Rose, 1992; Simons and Ikonen, 1997). The tight packing of the saturated acyl chains of the sphingolipids and the presence of cholesterol appears to result in a domain that is less fluid than the surrounding plasma membrane. Further, this unique lipid composition appears to be responsible for two key characteristics of membrane microdomains, 1) relative insolubility in non-ionic detergents and, 2) migration to low-density fractions in sucrose gradients. Thus, these "lipid rafts" appear to exist as islands of distinct liquid-ordered phase dispersed in the more disordered matrix of the lipid bilayer (Simons and Ikonen, 1997; Simons and Toomre, 2000).

It is thought that lipid rafts serve to co-localize membrane receptors and their cognate downstream signaling components in either pre-assembled complexes or in separate complexes that, upon ligand activation, co-segregate to form a transient signaling platform (Grzybek et al., 2005; Hoessli et al., 2000; Zajchowski and Robbins, 2002). We have shown that the mammalian Type I GnRH receptor (GnRHR) and downstream signaling intermediates including c-raf kinase and $G\alpha_q$ are detectable in low-density sucrose fractions prepared from the gonadotrope derived αT3-1 cell line (Navratil et al., 2003). Furthermore, raft localization of the GnRHR appears to be independent of cell type suggesting that segregation of the GnRHR into lipid rafts is intrinsic to the GnRHR. Unresolved, however, is the identity of the structural features of the GnRHR that dictate inclusion of this molecule in lipid microdomains.

Protein-targeting theory would predict that the segregation of raft-associated proteins into lipid microdomains is dependent on unique structural determinants found within the protein itself. Unfortunately, determination of these unique structural motifs remains elusive. At least in part, this is likely a consequence of the diversity of mechanisms that underlie protein targeting to lipid rafts (Anderson and Jacobson, 2002; Kimura et al., 2001; Schlegel and Lisanti, 2001; Yamabhai and Anderson, 2002; Zacharias et al., 2002). For transmembrane proteins such as cell-surface receptors, critical amino acid residues have been identified in membrane-proximal extracellular domains and in membrane spanning domains that function as molecular addresses for lipid rafts (Yamabhai and Anderson, 2002). In regard to the latter, Anderson and Jacobson (Anderson and Jacobson, 2002) have proposed that lipid binding properties of specific amino acid motifs located in membrane spanning regions of raft-associated

transmembrane proteins results in a "lipid shell" that directs the segregation of these proteins into lipid microdomains. Lipid or glycolipid modifications such as GPI anchors appear to be the essential targeting motif in several raft-associated proteins (Brown and London, 1998). In this scenario, the lipid modification (e.g. myristoylation, palmitoylation) may represent the anchor that directly interacts with membrane microdomains (Zacharias et al., 2002). Finally, for some transmembrane proteins, critical amino acid residues present in cytoplasmic domains function as molecular addresses (Anderson and Jacobson, 2002). In the case of GPCR's, this could represent motifs present in 1 of 3 intracellular loops or the cytoplasmic C-terminus (Gether, 2000). The latter is particularly intriguing as cysteine residues located in the intracellular C-terminus are targets for palmitoylation in multiple GPCR's (Munshi et al., 2001; O'Dowd et al., 1989; Tanaka et al., 1998).

Although classified as a member of the rhodopsin class of G-protein coupled receptors (GPCR), the mammalian GnRHR displays several unique structural features. Perhaps the most striking of these is an extremely short carboxyl-terminal cytoplasmic domain of only 1-2 amino acids (Sealfon et al., 1997; Stojilkovic et al., 1994). In more prototypical GPCRs, this domain is quite extensive and contains phosphorylation sites for GPCR kinases (GRK), second-messenger regulated kinases such as protein kinase A (PKA) and casein kinases (Pierce et al., 2002). In many GPCRs, phosphorylation of the C-terminus appears to be requisite for subsequent interaction with β-arrestin, which hinders further G-protein activation and targets the deactivated receptor for internalization (Ferguson, 2001; Zhang et al., 1997). This is thought to be the case with GnRH receptors found in non-mammalian vertebrates (e.g. catfish (Bogerd et al., 2002),

goldfish (Illing et al., 1999), chicken (Sun et al., 2001), Xenopus (Troskie et al., 2000)), which possess C-terminal tails and appear to be phosphorylated by GRKs, show rapid desensitization (Blomenrohr et al., 1999), and (Heding et al., 1998) undergo rapid agonist-induced internalization (Willars et al., 2000); however, there are differences in arrestin dependence among these receptors (McArdle et al., 2002b). The mammalian GnRHR shows a natural resistance to desensitization and internalization (Vrecl et al., 2000), which has been attributed to its lack of a C-terminal tail (McArdle et al., 1999).

As the absence of an intracellular carboxyl terminal domain is perhaps the most conspicuous feature of the GnRHR (Stojilkovic et al., 1994), we were intrigued with the possibility that raft localization of the mammalian Type I GnRHR might reflect both the gain (raft localization) and loss (C-terminal tail) of structural determinants. If correct, then this loss and gain theory would predict that placement of a heterologous C-terminal domain on the Type I (murine) GnRHR would redirect the localization of this receptor from a raft to non-raft domain. Consistent with this, we find that the intracellular C-terminus from the rat luteinizing hormone receptor (LHR) leads to trafficking of the mammalian type I GnRHR to a non-raft compartment. In contrast, the equivalent domain from a non-mammalian catfish (cf) GnRHR does not substantially alter raft localization of the mammalian GnRHR. Thus, while an intracellular C-terminus is sufficient to redirect the GnRHR to non-raft compartments, this is not a generalized feature of GPCR C-terminal tails.

MATERIALS AND METHODS

Materials

Anti-hemagglutinin (HA) antibody was purchased from Roche (Indianapolis, IN). Secondary antibodies, as well as those against p-ERK, and ERK-1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). D-Ala^6-desGly^{10}-GnRH-Pro^9-ethylamide (D-Ala^6-GnRH) and hCG were purchased from Sigma (St. Louis, MO). Glass bottom microwell dishes for confocal studies were obtained from Mat-Tek (Ashland, MA). Alexa 594 Concanavolin A (ConA) was purchased from Molecular Probes (Eugene, OR)

Cell Culture

Chinese Hamster Ovary (CHO) cells were maintained in high glucose DMEM containing 2 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and 1 X nonessential amino acids (Mediatech, Herndon, VA), with 10% FBS (Gemini Bioproducts, Woodland, CA). All cells were grown in 5% CO_2 at 37°C in a humidified environment. All transfections were performed with Polyfect Reagent (Qiagen, Valencia, CA), following the manufacturers instructions.

Plasmids

The pHA-GnRHR-GFP vector has been described previously (Navratil et al., 2003). The murine 3x-HA-GnRHR, along with the parent 3x-HA vector (pKH3) were generous gifts from Dr. Mark Roberson (Cornell University). For rat LHR or cfGnRHR C-terminal tail exchanges, PCR was performed on pHA-GnRHR-GFP using a CMV promoter-derived upstream primer, and a downstream primer that inserted an Eco RV site beginning at

position 2 of the codon for Gly^{323}. The resulting product was cloned into pGEM-TEasy (Promega, Madison, WI), and sequenced to confirm fidelity of PCR. A fragment was then excised with Ppu MI (within mGnRHR) and Eco RV (the resulting Eco RV half-site contains a single conservative substitution at position 3 of the Gly^{323} codon, stopping blunt after position 1 of codon Tyr^{324}). Tail fragments were generated by PCR using rat LHR cDNA or cfGnRHR cDNA (provided by J. Bogerd, University of Utrecht, The Netherlands), with upstream primers at the fusion junction site which completed an Ssp I half-site found beginning at position 2 of mGnRHR codon Tyr^{324} (the added sequence being removed following subsequent Ssp I digestion), included the codon for mGnRHR Phe^{325}, then switching to corresponding rLHR or cfGnRHR sequence, respectively. The products were cloned into pGEM-TEasy, sequenced, excised with Ssp I and Bam HI, and ligated with Ppu MI/Eco RV-cut upstream fragment into pHA-GnRHR GFP from which the C-terminal portion of the native receptor had been removed with Ppu MI/Bam HI (GnRHR-LF-GFP and GnRHR-CF-GFP). The truncated LHR-tailed GnRHR was constructed similarly, only a downstream tail primer inserted a Bam HI site after codon Arg^{657} (GnRHR-LT-GFP). For the LHR with murine GnRHR C-terminus, a fusion tail segment was produced by PCR using an LHR primer upstream of a Bgl I site at codon Ser^{595}, and one of two LHR/GnRHR fusion primers at the end of transmembrane domain 7, choosing for exchange points conserved residues 3 (Phe – LHR-GF) or 6 (Tyr - LHR-GY) amino acids from the end of mGnRHR, and placing a Bam HI site immediately downstream of the termination codon. The product was cloned into pGEM-TEasy, sequenced, isolated with Bgl I and Bam HI, and relocated along with an Eco RI/Bgl I LHR fragment from pLHR-GFP(Roess and Smith, 2003) into pDsRed2-N1 (BD

Clontech, Palo Alto, CA) (the resulting vectors contained the DsRed cDNA, but being downstream of the termination codon, it was not part of the expressed receptor protein). The 3x-HA-catfish GnRHR (cfGnRHR) was constructed by digesting the cfGnRHR cDNA in pcDNA3 with Xba I (blunted with Kenlow) and Bam HI, and placed into Eco RI (blunted)/Bam HI-cut pKH3. A schematic of the chimeric receptors is illustrated in **Figure 16**. All of the wild type (wt) and chimeric GnRH receptors were tagged with GFP at the C-terminus and an HA epitope at the N-terminus, except for the 3x-HA-GnRHR and 3x-HA-cfGnRHR which only had HA at the N-terminus.

Confocal Microscopy

CHO cells were grown on glass bottom microwell dishes and transfected with either GnRHR-LF-GFP, GnRHR-LT-GFP, or GnRHR-CF-GFP. After 48 hr, CHO cells were rinsed with PBS and labeled with 10 μg/ml of Alexa 594 ConA for 15 min at 4°C. Cells were washed twice with ice cold PBS and fixed for 15 min with 4% paraformaldehyde. Confocal microscopy was conducted using the 488 and 594 nm laser lines of a Zeiss LSM510 confocal laser-scanning microscope (CSLM) under a 63X oil objective.

Fluorescence Recovery After Photobleaching (FRAP) Measurements

CHO cells were grown on glass bottom microwell dishes and transfected with either the wt GnRHR-GFP, cfGnRHR-GFP, or GnRHR-LF-GFP for 48 hr. FRAP assays were performed at room temperature using a CLSM utilizing a protocol established by Tanimura et al. (Tanimura et al., 2003). First, cells were visualized using a 63X objective

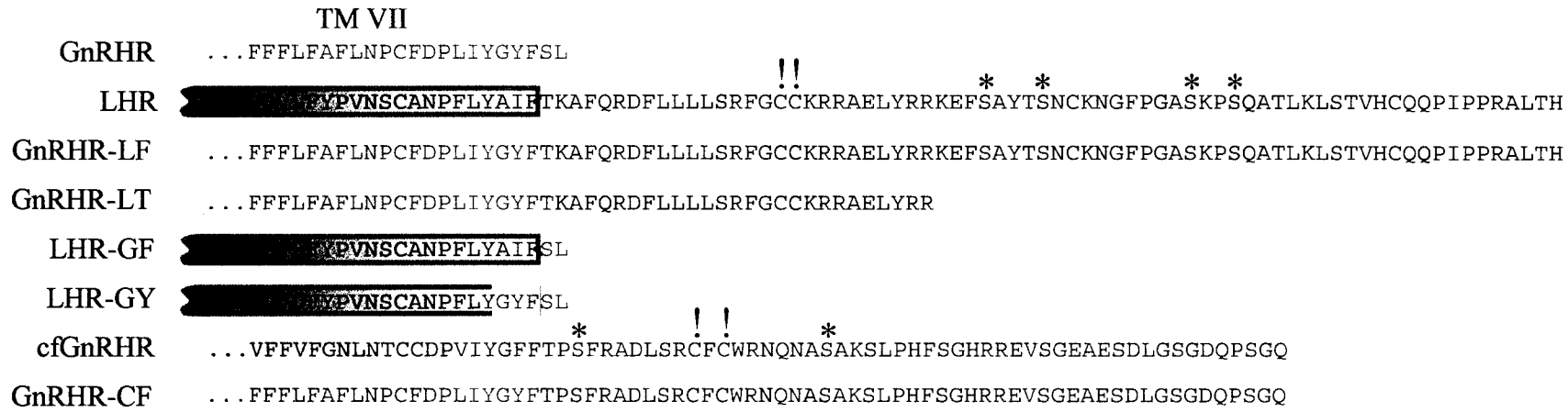


Figure 16. Schematic diagram of the wild-type and chimeric GnRHR and LHR fusion proteins. Chimeric GnRH receptors were constructed by the addition of either the full 70 amino acids of the LHR C-terminus (GnRHR-LF), a 27 amino acid truncated (t631) LHR C-terminus (GnRHR-LT) or the full 51 amino acid cfGnRHR C-terminus (GnRHR-CF). The LHR-GF and LHR-GY represent chimeras in which the LHR C-terminus is replaced with either the final 2 or final 5 amino acids from the murine GnRHR. (!) represent potential palmitoylation sites. (*) represent Ser phosphorylation sites.

and a 488 nm Argon laser line. Prior to photobleach, we take a whole cell scan at low laser power. Photobleaching is then carried out in a bleach region of interest (ROI) using 100% laser power, 100% transmission. Recovery is followed with low laser power scans taken every 2 sec for 100 seconds. All FRAP studies are completed within 15 min for a single dish to control for room temperature effects. Following data collection, fluorescence intensities are analyzed in the selected bleach ROI (Ib) and a reference ROI (Ir) at each time point. We selected reference regions of identical size and similar fluorescence intensities to our bleach region for each cell (n=10). Relative intensities (rI) at each time point are calculated by substracting bleach intensity from reference intensity (rI= Ib-Ir). Curves were plotted and a second-order polynomial was derived for each curve using Microsoft Excel. Each of the resulting quadratics was solved for recovery at 60 sec.

Lipid raft preparation and sucrose gradients

Monolayer cultures CHO cells (150 mm dish) were transfected for 48 hr with specified vectors. Cells were harvested in phosphate buffered saline (PBS) containing 5mM EDTA and centrifuged for 3 min at 300 x g. The cell pellet was resuspended in 375 µl of MES followed by the addition of 375 µl of a 2X TX-100 lysis buffer (0.2% TX-100 prepared in MES buffer). For the sample, the final lysis buffer concentration was 1X (TX-100 concentration = 0.1%) with a final volume was 750 µl. Cells were then homogenized by 3 passes through a 30 gauge needle. 750 µl of 90% sucrose (in MES buffer) was added to the samples to yield a 45% sucrose fraction in a final volume of 1.5 ml. A discontinuous sucrose gradient was then prepared by layering 1.5 ml of 35% and 5% sucrose in a 5 ml

ultracentrifuge tube. Isopycnic ultracentrifugation was then carried out at 46,000 rpm using a Beckman SW-55 rotor for 16-20 hr at 4°C. Following ultracentrifugation, 250 µl samples were collected representing a total of 18 fractions. Proteins that migrated to the interface of the 5% and 35% layers (approximately fractions 6 and 7) were considered to be raft-associated (Navratil et al., 2003; Song et al., 1996).

Western blots

Samples representing individual sucrose fractions were subjected to SDS polyacrylamide gel electrophoresis (acrylamide:bis-acrylamide ratio of 29:1) and electro-blotted to nitrocellulose (Osmonics, Westborough, MA). Membranes were blocked in 5% non-fat dried milk in Tris buffered saline (TBS). Anti-HA antibodies were used at a 1:1000 dilution with an incubation time of 1 hr. Blots were washed and then incubated with a 1:10,000 dilution of anti-mouse HRP for 1 hr at room temperature. All blots were washed for 60 min (6 x 10 min) with TBS after secondary antibody and then visualized by chemiluminescence using Pierce (Rockford, IL) SuperSignal reagents.

ERK Activation Assays

A monolayer of CHO cells (2×10^5) in 6-well tissue culture plates were transfected with the specified vectors. After 48 hr, cells were washed twice with PBS and incubated in serum-free DMEM for 6 hr. Then, either 0 or 100 nM GnRH was administered for a 15 min incubation. Cells were washed in ice cold PBS and lysed in RIPA buffer containing 20mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholate and 0.2 mM PMSF. 6X sample buffer (300 mM Tris-HCl, pH 6.8, 60%

glycerol, 30 mM DTT, 6% SDS) was added to yield a final concentration of 1X. Aliquots (15 µl) of each lysate were heated to 95°C for 5 min and subjected to SDS-PAGE and Western analysis. Nitrocellulose membranes were incubated for 2 hr with a phospho-ERK (1:1,000 dilution) followed by a 2 hr incubation with a 1:2,000 dilution of HRP conjugated secondary antibody. Phospho-ERK blots were then stripped at room temperature with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) heated to 50°C for 30 min. After stripping, membranes were washed twice for 15 min with TBS and blocked with 5% milk for 1 hr, then re-probed with a 1:10,000 dilution of an anti-ERK-1 antibody that recognizes ERK-1 and ERK-2 independent of phosphorylation state. Following washing in TBS, blots were incubated with a 1:2,000 dilution of anti-rabbit HRP and immunoreactive bands were visualized by chemiluminescence.

Internalization Assay

GnRHR internalization assays were performed 48 hr after transfection of CHO cells with either wt GnRHR-GFP, GnRHR-LF-GFP, or GnRHR-LT-GFP vectors in 12 well plates. Radioiodinated D-Ala^6-desGly^{10}-GnRH-Pro^9-ethylamine (D-Ala^6-GnRH) was radioiodinated using a glucose-oxidase procedure and purified by chromatography in QAE Sephadex as described by Wagner et al (Wagner et al., 1979). Briefly, cells are incubated with 72 fmol (2 x 10^5 cpm) of [^{125}I] D-Ala^6-GnRH in the presence or absence of 72 pmol of unlabeled D-Ala^6-GnRH. Cells were then incubated on ice for 4 hr and then warmed to 37°C for 0, 5, 10, 15, 30, 60, or 90 min. Internalization was stopped by immediate cooling to 0°C and rapidly washed with ice-cold PBS. Acid-sensitive

radioligand binding (cell-surface binding) was removed by addition of ice-cold acid solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) for 12 min. After removal of acid, cells are washed with ice-cold PBS and solubilized with 0.2 M NaOH and 1.0% SDS to measure acid- resistant (internalized) radioligand. Non-specific binding (binding in the presence of 72 pmol unlabeled D-Ala^6-GnRH) is subtracted at each time point. Surface binding is expressed as percentage of initial values and internalized radioligand is expressed as percentage of total cell-associated radioligand (internalized + cell surface) at each time point. Differences in the percentage of each receptor internalized at 0, 5, 10, 15, 30, 60, and 90 min after the addition of ligand were compared using least squares analysis of variance. Means between each receptor type at each specific time point were compared using Duncan's Multiple Range test, which was protected by a significant ($P < 0.05$) F-value for receptor type, time after addition of ligand, and the interaction between the two.

Hormone Binding Assays

Approximately 10 million CHO cells were transfected in 150 mm dishes with selected vectors. After 48 hr, cells were washed with PBS, centrifuged, and cell pellets were resuspended in assay buffer (10 mM Tris-HCl, .1% BSA, .01 mM $CaCl_2$) to a final concentration of 1 x 10^7 cells/50 µl. Triplicate 12 x 75 mm assay tubes were prepared containing 50 µl aliquots of cell suspension and 5 x 10^4 cpm of [^{125}I] D-Ala^6-GnRH (61.4 pM) in 50 µl assay buffer in the presence or absence of 50 µl of non-radioactive D-Ala^6-GnRH (340 nM) (1000 fold excess). The total volume for each tube was adjusted to 250 µl by addition of ice-cold assay buffer. Following a 4 hr incubation at 4°C, 3 ml of ice-

cold assay buffer was added and samples were immediately centrifuged for 15 min at 16,000 x g. The supernatents were decanted and radioactivity in the cell pellets was quantitated using an Apex automatic gamma counter (Micromedic Systems, Horsham, PA). Specific binding was determined by subtracting cpm in samples containing [^{125}I] D-Ala^6-GnRH in the presence of unlabeled D-Ala^6-GnRH from cpm in samples containing only [^{125}I] D-Ala^6-GnRH samples. The same procedure was followed for the LHR-GY and LHR-GF constructs only hCG was used as the [^{125}I]-radiolabeled and non-labeled ligand.

RESULTS

Chimeric GnRH receptors are trafficked to the plasma membrane, bind ligand and are capable of signaling to ERK

Appropriate trafficking and membrane localization of GnRHR-CF fusion proteins has been established (Heding et al., 1998; Heding et al., 2000; Lin et al., 1998). To determine if the LHR C-terminus disrupts membrane trafficking of the GnRHR, CHO cells were transiently transfected with either wt GnRHR-GFP, GnRHR-LF-GFP, GnRHR-LT-GFP or GnRHR-CF-GFP fusion proteins. At 48 hr post-transfection, cells were stained with Alexa 594 concanavalin A (con A), a red fluorescent derivative of Con A that binds to plasma membrane carbohydrates. Cells were then fixed and imaged utilizing the 488 and 594nm laser lines of a Zeiss LSM 510 confocal laser scanning microscope (CSLM). The Alexa 594-conjugated Con A clearly delineated the plasma membrane of CHO cells. A similar membrane distribution of green fluorescence was seen

for both the wt GnRHR-GFP, GnRHR-LF-GFP, GnRHR-LT-GFP, and the GnRHR-CF-GFP (**Figure 17**). Co-localization of the red and green fluorophores is revealed as yellow in the overlay image.

The CLSM analysis revealed membrane localization of the chimeric GnRH receptors. To determine if these receptors are capable of ligand binding, CHO cells were transiently transfected with wt. GnRHR-GFP, GnRHR-LF-GFP, or GnRHR-LT-GFP or the LHR chimerics with the GnRHR "C-terminus" LHR-GF and LHR-GY for 48 hr. Untransfected (UNT) CHO cells, which do not express GnRHR, were used as a negative control. Cells were incubated with [^{125}I]- D-Ala^{6}-GnRH in the presence or absence of cold D-Ala^{6}-GnRH for 4 hr at 4°C. This analysis revealed that the wt GnRHR-GFP, GnRHR-LF-GFP and GnRH-LT-GFP are all capable of binding [^{125}I] D-Ala 6 GnRH (**Figure 18A**). CHO cells transfected with wt LHR displayed binding of [^{125}I] hCG; however, neither the LHR-GF nor LHR-GY fusion proteins displayed any significant binding of radioactive ligand (**Figure 18B**). Thus, the extreme C-terminus of the GnRHR appears to be incapable of "rescuing" membrane trafficking of an LHR lacking the intracellular C-terminus. This is consistent with abrogation of membrane trafficking of LHR with simple truncation of the intracellular C-terminus (Rodriguez et al., 1992).

To confirm functional phenotype, we next assessed the ability of the chimeric GnRH receptors to activate MAP kinase in response to GnRH. CHO cells were transiently transfected with the wt GnRHR-GFP, GnRHR-LF-GFP, GnRHR-LT-GFP, or the GnRHR-CF-GFP for 48 hr. Untransfected CHO cells were used as a negative control. Transfected cells were serum starved for 6 hr and then incubated in the presence or absence of 100 nM GnRH for 15 min. Cells were lysed in RIPA buffer and subjected to

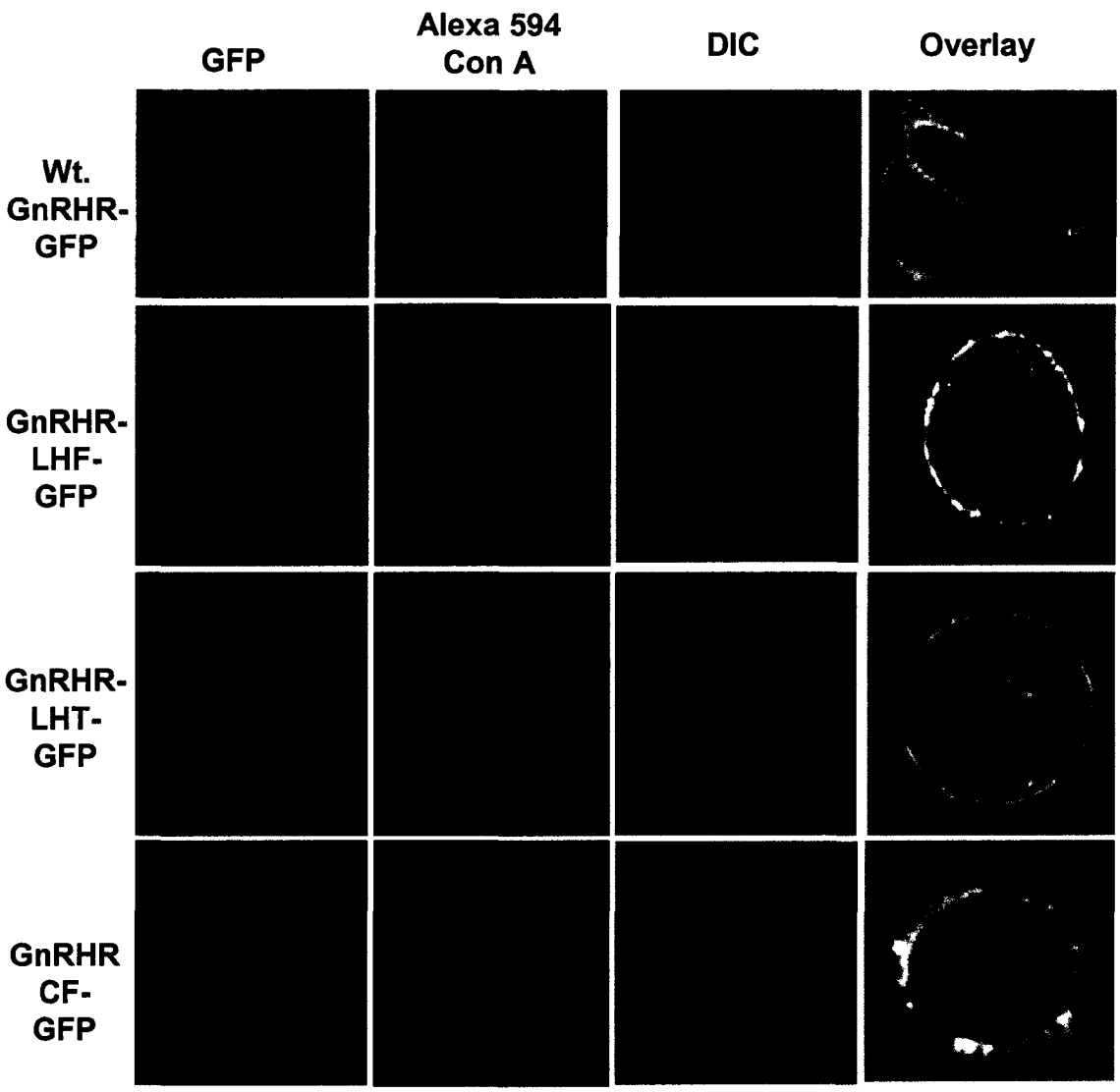


Figure 17. The GnRHR-LF, GnRHR-LT and GnRH-CF co-localize with a plasma membrane marker in CHO cells.
CHO cells expressing either a wt. GnRHR-GFP, GnRHR-LF-GFP, GnRHR-LT-GFP, or GnRHR-CF-GFP expression vectors were grown in glass bottom microwell dishes and stained with an Alexa 594-Conconavalin A (Con A) for 15 min at 4°C. Cells were then fixed with 4% paraformaldehde for 10 min. CLSM was conducted using the 63 X objective and the 488 argon laser line of a Zeiss LSM 510 confocal microscope.

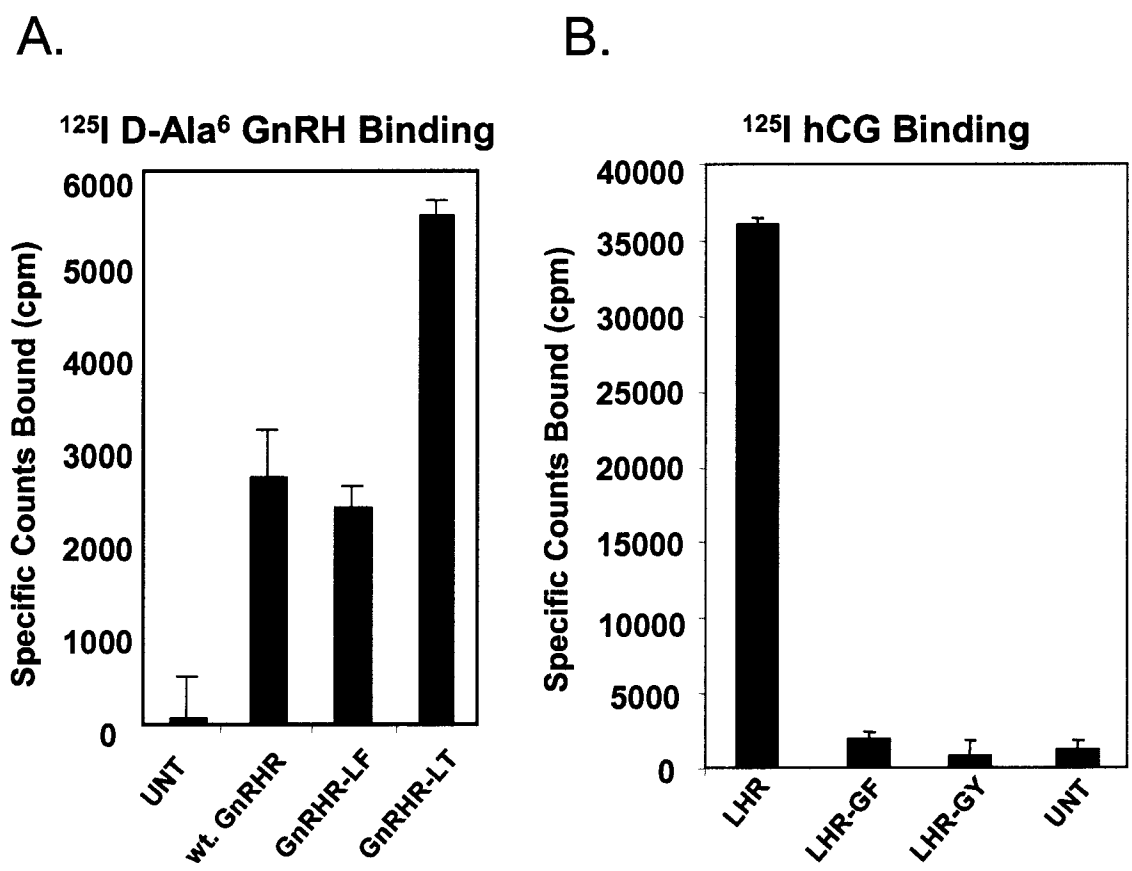


Figure 18. The intracellular C-terminus from the LHR does not affect the binding [^{125}I] D-Ala^6-GnRH to GnRHR fusion proteins.

A. CHO cells were transiently transfected with expression vectors for the wt. GnRHR-GFP, GnRHR-LF-GFP, or GnRHR-LT-GFP fusion proteins. Untransfected (UNT) CHO cells were used as a negative control. At 48 hr post-transfection, cells were harvested in ice cold PBS, centrifuged, and placed in assay tubes with [^{125}I] D-Ala^6-GnRH in the presence or absence of unlabeled D-Ala^6-GnRH (1 x 10^7 CHO cells/50 µl). All samples were done in triplicate. Binding of [^{125}I] D-Ala^6-GnRH (61.4 pM) was determined as counts bound in the absence of unlabeled D-Ala^6-GnRH subtracted from counts bound in the presence of unlabeled D- Ala^6-GnRH (340 nM). **B.** CHO cells were transiently transfected for 48 hr with LHR, LHR-GY or LHR-GF and binding was determined as in Panel A except that [^{125}I]-hCG and hCG served as the radioactive and non-radioactive ligands. Errors represent standard errors of the mean of at least 3 replicates.

western blot analysis probing for the dual phosphorylated forms of ERK (ERK 1 and 2). Consistent with our previous studies, 15 min exposure to GnRH increased ERK phosphorylation (p-ERK) in the wt. GnRHR {Navratil, Bliss, et al. 2003 5251 /id}{Ellsworth, White, et al. 2003 5231 /id}. A similar increase in p-ERK was evident in RIPA lysates prepared from CHO cells transfected with the GnRHR-LF-GFP, GnRHR-LT-GFP and the GnRHR-CF-GFP fusion proteins (**Fig 19A**).

The presence of an intracellular C-terminus increases the extent of GnRH receptor internalization

The relatively blunted internalization kinetics of the mammalian Type I GnRHR has been attributed to the absence of an intracellular C-terminus {McArdle, Davidson, et al. 1999 4990 /id}{McArdle, Franklin, et al. 2002 5164 /id}. Thus, the addition of heterologous C-terminal domains, including the cfGnRHR C-terminus, typically results in an increased rate and extent of receptor internalization {Heding, Vrecl, et al. 1998 3817 /id}. To test this in our system, CHO cells were transiently transfected for 48 hr with either the wt. GnRHR-GFP, GnRHR-LF-GFP, or the GnRHR-LT-GFP. Internalization of [^{125}I]D-Ala^{6}-GnRH was determined at 0, 5, 10, 15, 30, 60 and 90 min as previously described {Hashizume, Yang, et al. 2001 4989 /id}. GnRHR internalization reached a maximum of 32% at 90 min (**Fig. 19B**). At 60 and 90 min, the extent of internalization of the GnRHR harboring the full-length LHR C-terminus was significantly ($p<.05$) greater than the wt. Type I receptor (**Fig. 19B**). The presence of the truncated LHR C-terminus led to a further increase in the extent of internalization. It is interesting to note that the impact of the truncated LHR C-terminal domain exactly

recapitulates what has been previously reported for the wt. LHR. Specifically, the LHR C-terminal truncate displayed a higher rate and extent of internalization than the wt. receptor {Rodriguez, Xie, et al. 1992 2518 /id}.

The presence of an intracellular C-terminus differentially affects lipid raft distribution of the mammalian Type I GnRHR.

In contrast to the constitutive residence of the Type I GnRHR in lipid rafts (Navratil et al., 2003), the LHR is detectable in lipid rafts only after ligand activation (Roess and Smith, 2003). Upon confirming the ability of the tailed chimeric GnRH receptors to recapitulate key functional attributes including membrane trafficking, ligand binding, signaling and internalization, we next asked if localization of the GnRHR in lipid rafts might reflect the loss of structural determinants present in the intracellular C-terminal domains of more prototypical GPCR's. To address this issue, CHO cells were transfected with expression vectors for wt GnRHR, cfGnRHR, GnRHR-LF-GFP, GnRHR-LT-GFP, or GnRHR-CF-GFP. At 48 hr post-transfection, raft preparations were prepared using a detergent based method described earlier (Navratil et al., 2003). Raft fractions were separated using sucrose gradient centrifugation. As with our earlier studies, wt GnRHR was predominantly localized to low-density sucrose fractions (Navratil et al., 2003). In contrast, both the chimeric GnRHR-LF-GFP and GnRHR-LT-GFP were detectable only in high-density sucrose fractions (**Figure. 20**). Thus, the addition of the LHR intracellular C-terminus appears to fundamentally alter raft distribution of the murine GnRHR. Furthermore, this effect appears to be independent of C-terminal Ser phosphorylation sites. Interestingly, despite the presence of an extensive

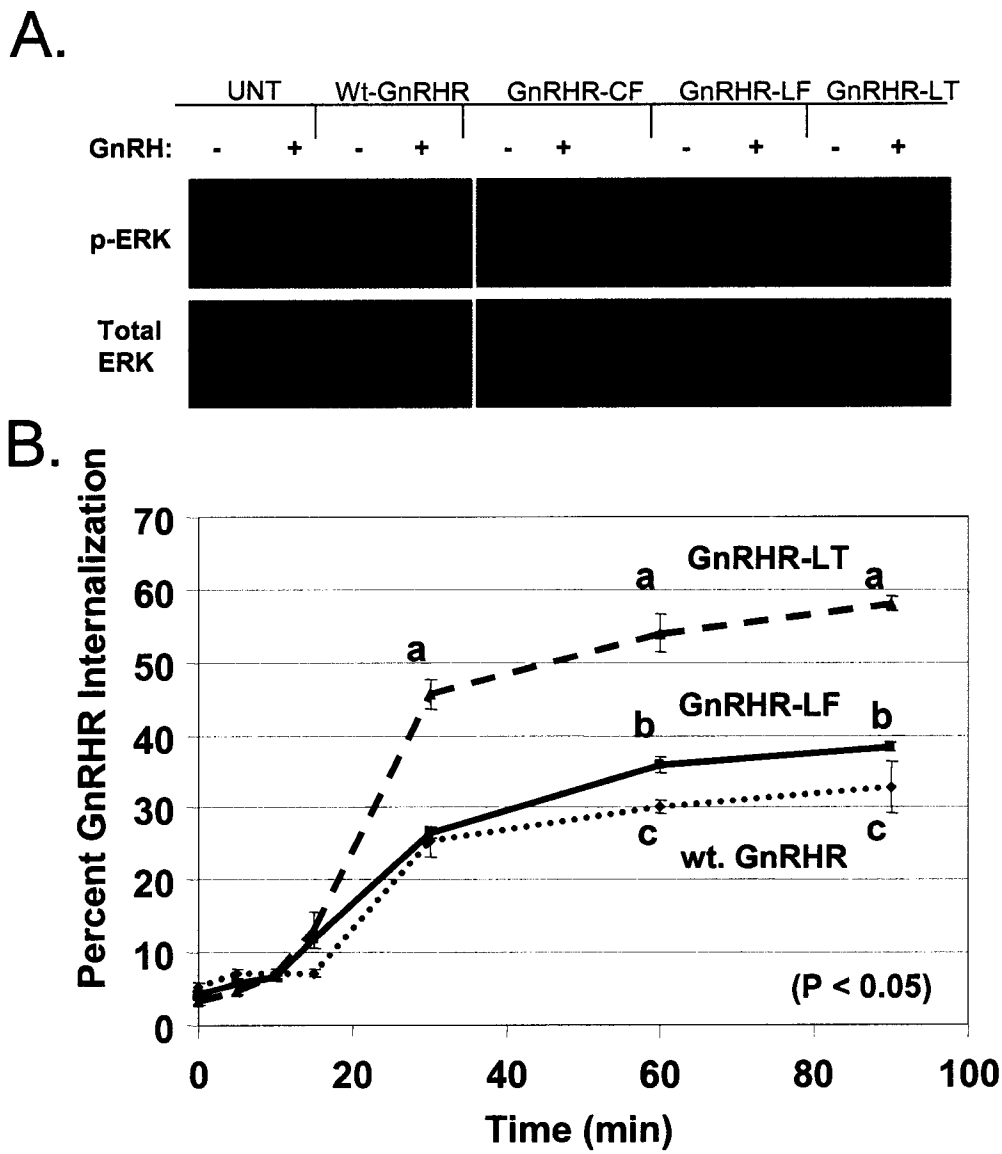


Figure 19. GnRH receptors harboring heterologous intracellular C-terminal domains are capable of signaling to ERK and display agonist induced internalization. A. CHO cells were transiently transfected with expression vectors for the wt. GnRHR-GFP, GnRHR-LF-GFP, GnRHR-LT-GFP, or GnRHR-CF-GFP fusion proteins. UNT CHO cells were used a negative control. At 48 hr post-transfection, cells were washed with PBS twice and incubated with serum free medium alone for 6 hr at 37°C. Cells were incubated with serum free medium containing 0 or 100 nM GnRH for 15 min. Samples were then lysed in RIPA buffer and lysates analyzed by Western blotting using antibodies specific for phosphorylated ERK. After probing with anti-phospho-ERK, blots were stripped and reprobed with an antibody that detects ERK 1 and 2 independent of phosphorylation. **B.** CHO cells were transiently transfected with expression vectors for the GnRHR-GFP, GnRHR-LF-GFP, or GnRHR-LT fusion proteins for 48 hr. Cells were then incubated with [125I] D-Ala6-GnRH in the presence or absence of unlabeled D-Ala6-GnRH for 4 hr at 4°C. Cells were warmed at 37°C for 0, 5, 10, 15, 30, 60, or 90 min. Surface binding is expressed as percentage of initial values and internalized radioligand is expressed as percentage of total cell-associated radioligand (internalized + cell surface) at each time point.

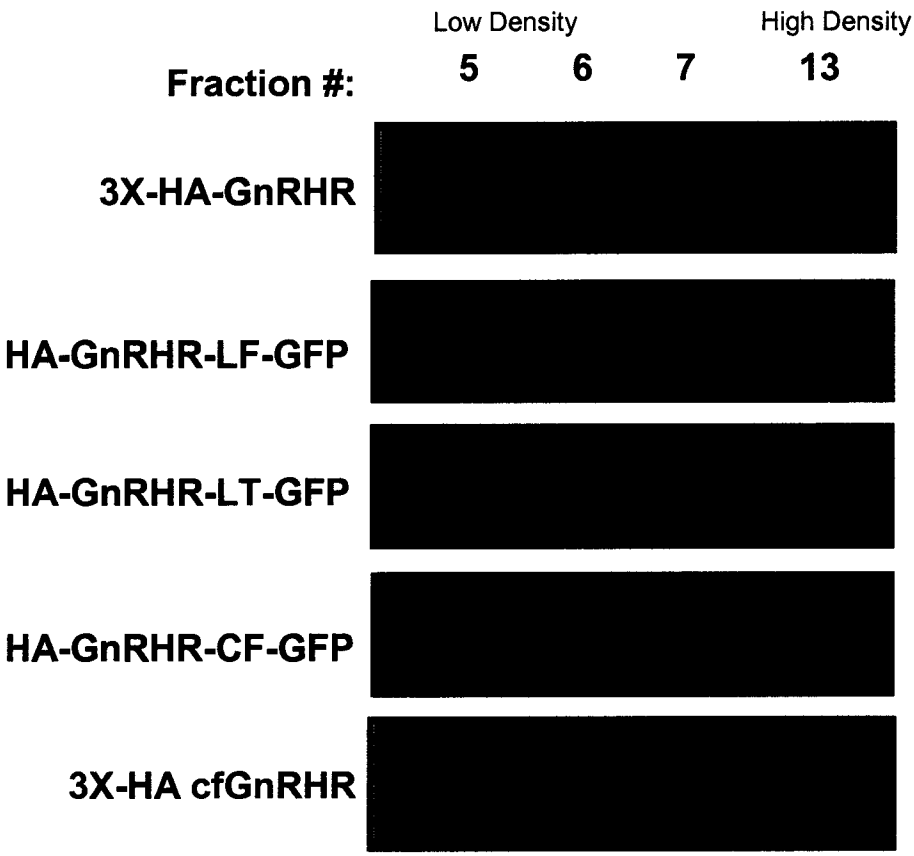
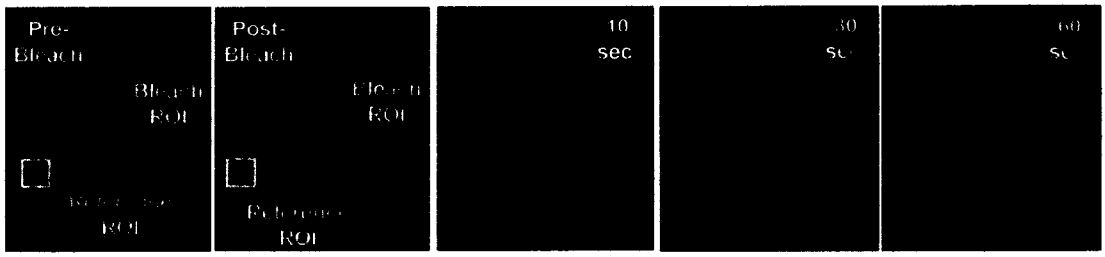


Figure 20. The intracellular C-termini from the LHR and cfGnRHR differentially affect raft localization of the murine GnRHR. CHO cells (150 mm dish) were transfected with expression vectors for the indicated fusion proteins for 48 hr. Raft samples were prepared using a TX-100 detergent buffer and separated by isopycnic ultracentrifugation through a non-linear sucrose gradient (45%, 35%, 5%). Fractions (250 µl) were collected from the top and separated by SDS-PAGE. Western blots were conducted using an antibody directed against an hemagglutinin (HA) epitope tag on the GnRHR, cfGnRHR, or GnRHR chimerics. Proteins that migrate to the 5%/35% interface (fractions 5-7) are considered raft associated.

intracellular C-terminus, the cfGnRHR, like its non-tailed mammalian counterpart, also localized to low-density sucrose fractions. Thus, raft trafficking may be a general feature of both mammalian (non-tailed) and non-mammalian (tailed) GnRH receptors. If correct, then in contrast to LHR, the C-terminal domain of the cfGnRHR would not, presumably, redirect raft trafficking of the mammalian Type I receptor. Consistent with this, the C-terminal domain from the cfGnRHR did not alter raft distribution of the mammalian Type I GnRHR (**Figure 20**).

Fluorescence Recovery after Photobleaching (FRAP) reveals differences in the lateral diffusion properties of tailed mammalian Type I GnRH receptors.

Protein-protein interactions and the surrounding lipid environment affect the lateral motion of integral membrane proteins (Kenworthy et al., 2004). We have found that the major fraction of unoccupied GnRH receptors in the plasma membrane are laterally mobile and display a relatively rapid rate of lateral movement (Nelson et al., 1999). Based on the ability of the LHR C-terminus to redirect the GnRHR to non-lipid raft domains, we reasoned that the in-membrane behavior of the GnRHR harboring the LHR C-terminus would display a different lateral diffusion phenotype. To address this, CHO cells were transfected with wt GnRHR-GFP, cf GnRHR-GFP, or GnRHR-LF-GFP expression vectors in glass bottom microwell dishes. FRAP analysis was carried out with CLSM (Tanimura et al., 2003). Consistent with our earlier work, we found that the mobile fraction of wt GnRH receptors is greater than 90% (**Figure 21**). The fraction of laterally mobile cfGnRH receptors in the plasma membrane was similar to the wt mammalian Type I receptor (**Figure 22**). In contrast to the percentage of laterally mobile



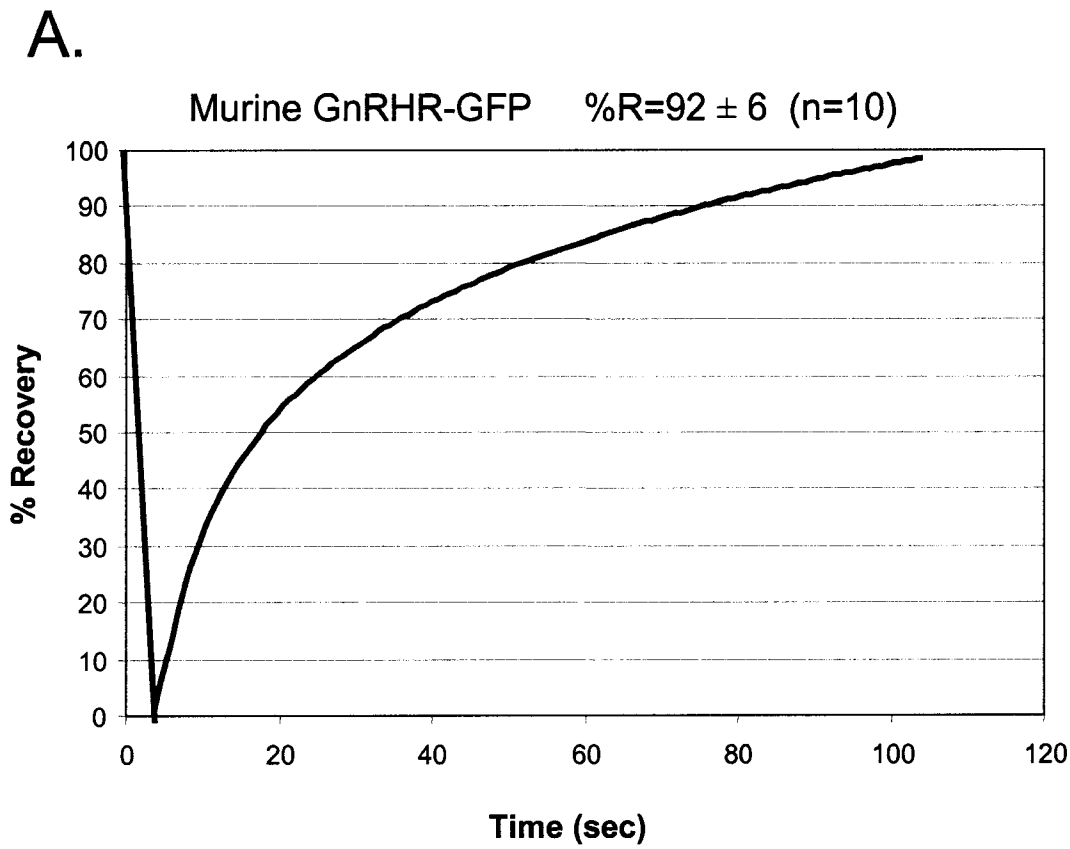
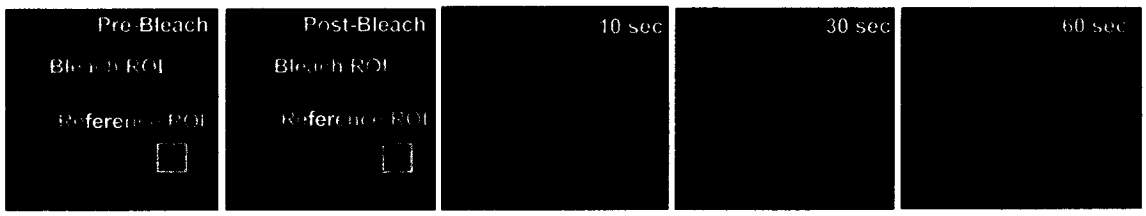


Figure 21. Fluorescence recovery after photobleaching (FRAP) reveals similar lateral diffusion characteristics of the mammalian GnRHR and cfGnRHR. CHO cells were transiently transfected with expression vector wt. GnRHR-GFP for 48 hr Cells were imaged utilizing a 63X objective and the 488 argon laser line of a Zeiss LSM510 CLSM. A bleach region of interest (ROI) was utilized in the FRAP measurements. The ROI was photobleached using 100 % power of the 488 line of an argon laser. Fluorescence recovery was then obtained in the ROI using low power laser scans at 2 sec intervals for 60 sec. Images and recovery curves from a representative experiments are shown. Fluorescence intensity and recovery was determined for both the bleach ROI and a reference ROI of the same size. The percent recovery (%R) was determined as described in *Materials and Methods.*



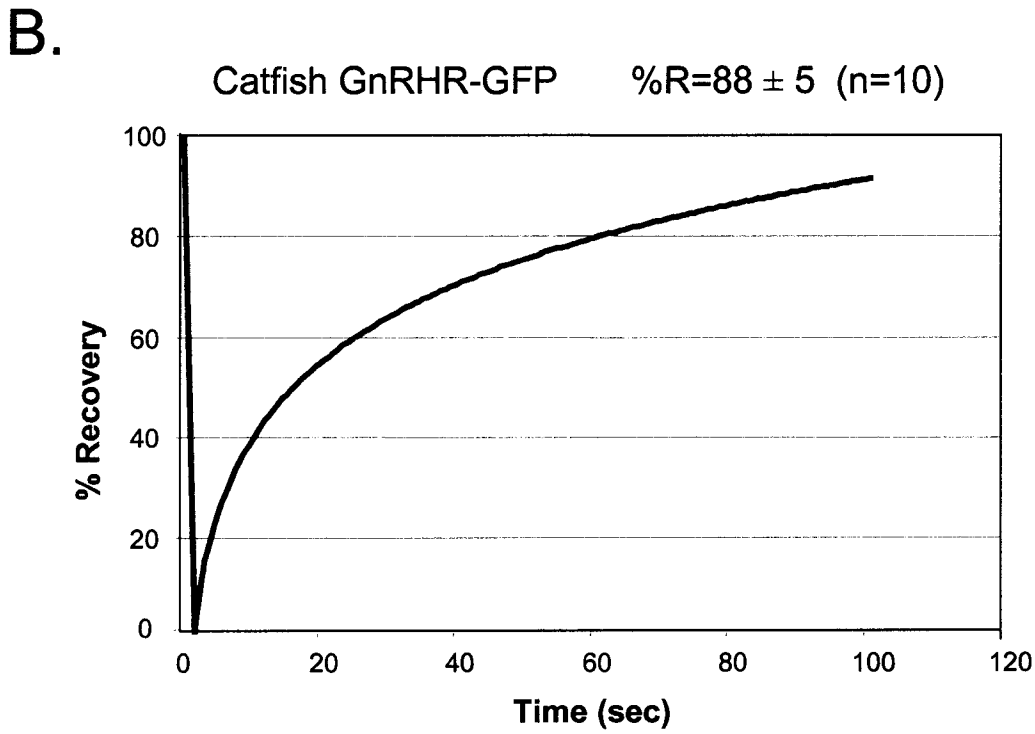
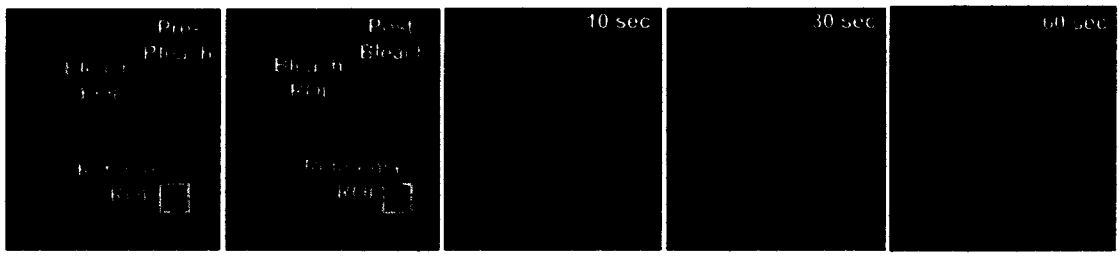


Figure 22. Fluorescence recovery after photobleaching (FRAP) reveals similar lateral diffusion characteristics of the mammalian GnRHR and cfGnRHR. CHO cells were transiently transfected with expression vector cfGnRHR-GFP for 48 hr Cells were imaged utilizing a 63X objective and the 488 argon laser line of a Zeiss LSM510 CLSM. A bleach region of interest (ROI) was utilized in the FRAP measurements. The ROI was photobleached using 100 % power of the 488 line of an argon laser. Fluorescence recovery was then obtained in the ROI using low power laser scans at 2 sec intervals for 60 sec. Images and recovery curves from a representative experiments are shown. Fluorescence intensity and recovery was determined for both the bleach ROI and a reference ROI of the same size. The percent recovery (%R) was determined as described in *Materials and Methods.*



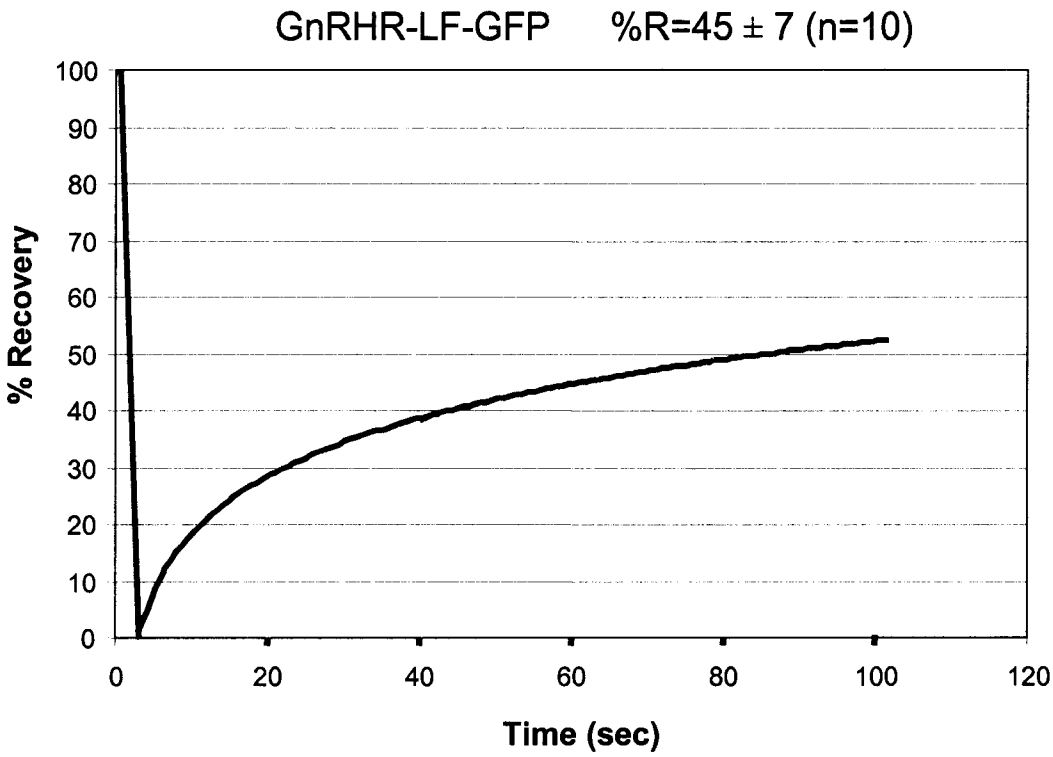


Figure 23. The intracellular C-terminus from the LHR alters the lateral diffusion phenotype of the mammalian GnRHR. CHO cells were transiently transfected with an expression vector for the GnRHR-LF-GFP fusion protein for 48 hr. FRAP analysis was conducted as described in **Fig 6.** Images and recovery curve from a representative experiment are shown. The percent recovery (%R) in the bleach ROI was determined as described in *Materials and Methods*.

GnRHR-LF-GFP fusion proteins was reduced (**Figure 23**). Thus, differential localization of the GnRHR, cfGnRHR and the chimeric GnRHR-LHR C-tail to lipid rafts is reflected as fundamental differences in the lateral diffusion properties of these molecules.

DISCUSSION

Over the past decade, the structural view of the plasma membrane has evolved from a continuous and random sea of lipids to a far more structurally dynamic organelle. Of particular interest are discrete membrane microdomains consisting of tightly packed sphingolipids and cholesterol – a unique lipid composition that leads to resistance to solubilization and a low-buoyant density in sucrose gradients as compared to the "bulk"plasma membrane (Brown and London, 2000; Simons and Ikonen, 1997). The latter accounts for the term lipid "raft" as a general descriptor of these membrane domains. Although our understanding of the molecular determinants that dictate inclusion or exclusion of proteins from lipid rafts is incomplete, it is evident that many raft-associated proteins are cell-surface receptors and components of intracellular signaling cascades (Becher and McIlhinney, 2005; Golub et al., 2004; Simons and Toomre, 2000; Zajchowski and Robbins, 2002). Consistent with this, we have found that the mammalian Type I GnRHR, an atypical member of the rhodopsin like family of GPCR's, is constitutively localized to low-density membrane microdomains rafts in both the gonadotrope-derived αT3-1 cell line and in heterologous CHO cells (Navratil et al., 2003). Thus, constitutive segregation into lipid rafts is independent of cell type and is intrinsic to the GnRHR itself. Given that the lack of an intracellular C-terminus is one of the most conspicuous features of the mammalian GnRHR (Sealfon et al., 1997;

Stojilkovic et al., 1994), we sought to determine if association with lipid rafts might reflect both a loss (C-terminal tail) and gain (raft association address) of structural characteristics.

In prototypical GPCRs, the intracellular C-terminal domain is quite extensive and contains phosphorylation sites for GPCR kinases (GRK), second-messenger regulated kinases such as protein kinase A (PKA) and casein kinases (Pierce et al., 2002). Based on these receptors, the paradigm that has developed suggests that phosphorylation of the C-terminus is requisite for subsequent interaction with β-arrestin, which hinders further G-protein activation and targets the deactivated receptor for internalization (Ferguson, 2001; Zhang et al., 1997). Consistent with the absence of an intracellular C-terminus, the mammalian GnRHR is not phosphorylated and internalization is not mediated via a β-arrestin dependent mechanism (Heding et al., 2000; Hislop et al., 2001; Willars et al., 2000). Thus, the "life-cycle" of the mammalian Type I GnRHR does not fit this paradigm.

Based on previous work, the construction of chimeric Type I GnRH receptors has offered significant insight into the role of C-terminal tails on signaling, trafficking and internalization (Lin et al., 1998); however, the potential impact of this structural motif on "in-membrane" localization has not been evaluated (Blomenrohr et al., 1999; Heding et al., 2000). To address this issue we fused either the full length or a truncated t631 C-terminus from the LHR onto the murine GnRHR. We were interested in the C-terminal domain from the LHR because, unlike the GnRHR, the LHR is excluded from lipid rafts in the absence of hormone (Roess and Smith, 2003). We also constructed a chimeric murine GnRHR that contained the intracellular C-terminal domain from the catfish

GnRHR. Although not directly demonstrated, the ability of cholesterol depletion to attenuate internalization has been used as prima facie evidence for lipid raft localization of non-mammalian GnRH receptors (Pawson et al., 2003a; Ronacher et al., 2004). Consistent with this interpretation, we find that the cfGnRHR, like its mammalian Type I counterpart, is evident in detergent resistant membrane fractions. Thus, we were positioned to evaluate the potential impact of intracellular C-terminal tails from GPCR's that either are (cfGnRHR) or are not (LHR) constitutively localized to lipid rafts. Clearly, however, the utility of this approach is dependent on the degree to which the chimeric receptors display key functional attributes. In short, the raft association phenotypes would be uninformative if the chimeric receptors are not trafficked to the plasma membrane. Importantly, our data suggests that, with the exception of the LHR harboring the C-terminus of the GnRHR, the chimeric receptors are trafficked to the plasma membrane, are capable of ligand binding and conveying an intracellular signal to the level of MAPK phosphorylation. Additionally, the internalization phenotype of the mammalian Type I GnRHR is altered depending on the identity of the heterologous intracellular C-terminus, such that the extent of internalization of GnRH receptors harboring the T631 truncated LHR C-terminus is greater than either the GnRHR-LF fusion protein or wt GnRHR. Enhanced internalization of the Type I GnRHR harboring the cfGnRHR C-terminal domain has been established (Heding et al., 1998).

We find that both the full-length and truncated forms of the intracellular C-terminal tail of the LHR redirects the GnRHR to non-raft domains. Interestingly, however, this is not simply the generic effect of adding a GPCR tail to the GnRHR. Specifically, when the intracellular C-terminus tail of the cfGnRHR is used to extend the

Type I GnRHR, this chimeric receptor, like the wt murine GnRHR, partitions into low-density fractions in sucrose gradients. Thus, the change in raft-localization conferred by LHR is, presumably, due to the presence of a specific structural feature within the C-terminal tail of LHR that overrides the intrinsic raft-localization of the GnRHR. At issue then is the key difference between the cfGnRHR and LHR C-termini that account for the differential impact of these domains on raft trafficking of the mammalian Type I GnRHR. At present, we cannot reach a definitive conclusion; however, several points can be made. First, the difference is probably not due to phosphorylation or palmitoylation, as the C-terminal tails of both LHR and cfGnRHR are likely to be similarly modified (Janovick et al., 2003; Pawson et al., 2003b; Zhu et al., 1995). In addition, removal of the 4 distal Ser phosphorylation sites did not alter the impact of the LHR C-terminus on trafficking of the GnRHR to non-raft microdomains. One intriguing difference between these C-terminal domains is the dependence on β-arrestin for internalization. While the LHR displays an arrestin dependent phenotype (Lazari et al., 1998), internalization of the cfGnRHR or GnRHR-CF appears to be arrestin independent (Hanyaloglu et al., 2001). Although historically thought of as mediating desensitization and internalization of GPCR's (Ferguson, 2001;Zhang et al., 1997), more global roles for β-arrestin family members including scaffolding and trafficking has emerged over the past several years (Morrison and Davis, 2003; Shenoy and Lefkowitz, 2003).

The lateral diffusion of proteins integrated into the plasma membrane is dependent on a number of factors including interactions with other proteins, interactions with the cortical cytoskeleton, the mode of membrane insertion and surrounding lipid environment (Kwik et al., 2003). Consistent with our earlier studies, we find that the

major fraction of unoccupied Type I GnRH receptors are laterally mobile in the plasma membrane and display a relatively rapid rate of lateral diffusion (Nelson et al., 1999). As such, it is interesting to note that the lateral diffusion characteristics of the cfGnRHR are equivalent to the Type I receptor. As both proteins appear to be trafficked to low-density raft domains then the in-membrane biophysical behavior of these receptors may reflect localization to a similar lipid microenvironment in the plasma membrane. In contrast, the addition of the LHR C-terminus not only redirects the Type I GnRHR to non-raft domains but also fundamentally alters the lateral motions of the GnRHR reflected as a significant reduction in the fraction of laterally mobile receptors on the cell surface. In fact, the lateral diffusion phenotype of the Type I GnRHR harboring the LHR C-terminus is similar to what has been previously reported for the wt LHR expressed as a GFP fusion protein (Horvat et al., 2001a). We should underscore that although limited lateral diffusion of membrane proteins has been used as a biophysical index of protein localization in confined plasma membrane microdomains (Jiao et al., 2005) this relationship is far from clear. For example, in contrast to the view that raft-associated proteins will display more constrained lateral movements in the plasma membrane, Kenworthy et al. (Kenworthy et al., 2004) found that raft proteins can display a high percent mobile fraction – in some cases equivalent to or greater than that determined for non-raft proteins. As such, it is likely problematic to infer a mode of biophysical behavior of a raft-associated protein based on biochemical characterization. Thus, in our view, the key observation is that differential sorting of the GnRHR, cfGnRHR and the chimeric GnRHR-LF to either low or high-density sucrose fractions is reflected as a distinct difference in the lateral diffusion properties of these molecules.

Given the role of fatty acyl modifications as a mechanism for raft association of a number of membrane proteins including GPCR's (Becher and McIlhinney, 2005), much of the work in this arena has focused on the intracellular C-terminal domain of GPCR's. Our data certainly support the notion that this domain can not only contribute to raft targeting but also override an intrinsic raft "address" in the non-tailed Type I GnRHR. It is, however, also clear that the role(s) of the GPCR C-terminal tails in raft trafficking is not simple. While the cfGnRHR, like the LHR, possesses an intracellular C-terminus, this receptor predominately localizes to low-density fractions. Similarly, the cfGnRHR C-terminus does not alter raft distribution of the Type I receptor. Thus, while the field has logically focused on C-terminal acylation as a mechanism for raft localization of GPCR's, it is important to underscore that other domains of these receptors are equally likely candidates for raft trafficking in both tailed and non-tailed receptors.

In summary, we find that lipid raft localization of the mammalian Type I GnRHR cannot simply be accounted for by the absence of an intracellular C-terminal domain. Thus, raft localization is likely not a "default" trafficking pathway resulting from the loss of an intracellular C-terminus. While the LHR C-terminus is capable of directing the GnRHR from raft to non-raft domains this is not the case for the intracellular C-terminus from the cfGnRHR. As such, it is tempting to speculate that lipid raft localization may be a generally conserved feature of both tailed and non-tailed GnRH receptors. As raft placement appears to be critical for GnRHR coupling to $G\alpha_q$ and, more recently, calmodulin in homologous cells (Navratil et al., 2003; Roberson et al., 2005) then elucidation of the motifs that lead to raft localization remains important to fully understanding the structural organization of GnRH receptors.

CHAPTER FIVE

CONCLUSIONS

The isolation of the first cDNA's encoding the mammalian GnRHR approximately 12 years ago ushered in new possibilities to explore our understanding of GnRH signaling in the anterior pituitary gland (Sealfon and Millar, 1995; Tsutsumi et al., 1992). As it had already been established that GnRH signals through $G_{\alpha q}$, the classification of the GnRHR as a GPCR was not necessarily a surprise; however, the GnRHR was structurally unusual. Perhaps most conspicuous was the absence of an intracellular C-terminus. Over the past decade, it has become apparent that the absence of this structural feature explains the unique desensitization and internalization phenotype of the mammalian Type I GnRHR (McArdle et al., 2002a).

In addition to the isolation of GnRHR cDNA's, several other developments were key to the progress in our understanding of the GnRHR. First, the establishment of an immortalized cell line of gonadotrope origin that expresses the endogenous GnRHR gene. These αT3-1 cells have proven critical as a homologous biological system to study the biochemical and biophysical behavior of the GnRHR (Windle et al., 1990). Second, the production of enhanced forms and color shifted variants of the intrinsically fluorescent protein termed GFP. Due to the absence of good anti-GnRHR antibodies, the

construction of GFP fusion proteins allowed, for the first time, real-time analysis of both bound and unbound forms of the GnRHR. Earlier work in our laboratory utilized this approach to characterize the lateral diffusion characteristics of the GnRHR and demonstrate that, while apparently not self-associated in the unbound state, GnRHR self-association occurs rapidly after the binding of agonist but not antagonist (Horvat et al., 2001b; Nelson et al., 1999). Thus, GnRHR self-association is a process that is unique to the agonist occupied receptor prompting the idea that GnRH binding to its receptor leads to the formation of a multi-protein complex or signaling platform at the plasma membrane. At the start of my doctoral work, a hypothesis was emerging that suggested that cholesterol/sphingolipid enriched domains in the plasma membrane termed lipid rafts may serve as key platforms for organizing cell-surface receptors and their cognate downstream signaling components (Simons and Toomre, 2000). My initial hypothesis was that lipid raft domains might contribute to membrane organization and signaling by the mammalian Type I GnRHR. The research I have presented is consistent with this hypothesis. Specifically, I have found that the GnRHR and key down stream signaling intermediates including c-raf kinase, $G_{\alpha q}$ and ERK are localized to lipid rafts. The placement of the GnRHR within these domains appears to be necessary for GnRH induced activation of phospholipase C, ERK and cFos gene expression. As such, cholesterol depletion and raft disruption appears to lesion the ability of GnRH to signal to the level of ERK phosphorylation. Based on the loss of IP_3 generation, this lesion likely resides upstream of PKC activation (Navratil et al., 2003). In summary, I suggest that the GnRHR exists as a constitutively raft associated GPCR and that this organization is critical for GnRH signaling to its key target sites in gonadotrope cells. Further studies

designed to explore the biochemical identity and dynamic properties of the GnRHR "raft proteome" would seem to be logical and important steps in furthering our understanding of the early events associated with GnRH signaling. Finally, since GnRHR localization to lipid rafts was equally evident in both αT3-1 cells and CHO cells, the trafficking of this protein appears to be independent of cell type and caveolin expression. Based on these observations, I suggest that localization of the GnRHR to lipid rafts is mediated by structural information that is intrinsic to the GnRHR itself. Thus, I next focused my efforts on trying to understand the structural motifs that might account for constitutive localization of the GnRHR to lipid rafts.

At this stage, it is important to point out that the concept of one GnRH-one GnRHR was rapidly changing. In fact, rather than one GnRH, multiple GnRH genes have been characterized. Depending on species, this may range from a single gene to as many as three genes (Neill, 2002b). Similarly, several mammalian species, including humans, have been shown to possess a second GnRHR termed the mammalian Type II GnRHR (Neill, 2002a). Interestingly, the presence of an intracellular C-terminus makes this receptor more structurally similar to the GnRHR from non-mammalian species such as chicken, xenopus and catfish. While a number of groups have shown that these "tailed" receptors display very different desensitization and internalization characteristics from the mammalian Type I GnRHR, essentially nothing was known as to their raft association phenotype (Pawson et al., 2003a). I was intrigued by the possibility that these receptors, unlike the mammalian Type I receptor, may display differential trafficking to lipid rafts - particularly as other C-tailed GPCR's, such as the LHR, are not raft associated in the unbound state (Roess and Smith, 2003). If correct, then I hypothesized

that raft association of the mammalian Type I GnRHR may, at least partially, reflect the loss of an important structural determinant. In this scenario then, trafficking of the GnRHR may be a default pathway reflecting the loss of an intracellular C-terminus.

The data I have generated would not appear to fully support this hypothesis. First, although tailed, the catfish GnRHR, like the mammalian Type I receptor, also resides in low-density domains. Thus, I was not surprised to find that fusion of the catfish GnRHR intracellular C-terminus to the mouse GnRHR did not alter raft localization of the mammalian receptor. In contrast, when I fused either the full length or a truncated form of the intracellular C-terminus from the non-raft associated LHR to the GnRHR, these chimeric receptors did not partition into lipid rafts. Based on these data, I concluded that while an intracellular C-terminus from a non-raft associated GPCR is capable of redirecting the GnRHR to non-raft compartments; this is not a generalized feature of GPCR C-terminal tails. Thus, constitutive raft localization of the GnRHR cannot simply be explained by the loss of an intracellular C-terminal domain. As such, the identity of the structural motifs that direct raft localization remains an important question in the biology of the mammalian Type I GnRHR. I am interested in the possibility that scaffolding proteins serving either as cellular chaperones or scaffolds may be key to raft placement of the GnRHR. If correct, then coupling mutagenesis studies with "knock-down" experiments directed at candidate proteins (e.g. flotillin) might represent a fruitful line of research.

It is clear that the lipid raft hypothesis is evolving. While the concept of membrane rafts is becoming generally accepted I fully recognize that there remains a great deal of controversy as to the physical properties and biochemical characteristics of

these domains. In particular, the tools and methods that have been applied to studying these domains, including sucrose density centrifugation, have intrinsic shortcomings and are relatively "blunt". Future research directed at understanding the membrane and lipid raft organization of the GnRHR must include increasingly refined and more precise detection methods including confocal microscopy to determine protein co-localization, FRET analyses to assess protein-protein interactions and careful application of biophysical approaches to characterize lateral diffusion and the possible inclusion of the GnRHR and associated proteins in transient confinement zones in the plasma membrane. Finally, while MβCD has been extensively applied as a raft disruption tool, it is clear that cholesterol depletion can have more global impacts on cell biology than simply disrupting lipid raft organization in the plasma membrane. Thus, it will be important that future studies involve more "surgical" approaches to disrupt raft association of the GnRHR. These may include blocking of raft associated docking sites such as PIP_2 enriched domains or, as we learn more about GnRHR protein partners, knock-down of raft scaffolding proteins. These approaches would ultimately yield more precise information as to the dynamic properties and identity of even transient GnRHR associated signaling complexes.

CHAPTER SIX

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