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Membrane Rafts and GnRH Receptor Signaling

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Abstract

The binding of hypothalamic gonadotropin-releasing hormone (GnRH) to the pituitary GnRH receptor (GnRHR) is essential for reproductive function by stimulating the synthesis and secretion of gonadotropic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Engagement of the GnRHR by GnRH initiates a complex series of signaling events that include the activation of various mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK). GnRHR signaling is thought to initiate within specialized microdomains in the plasma membrane termed membrane rafts. These microdomains are enriched in sphingolipid and cholesterol and are believed to be highly dynamic organizing centers for receptors and their cognate signaling molecules associated with the plasma membrane. Within this review we discuss the composition and role of membrane rafts in cell signaling and examine evidence that the mammalian type I GnRHR is constitutively and exclusively localized to these membrane microdomains in various experimental models. We conclude that membrane raft composition and organization potentially underlie the functional ability of GnRH to elicit the assembly of multi-protein signaling complexes necessary for downstream signaling to the ERK pathway that ultimately is critical for controlling fertility.

Keywords

GnRH receptor; GnRH; membrane rafts; ERK; signaling platform; gonadotrope

1. Introduction

As the central regulator of reproductive function, gonadotropin-releasing hormone (GnRH) is situated at the peak of the hypothalamic-pituitary-gonadal (HPG) axis. The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but is obligatory for synthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrope cells of the anterior pituitary gland. In the absence of pulsatile GnRH secretion to the anterior pituitary, proper reproductive function in mammals ceases [1–3]. Numerous genetic mutations exist that result in impaired hypothalamic production or function of GnRH and lead to hypogonadism and infertility in mammals [4–7]. In humans, one well-defined clinical example of hypogonadotropic-hypogonadism is Kallmann's syndrome where patients present with delayed or absent pubertal development. This syndrome arises as a failure of GnRH neurons to migrate from the olfactory placode to

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the hypothalamus correctly during embryogenesis resulting in decreased levels of GnRH and subsequent gonadotropins [8–10]. In addition to various naturally occurring mutations in humans, several experimental models also underscore the critical role of GnRH/GnRH receptor (GnRHR) signaling including mutation of the GnRHR gene [11,12], genetic ablation of gonadotropes [13], immunoneutralization of GnRH [14,15], and animal models of hypothalamic-pituitary disconnection [16,17]. Thus, fertility is not only dependent on proper GnRH production and release from the hypothalamus but also its subsequent binding to high affinity GnRHR on pituitary gonadotropes and the coordination of these events is of universal importance in controlling reproduction in mammals.

Studies of GnRH action have been greatly facilitated by the cloning of the GnRHR [18]. To date, three forms of GnRHR have been identified in vertebrates, GnRHR I, II, and III [19]. For the purposes of this review, we will focus on the mammalian type I GnRHR, which is the predominate form that is expressed in anterior pituitary gonadotropes. The type I GnRHR has been cloned from several species including murine, rat, human, sheep, pig, and horse [20]. The predicted amino acid structure is a 327 amino acid receptor containing 7 transmembrane domains and an extracellular ligand binding domain placing the GnRHR in the rhodopsin class of heptahelical G-protein coupled receptors (GPCR) but interestingly, the type I GnRHR is the only GPCR classified to date that lacks a carboxyl-terminal intracellular cytoplasmic domain. Unlike other GPCRs, the type I GnRHR C-terminal tail is only 1–2 amino acids [21]. In more prototypical GPCRs, like β -adrenergic receptors, this cytoplasmic domain is extensive and is targeted for phosphorylation by GPCR kinases (GRK) and second-messenger regulated kinases [22]. In many GPCRs, phosphorylation of the C-terminus appears to be required for subsequent interaction with β -arrestin, which hinders further G-protein activation and targets the deactivated receptor for internalization [23]. Experimental evidence suggests that due to the lack of an intracellular C-terminus, the mammalian GnRHR is not phosphorylated and internalization is not mediated via a β -arrestin-dependent mechanism. Indeed, the GnRHR has been described as a naturally occurring desensitization and internalization mutant [24]. Interestingly, the mammalian type I receptor does differ from GnRH receptors found in non-mammalian vertebrates such as catfish [25] and *Xenopus* [26]. In contrast to the mammalian type I GnRHR, the non-mammalian GnRHRs do possess prototypical C-terminal tails, are phosphorylated by GRK's and are characterized by more rapid internalization and desensitization kinetics [24,27].

More recent studies with the human GnRH receptor have found that, unlike its rodent counterpart, it is poorly trafficked to the plasma membrane. The majority of the human receptor appears to be intracellular and retained within the ER due to receptor misfolding [28]. Elegant work by Conn and colleagues has found that using the GnRH-mimetic indole IN3 rescues misfolded GnRH receptor from the ER. Spatial analysis of the human GnRH receptor in heterologous human cell lines further suggests that the receptor is not only associated with the ER but is also expressed and functional on the nuclear membrane [29].

Considerable efforts have also been devoted toward understanding the molecular basis of GnRH action on its cognate receptor in the anterior pituitary. It is well established that 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 (PIP₂) to produce two signaling intermediates, inositol 1,4,5, bisphosphate (IP₃) and diacylglycerol (DAG). IP₃ interaction with the IP₃ receptor induces an elevation of intracellular free Ca^{2+} via endoplasmic reticulum stores. DAG, along with Ca^{2+} in many instances, leads to an activation of one or more isoforms of protein kinase C (PKC), including α , ϵ , and ζ in gonadotrope cells [30,31]. These early events underlie GnRH activation of multiple mitogen activated protein kinase (MAPK) signaling cascades including p38 MAPK [32,33], c-Jun N-

terminal kinase (JNK) [34,35] and extracellular signal-regulated kinase (ERK) [36–39]. Due to space constraints, a comprehensive overview of the GnRH signaling network can be found in a recent review by Naor and colleagues [40]. For the purposes of this review, we will focus on GnRHR activation of the ERK cascade.

Regulation of the ERK signaling pathway can lead to the activation of numerous cellular targets involved in transcription, translation, and cytoskeletal remodeling [41]. Classically, activation of the ERK module proceeds through a three-tiered kinase cascade of MAPK kinase kinase (MAPKKK)-Raf-1, MAPK kinase (MAPKK)-MEK 1 and 2 and (MAPK)-ERK1 and ERK2 [41,42]. Work from our laboratory and others suggests that the phosphorylation of ERKs within the gonadotrope is dependent on PKC and influx of Ca^{2+} via L-type voltage gated calcium channels (VGCC) [35,38]. The latter observation is one of the initial clues that GnRHR engagement of ERK signaling in gonadotropes includes compartmentalization of signaling intermediates whereby the VGCC signal may indeed be spatially restricted within the cell. For example, it is well established that Ca^{2+} signaling is organized within discrete microdomains that allow for local Ca^{2+} concentrations to increase dramatically within the cytoplasm to regulate information processing within localized regions of numerous cell types [43]. Within the gonadotrope, our laboratory suggests that the compartmentalized influx of extracellular Ca^{2+} by VGCCs following GnRH binding involves the interaction of the Ca^{2+} binding protein calmodulin and Raf-1 and/or proline-rich-tyrosine kinase 2 (Pyk2) which appears to be essential for modulating ERK activation [44,45].

Following phosphorylation, ERKs are traditionally thought to translocate to the nucleus where these enzymes are involved in the rapid activation of a cohort of immediate early response genes in gonadotropes [46]. Several of these early response genes include c-fos, Egr-1, and ATF-3 [47–50], all of which had been linked to expression of several gonadotrope-specific genes such as FSH β , LH β , and the common α subunit to the glycoprotein hormones (α GSU) [36,50–52] as well as other genes such as secretogranin II [53] and dual-specificity phosphatases (DUSPs) [54]. These gonadotrope-specific genes not only define the differentiated character of the gonadotrope but also provide important clues regarding the dynamic nature of MAPK signaling through putative intracellular feedback loops mediated by DUSPs controlling the magnitude and duration of the MAPK response.

Recently work from our laboratory has examined the role of the ERK pathway in gonadotropes *in vivo* using an ERK conditional double knock out (DKO) mouse model. Interestingly, our pituitary-specific DKO mouse model reveals a sexually dimorphic requirement for ERK signaling in the maintenance of fertility [55]. Using this genetic ablation approach, female mice are infertile due to reduced levels of LH β leading to an anovulatory phenotype. Despite having modestly reduced levels of LH β , ERK DKO male mice remain fertile with normal gonadal function. This difference is likely due to the relatively small levels of LH that are needed for male fertility compared to the high levels of LH that are necessary to induce the preovulatory LH surge in females. Thus, the ERK DKO mouse's phenotype clearly illustrates the absolute requirement of ERK signaling in the maintenance of fertility within the complex reproductive cycle of female mice.

Consistent with our model of compartmentalized signaling within gonadotropes, the primary focus of this review will highlight the spatial organization of the type I mammalian GnRHR and downstream GnRHR signaling intermediates (Gaq, Raf-1 kinase, calmodulin, and ERK) localization within discrete compartments within the plasma membrane, termed membrane rafts [56]. The organization of the GnRHR in membrane rafts appears to be critical for the ability of GnRH to propagate a productive intracellular signal to the level of ERK activation. As such, we suggest that membrane rafts serve as the most proximate platform for the

initiation of GnRH/GnRHR-induced signaling characterized by the assembly of multi-protein signaling complexes that couple the GnRHR to the ERK pathway that ultimately is critical for controlling fertility *in vivo*.

2. Membrane Rafts

2.1 Membrane raft definition and composition

The original Singer-Nicolson model describes the plasma membrane as a freely diffusing fluid mosaic of lipids and proteins within a uniform bilayer [57]. Recent evidence expands this view of the plasma membrane to a more complex, heterogeneous assembly of spatially and functionally distinct domains that differ with respect to both lipid and protein composition [58,59]. This membrane raft hypothesis suggests that specific lipid species within a plasma membrane differ in their affinities for each other and for membrane proteins, and that these differences in affinity may facilitate the formation of biochemically and biophysically distinct lipid domains within the membrane [60].

Early descriptions of membrane rafts were based on the observation that glycosylphosphatidylinositol (GPI)-anchored proteins remained insoluble when cells were homogenized at low temperature in the presence of the non-ionic detergent Triton X-100, and were consistently recovered in association with low-bouyant density membrane fractions following sucrose density gradient centrifugation of cell homogenates [61]. Subsequent analysis of the composition of these detergent-resistant membranes (DRM's) showed that they were enriched in cholesterol and sphingolipids as well as numerous specific integral membrane proteins [62,63]. Due to the tight packing of the saturated acyl chains of the sphingolipids and the presence of cholesterol, membrane rafts are less fluid than the surrounding plasma membrane creating a liquid ordered (l_o) domain within a surrounding milieu of fluid phase, or liquid disordered (l_d) domains [64]. The tight packing of lipids within l_o domains creates a unique membrane environment for which integral and peripheral membrane proteins may show highly different affinities. Rafts thus provide a theoretical mechanism for regulation of protein-protein interactions at the level of the plasma membrane through the partitioning of individual proteins into, or out of, regions of l_o phase leading to the possibility that membrane rafts serve as signaling platforms for the recruitment of specific membrane proteins [63,65].

Consensus within the field defines membrane rafts as “small (10–200 nm) heterogeneous, highly dynamic, sterol and sphingolipid enriched domains that compartmentalize cellular processes” [66]. Additional observations suggest that while phase separation and formation of raft domains are likely to occur in cellular membranes, both their size and the time scales over which they exist are likely to be extremely small and short, respectively [67]. For example, membrane rafts can consist of highly transient molecular assemblies involving as few as 3 molecules with lifetimes of tens of milliseconds [68]. Although membrane rafts may generally be extremely small and short-lived, there is evidence that specific extracellular stimuli can lead to crosslinking or clustering of membrane proteins that induce the formation of large scale and more persistent l_o domains that in turn could act as platforms for the recruitment of specific proteins or the assembly of specific protein complexes [68]. This may be consistent with receptor clustering into (or out of) discrete rafts to facilitate post-receptor cell signaling.

2.2 Membrane raft markers

Unique subsets of membrane microdomains have been described based on specific morphological and biochemical characteristics. Caveolae are flask shaped invaginations of the plasma membrane that are similar in lipid composition to membrane rafts but are enriched in the marker protein caveolin, of which three isoforms exist, caveolin 1, 2 and 3

[69]. Caveolins are direct cholesterol-binding proteins that oligomerize to form an unusual hairpin structure within the plasma membrane and act as a scaffold to regulate signal transduction of numerous molecules including GPCR pathway components [70]. Caveolae are found in a majority of mammalian cells although interestingly, caveolae are absent in lymphocytes, neuronal cells and are not expressed in pituitary gonadotropes [56,71].

Flotillins (also known as reggies) are ubiquitously expressed proteins that are constitutively localized in membrane rafts in most cell types. Flotillins can interact with caveolin proteins within membrane raft domains; however flotillins are also expressed in cells that lack caveolin. The exact function of flotillins is unknown but several studies have implicated a role for flotillins in endocytosis, regulation of the actin cytoskeleton, and membrane trafficking [72]. Two isoforms of flotillins exist (flotillin-1 and flotillin-2) and both are expressed in anterior pituitary gonadotropes (Navratil, Bliss and Roberson, unpublished results). Minimally, flotillins can be used to define successful biochemical fractionation of membrane compartments and provide evidence for the association between this useful raft marker and signaling molecules partitioning into rafts [56].

Various techniques have been used for fluorescent labeling of membrane rafts *in vitro*. For example, fluorescently tagged cholera toxin B (CTB) subunit targets the ganglioside GM1 in living cells and can be used as a bona-fide marker for membrane microdomains since this lipid receptor is enriched in the extracellular leaflet of a subclass of membrane rafts [73]. Additionally, the C-terminal domain of perfringolysin O, domain 4 (D4), which is a cholesterol-binding cytolysin, has been shown to preferentially associate with cholesterol-enriched membrane domains including rafts. The D4-GFP fusion protein has been a potentially useful tool for real time imaging of membrane rafts in live cells [74]. The ability to visualize membrane rafts within live cells has helped to further define these structures as extremely dynamic microdomains.

2.3 Membrane raft targeting

Targeting of membrane raft-associated proteins into lipid microdomains is dependent on various structural determinants including cholesterol binding properties, transmembrane spanning segments, unique amino acid sequences, and lipid modification. Lipid modifications such as GPI-anchors, myristoylation, and palmitoylation appear to be the essential targeting motif in a number of raft-associated proteins [59,75]. In this scenario, the lipid modification may represent the anchor that directly interacts with membrane microdomains [75]. Also implicated in protein targeting to membrane rafts are the lengths of membrane spanning domains and the presence of specific extracellular glycosylations [76]. 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 [77]. Finally, for some transmembrane proteins, critical amino acid residues present in cytoplasmic domains function as molecular addresses. For GPCR's, this could represent motifs present in the 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 [78,79]. While it appears that multiple mechanisms account for segregation of membrane proteins into membrane microdomains, our understanding of this process in the context of the GnRHR is only beginning to be elucidated.

3. GnRHR in Membrane Rafts

3.1 GnRH receptor localization to membrane rafts

Previous work from our lab and others describes the mammalian (murine) type I GnRHR as a constitutive and exclusive resident of membrane rafts in whole cell homogenates of

various cell types including: dissociated murine pituicytes, gonadotrope-derived α T3-1 cells, and Chinese hamster ovary (CHO) cells stably expressing an epitope-tagged murine GnRHR [56,80]. These analyses were confirmed using both non-ionic detergent TX-100 and detergent-free whole cell homogenate preparations followed by sucrose density gradient centrifugation. The initial biochemical work has been strengthened with confocal imaging analysis demonstrating that the GnRHR associates with GM1 positive plasma membrane raft domains in the unoccupied state in gonadotrope α T3-1 cells [80]. Additionally, the non-mammalian catfish GnRH receptor, which contains an intracellular C-terminus, has also been shown to associate with membrane rafts following transfection in CHO cells. The targeting of the GnRHR, both mammalian and non-mammalian, to membrane rafts in multiple cell models suggests that the receptor may have an inherent preference for a l_o membrane environment [56]. This preferential localization is particularly intriguing as the mammalian GnRHR, while traditionally thought of as a product of gonadotropes, has been found in various peripheral tissues in humans, including the gonads, the placenta, and the central nervous system [81] as well in multiple reproductive neoplasias including, breast, prostate, and ovarian cancers [82]. The presumed segregation of GnRHR into membrane rafts in these peripheral and neoplastic tissues may help in the broader understanding of the physiological importance of the signaling behavior of the GnRHR outside of the pituitary.

It is notable that the constitutive and ligand-independent association of the murine GnRHR with low-density membrane rafts is atypical of GPCRs generally [83–85]. Most GPCRs show partial constitutive association with membrane rafts, and in many cases like the LH receptor [86], agonist treatment causes a significant change in the tendency of a receptor to partition into, or out of, a low-density membrane environment [70,87]. Whether the GnRHR has an intrinsic biophysical preference for a l_o membrane environment, or whether its behavior is dependent upon specific protein-protein interactions is not entirely clear at present; however, domain swap studies indicate that the absence of the C-terminal tail does not itself lead to constitutive raft association of the receptor [88]. For example, when a chimera receptor consisting of the murine GnRHR and the non-mammalian catfish GnRHR C-terminus was analyzed for membrane raft localization, the chimera receptor remained constitutively associated with membrane rafts. This data suggests that the unique presence of a short intracellular C-terminus on the murine GnRHR does not lead to a default association with membrane rafts. Interestingly, when the C-terminal domain of the non-raft associated LH receptor is added to the murine GnRHR, it is redirected to a non-membrane raft compartment. This suggests that the C-terminus of prototypical GPCRs can in fact contain targeting sequences that may contribute to membrane raft localization but is not a mechanism involved in the constitutive localization of the GnRHR to membrane microdomains because of the inherent lack of an intracellular C-terminus.

The definition of the structural features of the GnRHR that direct its association with lipid microdomains remains to be elucidated. Based on the membrane raft localization of both the mammalian and non-mammalian GnRHR, it is tempting to speculate that there is a conserved targeting feature in both of these GnRHRs. One possibility of a candidate raft trafficking motif within these GnRHRs could involve post-translational glycosylation. Evidence from the epidermal growth factor receptor suggests that N-linked glycosylations may be of importance for membrane raft targeting [76]. Additionally, it would be of particular interest to examine the possibility of a potential protein-protein interaction of the GnRHRs with the resident membrane raft proteins flotillins 1 and 2. Flotillin isoforms are highly expressed in gonadotropes and have the potential to serve as scaffolds in facilitating signal transduction and endocytotic events [89].

3.2 Compartmentalization of GnRH receptor signaling to membrane rafts

Biophysical analysis of the GnRHR within the plasma membrane suggests that agonist-induced receptor clustering may facilitate coupling of the receptor with the downstream intracellular signaling apparatus [90–92]. Consistent with this hypothesis, several key signaling intermediates known or presumed to play a role in the GnRHR-ERK pathway were also found to associate at least partially with low-density membranes including $G\alpha_{q/11}$, calmodulin, isoforms of the 14-3-3 family of adapter proteins, Raf-1, MEKs, and the ERKs themselves [56,93]. Previous work from our laboratory suggests that within five minutes of GnRH activation, a small population of phosphorylated ERK proteins associate directly with membrane raft microdomains. Additionally, immunoprecipitation studies of low density sucrose fractions following detergent resistant membrane extraction in α T3-1 cells reveal that ERK2 and the GnRHR may occupy the same membrane microenvironment. This observation was also confirmed in whole mouse pituitaries suggesting that the α T3-1 *in vitro* model has fidelity to the *in vivo* setting. We speculate that the ERK2 and GnRHR interaction in low density fractions is mediated by a scaffolding protein although a direct, transient association between ERKs and the GnRHR cannot be discounted. Our working hypothesis is that a GnRHR-raft associated “signaling platform” exists and this platform contains a scaffolding domain (potentially flotillin 1 and 2) that facilitates the association of the GnRHR and ERKs (and other downstream binding partners). This putative signaling platform provides a structural basis for cycling of ERKs to the membrane for activation within the MAPK cascade. Once phosphorylated, ERKs depart the membrane raft and translocate to the nucleus to elicit transcriptional regulation of various gonadotrope specific genes. At this time, the precise role of membrane raft associated ERKs beyond the role for ERKs within the nucleus is unknown but evidence suggests that ERKs can play a role in various extranuclear processes including cytoskeletal regulation, translational events, and membrane trafficking [94]. Collectively, these observations demonstrate the potential close proximity of the GnRHR and ERKs at the plasma membrane and raise the intriguing possibility that consistent with the compartmentalization of a VGCC calcium signal, GnRH-induced ERK activation in the gonadotrope represents a similarly compartmentalized event organized through association with plasma membrane rafts.

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 or depletion of cholesterol from plasma membranes leads to a loss of GnRH activation of the ERK cascade and expression of one of the downstream targets of ERK signaling, the *c-fos* gene. Importantly, this loss of signaling was not due to a loss of cell-surface binding and was reversible by cholesterol repletion and reconstitution of the membrane raft compartment. 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 cholesterol-depleted cells; this same paradigm had little effect on the efficacy of phorbol ester (PMA) - induced ERK phosphorylation. Thus, the ERK signaling cascade in cholesterol-depleted α T3-1 cells is sufficiently intact to transduce a PMA signal but not a GnRH signal. Analysis of inositol phosphate production indicated that cholesterol depletion uncouples the GnRHR-G-protein complex from PLC β . As such, the primary lesion in the GnRH response following membrane raft disruption likely lies upstream of PKC [56]. This is not entirely surprising because membrane rafts are enriched in phosphatidylinositol 4,5-bisphosphate (PIP₂), the substrate of PLC [95,96]. Compelling evidence suggests that plasma membrane GPCRs and PIP₂ are organized in a signaling microdomain that links the plasma membrane structure to the endoplasmic reticulum via generation of IP₃ that can trigger Ca²⁺ release from intracellular stores. These observations suggest that the ability of the GnRHR to signal appropriately to ERK is affected by the biophysical properties of the

plasma membrane (as represented by sterol content), and requires organization within l_o membrane rafts.

Despite the large body of published work focusing on the role of membrane rafts in facilitating signaling from a number of different receptor/ligand combinations in various cell types, many aspects of the membrane raft hypothesis require careful consideration, including how we interpret data regarding the biochemical characterization and composition of rafts, their transient nature and specific function, and methodologies for experimental perturbation of raft domains [97,98]. Much of this controversy appears to reflect the technical difficulty involved in either isolation of raft domains from the plasma membrane of living cells or direct visualization of rafts at physiological temperatures. Two widely used methods for assessing the localization and function of a protein within membrane rafts involve the use of cold non-ionic detergents for detergent resistant membrane extraction and the use of pharmacological cholesterol binding agents used for the disruption of membrane rafts lipid composition. While these techniques may provide useful information regarding the general preference of a protein for a specific membrane environment, they do not allow for temporal and spatial resolution or disclosing the time scales over which rafts may form and disperse in cells. Additionally, sucrose gradient centrifugation does not allow for distinguishing the *type* of membrane that is found within low density fractions from whole cell lysates. It is possible to detect endocytic membrane compartments as well as organelle membranes including nuclear, endoplasmic reticulum (ER), and mitochondria within low density sucrose fractions. In the case of the murine GnRHR, imaging analysis from our laboratory suggests that majority of the murine GnRH receptors appear to be localized to the plasma membrane of α T3-1 cells although we cannot discount the possibility that we are potentially detecting GnRH receptors from internalized endosomes (following agonist treatment) or those GnRH receptors that are retained within the ER.

In lieu of biochemical approaches, numerous sophisticated biophysical techniques have been developed in recent years for direct characterization of membrane rafts *in situ*. These include confocal microscopic imaging of cells treated with phase-sensitive membrane reporters, FRET analysis of protein or lipid juxtapositions within cellular or model membranes, single particle tracking, and proteomic studies [99–102]. These techniques have been instrumental in the development of the current view of membrane rafts as small and highly dynamic membrane heterogeneities that may nevertheless play important roles in signal transduction, especially through ligand-dependent clustering and stabilization.

4. Conclusions

Appropriate cell surface expression of the GnRHR by gonadotropes is obviously essential for GnRH action and the competence of the hypothalamic-pituitary-gonadal axis and mammalian fertility. Clearly, certain forms of hypogonadotropic-hypogonadism result from aberrant intracellular trafficking and lack of appropriate expression of the human GnRHR on the plasma membrane of pituitary gonadotropes [103,104]. This review suggests that murine GnRHR function may be further regulated at the level of the plasma membrane through its association with cholesterol-enriched membrane microdomains; a hypothesis that is consistent with our model of compartmentalized VGCC calcium signaling in gonadotropes as a required element of GnRH-induced ERK signaling. Towards this end, our experimental data suggests that the GnRHR in membrane rafts appears to be critical for the ability of GnRH to propagate a productive intracellular signal at the level of ERK activation and potentially more broadly at the level of PLC. We propose that membrane rafts serve as a proximate platform for the initiation of GnRH/GnRHR-induced signaling that is involved in the assembly of multi-protein signaling complexes that potentially couple the GnRHR to the

various MAPK pathways, including ERK, that ultimately are critical for controlling fertility *in vivo*.

Despite our data that highlights the localization of the GnRHR into membrane rafts; clearly important questions still remain in determining the full scope of the signaling proteins and intracellular signaling cascades that are dependent on these domains within the gonadotrope. Towards this end, on-going work in our laboratory is focused on clarifying the *in vivo* gonadotrope raft proteome using a variety of approaches including imaging and mass spectrometry analysis. The ultimate goal of these studies is to gain a deeper understanding of the organization and physiological relevance of the GnRHR localization to membrane raft compartments and its regulation of various MAPK signaling modules (ERK, JNK, p38) in the context of a living animal.

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