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The subcellular dynamics of GPCR signaling

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Abstract

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors and mediate the effects of a multitude of extracellular cues, such as hormones, neurotransmitters, odorants and light. Because of their involvement in numerous physiological and pathological processes and their accessibility, they are extensively exploited as pharmacological targets. Biochemical and structural biology investigations have clarified the molecular basis of GPCR signaling to a high level of detail. In spite of this, how GPCRs can efficiently and precisely translate extracellular signals into specific and well-orchestrated biological responses in the complexity of a living cell or organism remains insufficiently understood. To explain the high efficiency and specificity observed in GPCR signaling, it has been suggested that GPCR might signal in discrete nanodomains on the plasma membrane or even form stable complexes with G proteins and effectors. However, directly testing these hypotheses has proven a major challenge. Recent studies taking advantage of innovative optical methods such as fluorescence resonance energy transfer (FRET) and single-molecule microscopy have begun to dig into the organization of GPCR signaling in living cells on the spatial (nm) and temporal (ms) scales on which cell signaling events are taking place. The results of these studies are revealing a complex and highly dynamic picture, whereby GPCRs undergo transient interaction with their signaling partners, membrane lipids and the cytoskeleton to form short-lived signaling nanodomains both on the plasma membrane and at intracellular sites. Continuous exchanges among such nanodomains via later diffusion as well as via membrane trafficking might provide a highly sophisticated way of controlling the timing and location of GPCR signaling. Here, we will review the most recent advances in our understanding of the organization of GPCR signaling in living cells, with a particular focus on its dynamics.

Keywords

GPCR, signal compartmentalization, nanodomains, FRET, single-molecule microscopy

1. Introduction

During evolution from simple unicellular to complex multicellular organisms, cells have developed increasingly sophisticated strategies to sense the extracellular environment and communicate with each other. The large superfamily of G protein-coupled receptors (GPCRs) arguably represent the most successful result of this amazing evolutionary endeavor, which enabled our cells to sense and decode a large number of extracellular cues, encompassing light, odorants, hormones and neurotransmitters (Pierce et al., 2002; Lefkowitz, 2004). Given their accessibility, diversity and well-defined pharmacology, GPCRs have also served as major drug targets. As a result, at least one third of all drugs currently on the market target these receptors (Hauser et al., 2017). Moreover, there is large scope for further development since only a fraction of all potentially targetable GPCRs are currently exploited for pharmacological purposes.

Given the fundamental biological role and importance of GPCRs as drug targets, all major steps in GPCR signaling have been intensively investigated. By as early as the late 70's, pioneering work on the mechanisms of hormone action initiated by Earl Sutherland and Ted Rall in the late 50's and later taken over by Alfred G. Gilman and Martin Rodbell had already clarified that hormones like adrenaline and glucagon act via binding to a specific receptor located on the plasma membrane, triggering the activation of G proteins in a process that requires GTP, ultimately leading to the production of cyclic AMP (cAMP) by adenylyl cyclase – for a historical perspective see (Beavo and Brunton, 2002). In 1986, the sequence of the β_2-adrenergic receptor was elucidated by the group of Robert Lefkowitz, revealing an unexpected similarity with rhodopsin (Dixon et al., 1986). More recently, there has been enormous progress in the clarification of the structural basis of GPCR signaling, with the successful elucidation of the three dimensional structures of several GPCRs in different conformational states (Cherezov et al., 2007; Rasmussen et al., 2007) as well as in complex with both G proteins (Rasmussen et al., 2011; Koehl et al., 2018; Liang et al., 2017; Zhang et al., 2017) and arrestins (Kang et al., 2015; Zhou et al., 2017).

Despite these tremendous advances, we are just beginning to understand how GPCRs function and are regulated within the complexity of an intact cell or organism to produce specific effects. Indeed, recent data, mainly obtained with innovative microscopy approaches, indicate that GPCRs are far more complex and dynamic than previously thought, being able to signal at distinct signaling nanodomains both at the cell surface and on intracellular membranes. Understanding this complexity is going to be crucial to answer fundamental and still unresolved questions such as how GPCRs can produce specific effects and might pave the way to innovative pharmacological approaches.

2. Early evidence for signal compartmentalization

Whereas the highly successful model of GPCR signaling derived from the early biochemical studies described well the general mechanisms of GPCR signaling, it soon emerged that such a model was insufficient to fully explain the effects observed in intact cells and tissues – for a comprehensive review see (Beavo and Brunton, 2002; Steinberg and Brunton, 2001). For example, already in early studies in perfused hearts it was noted that whereas both epinephrine and prostaglandin E1 induce similar increases of cAMP and activate protein kinase A (PKA), only stimulation of adrenergic receptors with epinephrine was able to significantly activate glycogen phosphorylase, increase heart contractility and induce troponin I phosphorylation (Keely, 1979; Brunton et al., 1979). Subsequently it was found that the β-adrenergic agonist isoproterenol but not PGE1 increases the amount of cAMP and PKA activity in the particulate fraction of rabbit heart lysates, which mainly contain type-II isoforms of PKA (Hayes et al., 1980). These and similar findings obtained in isolated cardiomyocytes led Buxton and Brunton to hypothesize that β-adrenergic and PGE1 receptors might induce cAMP accumulation and PKA activation in distinct subcellular microdomains, leading to different biological effects (Buxton and Brunton, 1983).

In parallel, experiments suggested that adenylyl cyclases and other signaling proteins might not be randomly distributed on biological membranes. For instance, pioneering work by Tolkovsky and Levitzki provided indirect evidence that on turkey erythrocyte membranes adenosine receptors might be pre-coupled to adenylyl cyclases (note that the role of G proteins was not known at that time), whereas β-adrenergic receptors would activate adenylyl cyclases by random collision (Tolkovsky and Levitzki, 1978; Tolkovsky and Levitzki, 1978).

Several early studies concentrated on the role of lipid domains on the plasma membrane. These studies suggested that GPCR signaling might preferentially occur in lipid rafts and caveolae (Insel et al., 2005). Lipid rafts were initially identified biochemically as small (micrometer-sized) sphingolipid- and cholesterol-rich membrane domains that are resistant to detergent extraction at low temperature (Simons and Ikonen, 1997). A fraction of these domains contains also caveolins and, morphologically, corresponds to small invaginations of the plasma membrane, known as caveolae, which can be visualized by electron microscopy (Simons and Toomre, 2000). Based on biochemical evidence, several groups proposed that receptors, G proteins and adenylyl cyclases preferentially accumulate within lipid rafts and caveolae, suggesting a possible functional role for the resulting inhomogeneous distribution of GPCRs and their signaling partners on the plasma membrane (Insel et al., 2005). For instance, it has been proposed that, in cardiomyocytes, β_2-adrenergic receptors are preferentially localized in caveolae and T-tubules, which have a membrane composition similar to caveolae, whereas β_1-adrenergic receptors would be mainly excluded from these structures (Xiang

et al., 2002; Nikolaev et al., 2010). This has been suggested to play an important role in determining the specificity of signaling downstream of β_1- vs. β_2-adrenergic receptors, which have distinct biological effects on cardiomyocytes. In particular, the spatial proximity of β_2-adrenergic receptors, G_s proteins and adenylyl cyclases in caveolae has been suggested to be required for β_2-adrenergic receptors to produce physiological responses (Xiang et al., 2002; MacDougall et al., 2012; Wright et al., 2014).

3. New imaging approaches to address an old problem

Although the concept of signal compartmentalization is now widely accepted and supported by growing evidence, there has initially been considerable resistance to accept a non-random distribution of signaling molecules, possibly due to the popularity of the Singer and Nicolson's fluid mosaic model of the plasma membrane (Singer and Nicolson, 1972). Moreover, directly demonstrating the existence of signaling domains in living cells has proven challenging. A major problem was the lack of adequate tools to localize signaling events in living cells, as the classical biochemical and pharmacological methods typically used in these studies require cell disruption and have no spatial and very low temporal resolution. These limitations have been at least partially overcome by the introduction of innovative microscopy methods that allow scientists to directly visualize receptor signaling in living cells. A first major advance was represented by the introduction of reporters based on fluorescence resonance energy transfer (FRET) (Milligan and Bouvier, 2005; Lohse et al., 2012; Lefkimmiatis and Zaccolo, 2014; Calebiro and Maiellaro, 2014). These methods were crucial to provide direct evidence for the existence of signaling domains on the plasma membrane as well as inside cells (Calebiro et al., 2010; Irannejad et al., 2013; Irannejad et al., 2017; Godbole et al., 2017; Surdo et al., 2017; Castro et al., 2010; Maiellaro et al., 2016). Among other findings, this revealed that despite cAMP being a small water-soluble molecule, cAMP and PKA signals can be highly confined on the plasma membrane as well as at other intracellular compartments (Surdo et al., 2017; Castro et al., 2010; Maiellaro et al., 2016). This organization is likely particularly relevant in highly specialized cells such as cardiomyocytes and neurons, where neurotransmitter and hormone signals have to be rapidly converted into coordinated cellular responses such as synaptic plasticity or heart contraction.

More recently, the rapid development of innovative methods based on single-molecule microscopy has allowed probing the organization and dynamics of GPCR signaling nanodomains with unprecedented spatiotemporal resolution – for a detailed review see (Calebiro and Sungkaworn, 2017)). These methods hold great promise to directly study the dynamic organization of GPCR signaling on the spatial and temporal scales where GPCR signaling events are taking place.

4. The importance of the cytoskeleton

The cytoskeleton that is closely associated with the plasma membrane is often termed membrane skeleton. It consists of actin filaments, microtubules, and associated proteins. The membrane skeleton is somewhat different from the bulk cytoskeleton: it interacts with the plasma membrane and proteins that are located at the plasma membrane, playing a role in numerous cellular functions, such as endocytosis and exocytosis. Moreover, it provides anchors for the localization of transmembrane proteins. The membrane skeleton covers almost the entire cytoplasmic surface of the plasma membrane, and is intimately associated with clathrin-coated pits and caveolae (Morone et al., 2006). Based on results of early single-particle tracking (SPT) experiments with gold nanoparticles (Sako and Kusumi, 1994) and optical tweezers (Edidin et al., 1991; Sako and Kusumi, 1995), it was proposed that the membrane skeleton partitions the plasma membrane, influencing the diffusion of membrane-associated molecules (Jacobson et al., 1995; Kusumi et al., 2005). These and later measurements with fluorescently labelled proteins indicated that membrane molecules are temporarily confined into membrane compartments of 40–300 nm, consistent with the size of the membrane skeleton mesh (Murase et al., 2004). By observing membrane proteins and lipids embedded in the plasma membrane, it was found that they both undergo so called 'hop' diffusion, characterized by alternating phases of free diffusion and transient confinement (Suzuki et al., 2005; Fujiwara et al., 2002; Hiramoto-Yamaki et al., 2014). 'Hop' diffusion has been demonstrated for different types of membrane receptors, including GPCRs, which were investigated in a pioneering single-particle tracking study by the group of Akihiro Kusumi (Suzuki et al., 2005). In this study, tracking of μ-opiod receptors labelled with gold nanoparticles at the impressive temporal resolution of 25 μs revealed that these receptors jump between adjacent membrane compartments, in which they are temporally trapped. These findings led to the formulation of the 'fence-and-picket' model of the plasma membrane. According to this model, the membrane skeleton ('fences') and integral membrane proteins associated with it ('pickets') provide physical barriers to the diffusion of both membrane proteins and lipids, leading to compartmentalization of the plasma membrane in small nanodomains (Fujiwara et al., 2002; Kusumi et al., 2011). Importantly, the confinement of interacting molecules in such nanodomains has been suggested to increase the probability of their encounters, thus potentially increasing the rate of biochemical reactions (Saxton, 2002).

The cytoskeleton does not only provide barriers to receptor diffusion, but also provides anchor points for receptors and other membrane proteins, further contributing to the formation of signaling nanodomains and controlling their spatial arrangement on the plasma membrane. This has been probably best studied for ionotropic receptors at chemical synapses. It has been shown that

ionotropic glutamate receptors are constantly diffusing within the plasma membrane, which promotes the exchange of receptors between synaptic and extrasynaptic sites (Triller and Choquet, 2003). However, the local entrapment of receptors at postsynaptic densities is essential for synaptic function. This is achieved via a network of molecular interactions with the membrane skeleton and associated transmembrane proteins (Sheng and Sala, 2001; Garner et al., 2000). For instance, single-particle tracking in living cells and super-resolution imaging in fixed cells have revealed that the nanoscale localization and lateral mobility of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors can greatly influence synaptic transmission (Compans et al., 2016). In the case of AMPA receptors, the interactions with the cytoskeleton have been shown to be mediated by stargazin (also known as TARP γ2), an auxiliary subunit of the AMPA receptor, which, in turn, interacts with the postsynaptic density protein 95 (PSD95). PSD95 is a scaffold that plays a crucial role in the organization of post-synaptic densities. This involves interactions with the actin cytoskeleton mediated by binding of PDS95 to α-actinin (Matt et al., 2018). Since the interactions between AMPA receptor and stargazin are transient, this allows a dynamic exchange of AMPA receptors between synaptic and extrasynaptic sites (Bats et al., 2007). Moreover, it has been shown that glutamate stimulation decreases the stargazin-mediated immobilization of AMPA receptors at post-synaptic sites, allowing a faster replacement of desensitized receptors with new ones. Thus, this might provide a mechanism to fine tune synaptic sensitivity to repeated stimulation.

Although the mechanisms responsible for the organization of GPCRs on the plasma membrane are less understood than in the case of ionotropic receptors, there is evidence that also GPCRs might interact with the actin cytoskeleton. For example, single-molecule experiments in simple cell models have shown that $GABA_B$ receptors undergo dynamic interactions with the actin cytoskeleton, likely mediated by an as yet unknown scaffold, causing their preferential arrangement along actin fibers (Calebiro et al., 2013). As in the case of AMPA receptors, agonist stimulation with GABA was also found to weaken the interaction of $GABA_B$ receptors with the cytoskeleton, leading to an increase of their lateral mobility. Whereas the occurrence and functional relevance of this organization in neurons is presently unknown, it is tempting to speculate that it might be involved in controlling the precise localization and/or function of $GABA_B$ receptors at synapses (Calebiro et al., 2013). Another receptor that has been recently investigated in this respect is the somatostatin receptor type 2 (SSTR2), which had been shown to interact with the acting-binding scaffold filamin A (Peverelli et al., 2014). Recent single-molecule data by our group indicate that SSTR2 undergoes transient interactions with filamin A, which lead to a preferential localization of SSTR2 along actin fibers and participate in restraining SSTR2 diffusion on the plasma membrane. These interactions are increased

by agonist stimulation and are apparently required for efficient SSTR2 recruitment to clathrin-coated pits and internalization in response to agonist stimulation (Treppiedi et al., 2018).

5. Hot-spots for GPCR signaling on the plasma membrane

As mentioned above, despite several lines of evidence suggesting the possible existence of GPCR signaling nanodomains on the plasma membrane, their demonstration has proven extremely challenging. In fact, it has been only recently, with the further development of methods based on single-molecule and super-resolution microscopy that their direct visualization has become possible. These methods are not only able to resolve the organization of receptors and their signaling partners but can also localize downstream signaling events. For instance, in a recent elegant study, Mo et al. developed a new type of sensors – based on changes in the fluorescence fluctuation of the fluorescent protein TagRFP-T when in close proximity to another fluorescent protein called Dronpa – that allowed them to resolve PKA activity on the plasma membrane of living cells with a resolution up to three times better than the diffraction limit (Mo et al., 2017). This revealed the existence of PKA signaling nanodomains, which likely result from PKA clustering at the plasma membrane mediated via interaction with PKA anchoring proteins such as AKAP79.

More recently, our group succeeded for the first time in directly visualizing individual receptors and G proteins as they diffuse, interact and signal on the surface of intact cells (Sungkaworn et al., 2017). This study – which focused on α_{2A}-adrenergic receptor/G_i and β_2-adrenergic receptors/G_s as model receptor/G protein pairs – has led to a number of important observations. Notably, we could directly measure the duration of receptor–G protein interactions in intact cells, showing that they are transient and last approximately 1-2 seconds. Whereas we observed the occurrence of transient receptor–G protein interactions also in the absence of ligands, which were linked to the basal constitutive activity of the receptors, we did not observe preformed, stable receptor–G protein complexes, as proposed by some previous studies. However, differences might exist in the degree and stability of pre-association among different receptors and/or G proteins. For instance, using bioluminescence resonance energy transfer (BRET) and time-resolved fluorescence resonance energy transfer (FRET), the protease-activated receptor 1 (PAR1) was found in a previous study to pre-associate with G_i but not with G_{12} (Ayoub et al., 2010). Second, we found that agonists mainly act by increasing the association rate (k_{on}) between receptors and G proteins, as expected in the case of protein interactions that proceed through major conformational changes. Third, we discovered that receptor–G protein interactions and G protein activation do not occur randomly on the plasma membrane, but rather at dynamic nanodomains that we termed 'hot spots' (see Figure 1). Based on our recent results and simulations, we hypothesize that, by increasing the local effective

concentration of receptors and G proteins, these hot spots increase the speed and efficiency of their interactions, while allowing GPCR signals to occur locally. These data help to clarify one of the most fundamental and debated aspect of GPCR signaling, i.e. whether receptors are pre-coupled to G proteins or interact with them via random collisions. At least in principle, stable receptor complexes could permit fast and local signaling, but at the expense of signal amplification. In contrast, pure random coupling would favor signal amplification but would also bring low speed and efficiency. By establishing a sort of 'dynamic pre-coupling' – i.e. via allowing transient receptor–G protein interactions in the basal state, and keeping the involved receptors and G proteins near to each other thanks to the barriers provided by the cytoskeleton – nature seems to have found the ideal balance between signal amplification and speed. These and similar mechanisms might play a crucial role in determining the high efficiency and specificity observed among GPCRs and might provide a means of controlling GPCR signaling in space and time.

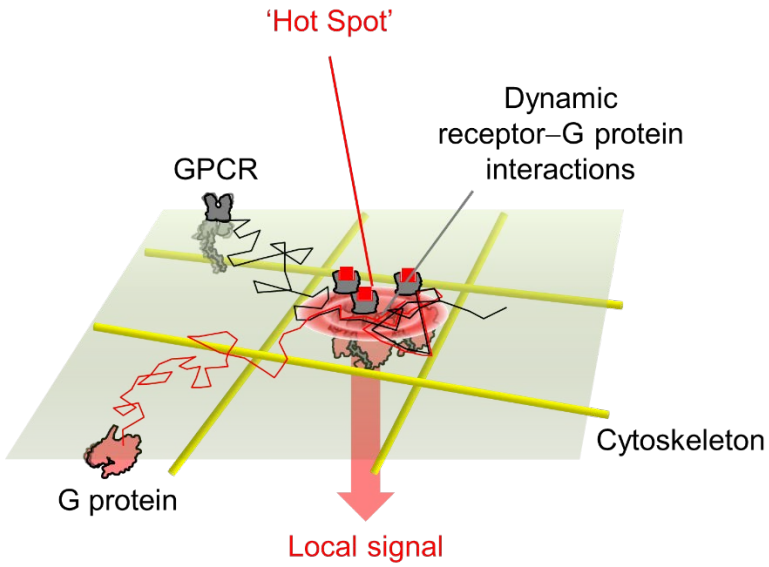

Figure 1. 'Hot spots' for GPCR signaling on the plasma membrane. Dynamic interactions between receptors, G proteins and barriers provided by the cytoskeleton lead to the formation of dynamic nanodomains on the plasma membrane that increase the efficiency of G protein activation, while allowing GPCRs to induce local signals.

6. Lipid nanodomains revisited

In spite of the biochemical evidence supporting the existence of lipid rafts, the size, dynamics and even existence of lipid domains on the plasma membrane has proven hard to demonstrate in living cells. This has sparkled an intense debate on the exact nature, role and functional relevance of lipid rafts (Munro, 2003; Eggeling et al., 2009; Eggeling, 2015). Traditionally, it was assumed that lipid rafts are rather stable plasma membrane compartments containing sphingolipids, cholesterol and a

unique set of resident (mainly GPI-anchored) proteins which float as 'rafts' on the plasma membrane and might provide specialized platforms for receptor signaling (Simons and Ikonen, 1997). This idea was mainly based on the fact that rafts could be isolated as detergent-resistant lipid patches and the observation of phase separation in model membranes (Eggeling, 2015). However, the most recent imaging studies that attempted to directly visualize lipid rafts in living cells failed to detect stable lipid domains on the plasma membrane (Eggeling et al., 2009).

Early attempts to directly visualize the spatial arrangement of fluorescently labelled GPI-anchored proteins by fluorescence microscopy in living cells showed fairly homogenous membrane staining, suggesting that either lipid rafts did not exist *in vivo* or were smaller than the lateral resolution of conventional fluorescence microscopy, which is about 200 nm (Mayor and Maxfield, 1995). The latter hypothesis was supported by FRET measurements suggesting that GPI-anchored proteins may associate in clusters smaller than 70 nm (Varma and Mayor, 1998). A subsequent study by the same group further narrowed down the estimated size of such clusters to less than 5 nm and a maximum of 4 GPI-anchored proteins per cluster (Sharma et al., 2004).

As a complementary approach, several groups have measured the diffusion of fluorescently labelled lipids and GPI-anchored proteins by either single-particle tracking or fluorescence correlation spectroscopy (FCS) in an attempt to understand their spatiotemporal organization at the plasma membrane. The results have shown that both GPI-anchored proteins and lipids undergo transient confinement in small nanodomains on the plasma membrane (Fujiwara et al., 2002; Lenne et al., 2006). However, multiple factors could contribute to such behavior, complicating the interpretation of the results. A first important factor is the cytoskeleton, which, as mentioned above, has been shown to provide barriers to both protein and lipid diffusion in the plasma membrane. However, there is some evidence that the confinement of membrane lipids and GPI-anchored proteins is not always dependent on the presence of an intact cytoskeleton and could be altered by manipulating the lipid composition of the plasma membrane, supporting a role for lipid–protein interactions in the transient confinement of GPI-anchored proteins (Lenne et al., 2006).

To more precisely measure the residency time of fluorescent lipids in membrane nanodomains, the groups of Stefan Hell and Christian Eggeling have developed novel approaches based on stimulated emission depletion (STED) microscopy, which enable them to control and narrow down the size of the excited volume in FCS measurements (Eggeling et al., 2009; Honigmann et al., 2014). This allowed them to investigate the diffusion of membrane molecules on different spatial scales. Overall, their results indicate that sphingolipids exhibit transient arrests as they diffuse on the plasma membrane, which are most likely caused by transient interactions with immobile or slow-diffusing membrane proteins. These trapping events last for approximately 10 ms and occur in areas

that are smaller than the resolution of the employed method, which is about 20 nm. These interaction sites are stable over a few seconds, during which they do not seem to diffuse within the plasma membrane. This behavior seems to be specific for sphingolipids, and to a much lesser extent phosphoethanolamine, with no correlation with the preference of the investigated lipid analogs for liquid-ordered membrane environments. Whereas these observations further support the occurrence of short-lived lipid–protein complexes, they seem to rule out the classical model of lipid rafts as stable and ordered lipid patches floating within the plasma membrane.

Another point to consider is that whereas lipid phase separation might affect protein dynamics on the plasma membrane, protein–protein interactions, such as those leading to clustering of GPI-anchored proteins during their activation, might also favor the formation of larger and possibly more stable lipid–protein complexes or nanodomains (Kusumi et al., 2012).

Overall, these recent data point to a much more dynamic picture than previously imagined, whereby membrane proteins and lipids undergo transient interactions that might still be relevant for the spatiotemporal organization of receptor signaling, but which do not necessarily lead to the formation of stable lipid–protein domains.

7. GPCR signaling at intracellular domains

Whereas signaling by GPCRs has long been thought to be restricted to the plasma membrane, a growing body of evidence indicates that GPCRs can also signal on intracellular membranes. The use of advanced optical methods such as FRET and conformation-sensitive biosensors was not only instrumental for these recent discoveries but also allowed scientists to identify the intracellular compartments where GPCR signaling is taking place. A first study by our group on endogenous thyroid stimulating hormone (TSH) receptors in thyroid cells revealed that these prototypical GPCRs for glycoprotein hormones remain active after internalization, leading to persistent cAMP signaling (Calebiro et al., 2009). Similar results were obtained by the group of Jean-Pierre Vilardaga studying the parathyroid hormone (PTH) receptor (Ferrandon et al., 2009). These early studies provided the first demonstration that GPCRs can continue signaling via cAMP at intracellular sites after internalization. Moreover, they suggested that TSH and PTH receptors were probably signaling in distinct compartments, i.e. a perinuclear compartment associated with the Golgi complex and early endosomes, respectively. However, it was only with the introduction of biosensors based on conformation-sensitive nanobodies that it was possible to directly visualize the subcellular sites of receptor and G protein activation in living cells. Using this elegant approach, the group of Mark von Zastrow was able to show that β_2-adrenergic receptors remain active in early endosomes, where they induce local G_s protein activation (Irannejad et al., 2013). More recently, our group further

investigated the nature and dynamics of the intracellular compartment where TSH receptors are signaling. For this purpose, we used a combination of FRET sensors measuring cAMP levels and PKA activity, which we tethered to different subcellular compartments, together with the previously developed conformation-sensitive biosensor for G_s protein activation (Godbole et al., 2017). The results of this study demonstrated that upon TSH stimulation, the TSH receptor and its ligand traffic retrogradely to the trans-Golgi network, where they induce local G_s protein activation, cAMP production and PKA activation. Importantly, this leads to a delayed phase of cAMP/PKA signaling at the Golgi/trans-Golgi network, which is required for TSH to efficiently induce phosphorylation of the cAMP response element-binding protein (CREB) and transcription of early genes (Godbole et al., 2017). Interestingly, signaling within the Golgi complex has also been recently demonstrated for the β_1-adrenergic receptor, even though in this case it has been proposed that it is the ligand (adrenalin) to reach the receptors, which are already located on membranes of the Golgi complex, via facilitated transport across cellular membranes (Irannejad et al., 2017). In the meantime, studies form several groups have demonstrated signaling at intracellular sites for a number of receptors (Kotowski et al., 2011; Feinstein et al., 2013; Kuna et al., 2013; Merriam et al., 2013; Ismail et al., 2016; Lyga et al., 2016), suggesting that this might be a rather common feature among GPCRs. Moreover, there is some evidence that GPCRs might also signal on the nuclear envelope (Tadevosyan et al., 2012) as well as in mitochondria (Hebert-Chatelain et al., 2016; Suofu et al., 2017).

These novel and exciting findings point to a previously unsuspected level of complexity in GPCR signaling. The fact that individual GPCRs can induce local signals in distinct subcellular compartments likely plays a major role in determining their biological effects. This provides a new basis to explain the high diversity found within the GPCR superfamily, in spite of the fact that all these receptors converge on just a few common signaling pathways. At the same time, they reveal an extremely dynamic picture. Indeed, data on both TSH (Godbole et al., 2017) and β_2-adrenergic receptors (Irannejad et al., 2013) indicate that GPCR signaling is highly controlled in space and time during receptor internalization and intracellular trafficking, apparently occurring in short 'bursts' once receptors enter well-defined membrane sub-domains of early endosomes or the trans-Golgi network. This high degree of integration between intracellular trafficking and signaling likely provides a key mechanism to fine tune GPCR signaling, which, once understood in its complexity, might also offer novel opportunities to modulate GPCRs for therapeutic purposes.

8. Concluding remarks

Altogether, the new findings obtained with advanced optical methods in living cells are deeply changing our views on the spatiotemporal organization of GPCR signaling cascades. Above all, they

have revealed a highly complex and dynamic picture, whereby GPCRs can rapidly form transient signaling nanodomains on the plasma membrane as well as at intracellular sites (Figure 2). We are only beginning to characterize these nanodomains, investigate which factors lead to their formation and understand their impact on GPCR signaling. As it is often the case, the development of innovative methods with increased power and resolution, has been instrumental for these discoveries. The current rapid progress in the fields of single-molecule and super-resolution microscopy combined with a growing awareness of the need for large multidisciplinary efforts to tackle biological complexity is likely to offer novel, exciting opportunities in the near future to further investigate the mechanisms and relevance of the spatiotemporal dynamics found in GPCR signaling.

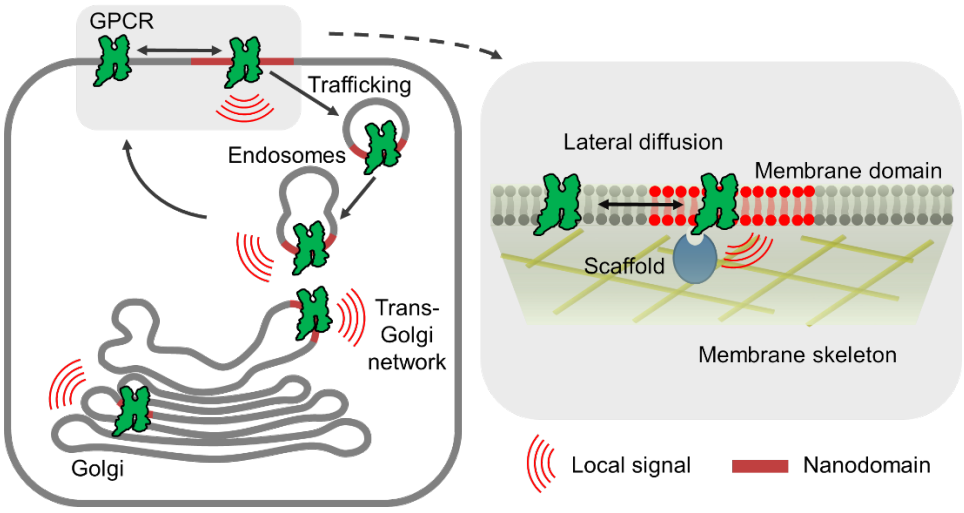

Figure 2. Dynamic nanodomains for GPCR signaling. Recent studies have revealed a highly complex and dynamic picture, whereby GPCRs can signal at dynamic nanodomains located both on the plasma membrane and on membranes of intracellular compartments such as early endosomes, the trans-Golgi network or the Golgi complex. Dynamic interactions of receptors and other signaling proteins with membrane lipids, the cytoskeleton and protein scaffolds likely play important role in the formation of these nanodomains. Lateral diffusion and trafficking control the localization of GPCRs within the nanodomains, which might provide a means to modulate the timing and location of GPCR signaling.

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