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Calebiro, Davide; Koszegi, Zsombor

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## **The subcellular dynamics of GPCR signaling**

Daive Calebiro<sup>1,2\*</sup> and Zsombor Koszegi<sup>1,2</sup>

<sup>1</sup>Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK

<sup>2</sup>Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, UK

\*Corresponding author:

Prof. Davide Calebiro MD PhD DSc

University of Birmingham

Institute of Metabolism and Systems Research

College of Medical and Dental Sciences

Edgbaston

B15 2TT Birmingham

United Kingdom

Email: [D.Calebiro@bham.ac.uk](mailto:D.Calebiro@bham.ac.uk)

## **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 lateral 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 **1. Introduction**

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

12 Given the fundamental biological role and importance of GPCRs as drug targets, all major steps in  
13 GPCR signaling have been intensively investigated. By as early as the late 70's, pioneering work on  
14 the mechanisms of hormone action initiated by Earl Sutherland and Ted Rall in the late 50's and later  
15 taken over by Alfred G. Gilman and Martin Rodbell had already clarified that hormones like  
16 adrenaline and glucagon act via binding to a specific receptor located on the plasma membrane,  
17 triggering the activation of G proteins in a process that requires GTP, ultimately leading to the  
18 production of cyclic AMP (cAMP) by adenylyl cyclase – for a historical perspective see (Beavo and  
19 Brunton, 2002). In 1986, the sequence of the  $\beta_2$ -adrenergic receptor was elucidated by the group of  
20 Robert Lefkowitz, revealing an unexpected similarity with rhodopsin (Dixon et al., 1986). More  
21 recently, there has been enormous progress in the clarification of the structural basis of GPCR  
22 signaling, with the successful elucidation of the three dimensional structures of several GPCRs in  
23 different conformational states (Cherezov et al., 2007; Rasmussen et al., 2007) as well as in complex  
24 with both G proteins (Rasmussen et al., 2011; Koehl et al., 2018; Liang et al., 2017; Zhang et al.,  
25 2017) and arrestins (Kang et al., 2015; Zhou et al., 2017).

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

34

## 35 **2. Early evidence for signal compartmentalization**

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

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

56 Several early studies concentrated on the role of lipid domains on the plasma membrane. These  
57 studies suggested that GPCR signaling might preferentially occur in lipid rafts and caveolae (Insel et  
58 al., 2005). Lipid rafts were initially identified biochemically as small (micrometer-sized) sphingolipid-  
59 and cholesterol-rich membrane domains that are resistant to detergent extraction at low  
60 temperature (Simons and Ikonen, 1997). A fraction of these domains contains also caveolins and,  
61 morphologically, corresponds to small invaginations of the plasma membrane, known as caveolae,  
62 which can be visualized by electron microscopy (Simons and Toomre, 2000). Based on biochemical  
63 evidence, several groups proposed that receptors, G proteins and adenylyl cyclases preferentially  
64 accumulate within lipid rafts and caveolae, suggesting a possible functional role for the resulting  
65 inhomogeneous distribution of GPCRs and their signaling partners on the plasma membrane (Insel et  
66 al., 2005). For instance, it has been proposed that, in cardiomyocytes,  $\beta_2$ -adrenergic receptors are  
67 preferentially localized in caveolae and T-tubules, which have a membrane composition similar to  
68 caveolae, whereas  $\beta_1$ -adrenergic receptors would be mainly excluded from these structures (Xiang

69 et al., 2002; Nikolaev et al., 2010). This has been suggested to play an important role in determining  
70 the specificity of signaling downstream of  $\beta_1$ - vs.  $\beta_2$ -adrenergic receptors, which have distinct  
71 biological effects on cardiomyocytes. In particular, the spatial proximity of  $\beta_2$ -adrenergic receptors,  
72  $G_s$  proteins and adenylyl cyclases in caveolae has been suggested to be required for  $\beta_2$ -adrenergic  
73 receptors to produce physiological responses (Xiang et al., 2002; MacDougall et al., 2012; Wright et  
74 al., 2014).

75

### 76 **3. New imaging approaches to address an old problem**

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

98 More recently, the rapid development of innovative methods based on single-molecule microscopy  
99 has allowed probing the organization and dynamics of GPCR signaling nanodomains with  
100 unprecedented spatiotemporal resolution – for a detailed review see (Calebiro and Sungkaworn,  
101 2017)). These methods hold great promise to directly study the dynamic organization of GPCR  
102 signaling on the spatial and temporal scales where GPCR signaling events are taking place.

103

104 **4. The importance of the cytoskeleton**

105 The cytoskeleton that is closely associated with the plasma membrane is often termed membrane  
106 skeleton. It consists of actin filaments, microtubules, and associated proteins. The membrane  
107 skeleton is somewhat different from the bulk cytoskeleton: it interacts with the plasma membrane  
108 and proteins that are located at the plasma membrane, playing a role in numerous cellular functions,  
109 such as endocytosis and exocytosis. Moreover, it provides anchors for the localization of  
110 transmembrane proteins. The membrane skeleton covers almost the entire cytoplasmic surface of  
111 the plasma membrane, and is intimately associated with clathrin-coated pits and caveolae (Morone  
112 et al., 2006). Based on results of early single-particle tracking (SPT) experiments with gold  
113 nanoparticles (Sako and Kusumi, 1994) and optical tweezers (Edidin et al., 1991; Sako and Kusumi,  
114 1995), it was proposed that the membrane skeleton partitions the plasma membrane, influencing  
115 the diffusion of membrane-associated molecules (Jacobson et al., 1995; Kusumi et al., 2005). These  
116 and later measurements with fluorescently labelled proteins indicated that membrane molecules  
117 are temporarily confined into membrane compartments of 40–300 nm, consistent with the size of  
118 the membrane skeleton mesh (Murase et al., 2004). By observing membrane proteins and lipids  
119 embedded in the plasma membrane, it was found that they both undergo so called ‘hop’ diffusion,  
120 characterized by alternating phases of free diffusion and transient confinement (Suzuki et al., 2005;  
121 Fujiwara et al., 2002; Hiramoto-Yamaki et al., 2014). ‘Hop’ diffusion has been demonstrated for  
122 different types of membrane receptors, including GPCRs, which were investigated in a pioneering  
123 single-particle tracking study by the group of Akihiro Kusumi (Suzuki et al., 2005). In this study,  
124 tracking of  $\mu$ -opioid receptors labelled with gold nanoparticles at the impressive temporal resolution  
125 of 25  $\mu$ s revealed that these receptors jump between adjacent membrane compartments, in which  
126 they are temporally trapped. These findings led to the formulation of the ‘fence-and-picket’ model  
127 of the plasma membrane. According to this model, the membrane skeleton (‘fences’) and integral  
128 membrane proteins associated with it (‘pickets’) provide physical barriers to the diffusion of both  
129 membrane proteins and lipids, leading to compartmentalization of the plasma membrane in small  
130 nanodomains (Fujiwara et al., 2002; Kusumi et al., 2011). Importantly, the confinement of  
131 interacting molecules in such nanodomains has been suggested to increase the probability of their  
132 encounters, thus potentially increasing the rate of biochemical reactions (Saxton, 2002).

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

137 ionotropic glutamate receptors are constantly diffusing within the plasma membrane, which  
138 promotes the exchange of receptors between synaptic and extrasynaptic sites (Triller and Choquet,  
139 2003). However, the local entrapment of receptors at postsynaptic densities is essential for synaptic  
140 function. This is achieved via a network of molecular interactions with the membrane skeleton and  
141 associated transmembrane proteins (Sheng and Sala, 2001; Garner et al., 2000). For instance, single-  
142 particle tracking in living cells and super-resolution imaging in fixed cells have revealed that the  
143 nanoscale localization and lateral mobility of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid  
144 (AMPA) receptors can greatly influence synaptic transmission (Compans et al., 2016). In the case of  
145 AMPA receptors, the interactions with the cytoskeleton have been shown to be mediated by  
146 stargazin (also known as TARP  $\gamma$ 2), an auxiliary subunit of the AMPA receptor, which, in turn,  
147 interacts with the postsynaptic density protein 95 (PSD95). PSD95 is a scaffold that plays a crucial  
148 role in the organization of post-synaptic densities. This involves interactions with the actin  
149 cytoskeleton mediated by binding of PSD95 to  $\alpha$ -actinin (Matt et al., 2018). Since the interactions  
150 between AMPA receptor and stargazin are transient, this allows a dynamic exchange of AMPA  
151 receptors between synaptic and extrasynaptic sites (Bats et al., 2007). Moreover, it has been shown  
152 that glutamate stimulation decreases the stargazin-mediated immobilization of AMPA receptors at  
153 post-synaptic sites, allowing a faster replacement of desensitized receptors with new ones. Thus,  
154 this might provide a mechanism to fine tune synaptic sensitivity to repeated stimulation.

155 Although the mechanisms responsible for the organization of GPCRs on the plasma membrane are  
156 less understood than in the case of ionotropic receptors, there is evidence that also GPCRs might  
157 interact with the actin cytoskeleton. For example, single-molecule experiments in simple cell models  
158 have shown that GABA<sub>b</sub> receptors undergo dynamic interactions with the actin cytoskeleton, likely  
159 mediated by an as yet unknown scaffold, causing their preferential arrangement along actin fibers  
160 (Calebiro et al., 2013). As in the case of AMPA receptors, agonist stimulation with GABA was also  
161 found to weaken the interaction of GABA<sub>b</sub> receptors with the cytoskeleton, leading to an increase of  
162 their lateral mobility. Whereas the occurrence and functional relevance of this organization in  
163 neurons is presently unknown, it is tempting to speculate that it might be involved in controlling the  
164 precise localization and/or function of GABA<sub>b</sub> receptors at synapses (Calebiro et al., 2013). Another  
165 receptor that has been recently investigated in this respect is the somatostatin receptor type 2  
166 (SSTR2), which had been shown to interact with the actin-binding scaffold filamin A (Peverelli et al.,  
167 2014). Recent single-molecule data by our group indicate that SSTR2 undergoes transient  
168 interactions with filamin A, which lead to a preferential localization of SSTR2 along actin fibers and  
169 participate in restraining SSTR2 diffusion on the plasma membrane. These interactions are increased



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

172

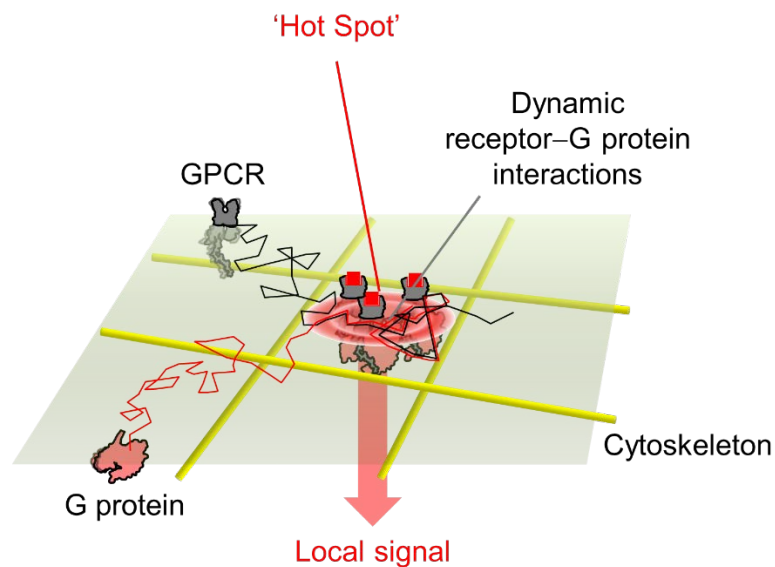
### 173 **5. Hot-spots for GPCR signaling on the plasma membrane**

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

186 More recently, our group succeeded for the first time in directly visualizing individual receptors and  
187 G proteins as they diffuse, interact and signal on the surface of intact cells (Sungkaworn et al., 2017).  
188 This study – which focused on  $\alpha_{2A}$ -adrenergic receptor/ $G_i$  and  $\beta_2$ -adrenergic receptors/ $G_s$  as model  
189 receptor/G protein pairs – has led to a number of important observations. Notably, we could directly  
190 measure the duration of receptor–G protein interactions in intact cells, showing that they are  
191 transient and last approximately 1-2 seconds. Whereas we observed the occurrence of transient  
192 receptor–G protein interactions also in the absence of ligands, which were linked to the basal  
193 constitutive activity of the receptors, we did not observe preformed, stable receptor–G protein  
194 complexes, as proposed by some previous studies. However, differences might exist in the degree  
195 and stability of pre-association among different receptors and/or G proteins. For instance, using  
196 bioluminescence resonance energy transfer (BRET) and time-resolved fluorescence resonance  
197 energy transfer (FRET), the protease-activated receptor 1 (PAR1) was found in a previous study to  
198 pre-associate with  $G_i$  but not with  $G_{12}$  (Ayoub et al., 2010). Second, we found that agonists mainly act  
199 by increasing the association rate ( $k_{on}$ ) between receptors and G proteins, as expected in the case of  
200 protein interactions that proceed through major conformational changes. Third, we discovered that  
201 receptor–G protein interactions and G protein activation do not occur randomly on the plasma  
202 membrane, but rather at dynamic nanodomains that we termed ‘hot spots’ (see Figure 1). Based on  
203 our recent results and simulations, we hypothesize that, by increasing the local effective

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

216



217

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

222

## 223 6. Lipid nanodomains revisited

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

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

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

243 As a complementary approach, several groups have measured the diffusion of fluorescently labelled  
244 lipids and GPI-anchored proteins by either single-particle tracking or fluorescence correlation  
245 spectroscopy (FCS) in an attempt to understand their spatiotemporal organization at the plasma  
246 membrane. The results have shown that both GPI-anchored proteins and lipids undergo transient  
247 confinement in small nanodomains on the plasma membrane (Fujiwara et al., 2002; Lenne et al.,  
248 2006). However, multiple factors could contribute to such behavior, complicating the interpretation  
249 of the results. A first important factor is the cytoskeleton, which, as mentioned above, has been  
250 shown to provide barriers to both protein and lipid diffusion in the plasma membrane. However,  
251 there is some evidence that the confinement of membrane lipids and GPI-anchored proteins is not  
252 always dependent on the presence of an intact cytoskeleton and could be altered by manipulating  
253 the lipid composition of the plasma membrane, supporting a role for lipid-protein interactions in the  
254 transient confinement of GPI-anchored proteins (Lenne et al., 2006).

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

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

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

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

278

## 279 **7. GPCR signaling at intracellular domains**

280 Whereas signaling by GPCRs has long been thought to be restricted to the plasma membrane, a  
281 growing body of evidence indicates that GPCRs can also signal on intracellular membranes. The use  
282 of advanced optical methods such as FRET and conformation-sensitive biosensors was not only  
283 instrumental for these recent discoveries but also allowed scientists to identify the intracellular  
284 compartments where GPCR signaling is taking place. A first study by our group on endogenous  
285 thyroid stimulating hormone (TSH) receptors in thyroid cells revealed that these prototypical GPCRs  
286 for glycoprotein hormones remain active after internalization, leading to persistent cAMP signaling  
287 (Calebiro et al., 2009). Similar results were obtained by the group of Jean-Pierre Vilardaga studying  
288 the parathyroid hormone (PTH) receptor (Ferrandon et al., 2009). These early studies provided the  
289 first demonstration that GPCRs can continue signaling via cAMP at intracellular sites after  
290 internalization. Moreover, they suggested that TSH and PTH receptors were probably signaling in  
291 distinct compartments, i.e. a perinuclear compartment associated with the Golgi complex and early  
292 endosomes, respectively. However, it was only with the introduction of biosensors based on  
293 conformation-sensitive nanobodies that it was possible to directly visualize the subcellular sites of  
294 receptor and G protein activation in living cells. Using this elegant approach, the group of Mark von  
295 Zastrow was able to show that  $\beta_2$ -adrenergic receptors remain active in early endosomes, where  
296 they induce local  $G_s$  protein activation (Irannejad et al., 2013). More recently, our group further

297 investigated the nature and dynamics of the intracellular compartment where TSH receptors are  
298 signaling. For this purpose, we used a combination of FRET sensors measuring cAMP levels and PKA  
299 activity, which we tethered to different subcellular compartments, together with the previously  
300 developed conformation-sensitive biosensor for G<sub>s</sub> protein activation (Godbole et al., 2017). The  
301 results of this study demonstrated that upon TSH stimulation, the TSH receptor and its ligand traffic  
302 retrogradely to the trans-Golgi network, where they induce local G<sub>s</sub> protein activation, cAMP  
303 production and PKA activation. Importantly, this leads to a delayed phase of cAMP/PKA signaling at  
304 the Golgi/trans-Golgi network, which is required for TSH to efficiently induce phosphorylation of the  
305 cAMP response element-binding protein (CREB) and transcription of early genes (Godbole et al.,  
306 2017). Interestingly, signaling within the Golgi complex has also been recently demonstrated for the  
307  $\beta_1$ -adrenergic receptor, even though in this case it has been proposed that it is the ligand (adrenalin)  
308 to reach the receptors, which are already located on membranes of the Golgi complex, via facilitated  
309 transport across cellular membranes (Irannejad et al., 2017). In the meantime, studies from several  
310 groups have demonstrated signaling at intracellular sites for a number of receptors (Kotowski et al.,  
311 2011; Feinstein et al., 2013; Kuna et al., 2013; Merriam et al., 2013; Ismail et al., 2016; Lyga et al.,  
312 2016), suggesting that this might be a rather common feature among GPCRs. Moreover, there is  
313 some evidence that GPCRs might also signal on the nuclear envelope (Tadevosyan et al., 2012) as  
314 well as in mitochondria (Hebert-Chatelain et al., 2016; Suofu et al., 2017).

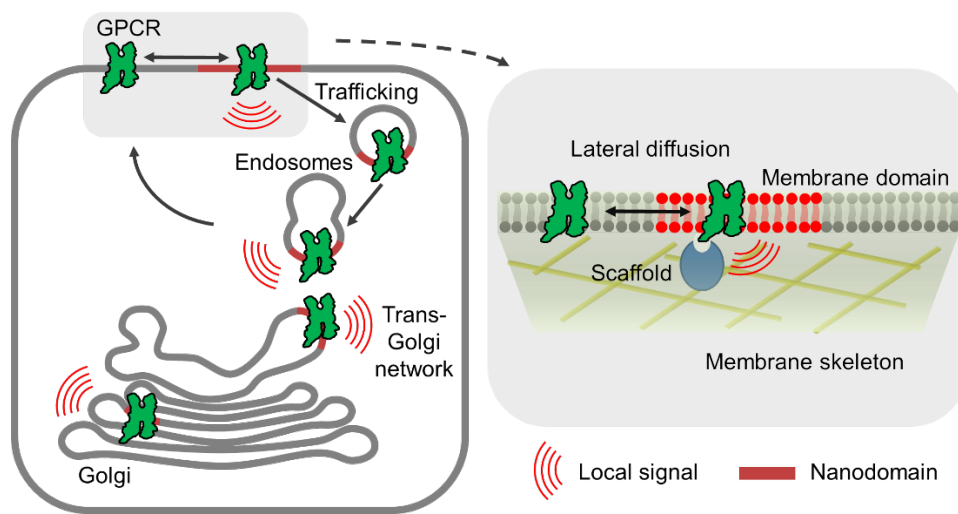
315 These novel and exciting findings point to a previously unsuspected level of complexity in GPCR  
316 signaling. The fact that individual GPCRs can induce local signals in distinct subcellular compartments  
317 likely plays a major role in determining their biological effects. This provides a new basis to explain  
318 the high diversity found within the GPCR superfamily, in spite of the fact that all these receptors  
319 converge on just a few common signaling pathways. At the same time, they reveal an extremely  
320 dynamic picture. Indeed, data on both TSH (Godbole et al., 2017) and  $\beta_2$ -adrenergic receptors  
321 (Irannejad et al., 2013) indicate that GPCR signaling is highly controlled in space and time during  
322 receptor internalization and intracellular trafficking, apparently occurring in short 'bursts' once  
323 receptors enter well-defined membrane sub-domains of early endosomes or the trans-Golgi  
324 network. This high degree of integration between intracellular trafficking and signaling likely  
325 provides a key mechanism to fine tune GPCR signaling, which, once understood in its complexity,  
326 might also offer novel opportunities to modulate GPCRs for therapeutic purposes.

327

## 328 **8. Concluding remarks**

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

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



341  
 342 Figure 2. Dynamic nanodomains for GPCR signaling. Recent studies have revealed a highly complex and  
 343 dynamic picture, whereby GPCRs can signal at dynamic nanodomains located both on the plasma membrane  
 344 and on membranes of intracellular compartments such as early endosomes, the trans-Golgi network or the  
 345 Golgi complex. Dynamic interactions of receptors and other signaling proteins with membrane lipids, the  
 346 cytoskeleton and protein scaffolds likely play important role in the formation of these nanodomains. Lateral  
 347 diffusion and trafficking control the localization of GPCRs within the nanodomains, which might provide a  
 348 means to modulate the timing and location of GPCR signaling.

349  
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## References

- 353 Pierce, K.L., Premont, R.T. and Lefkowitz, R.J., 2002. Seven-transmembrane receptors, *Nat Rev Mol*  
354 *Cell Biol.* 3, 639-50.
- 355 Lefkowitz, R.J., 2004. Historical review: a brief history and personal retrospective of seven-  
356 transmembrane receptors, *Trends Pharmacol Sci.* 25, 413-22.
- 357 Hauser, A.S., Attwood, M.M., Rask-Andersen, M., Schiöth, H.B. and Gloriam, D.E., 2017. Trends in  
358 GPCR drug discovery: new agents, targets and indications, *Nature Reviews Drug Discovery.*  
359 16, 829.
- 360 Beavo, J.A. and Brunton, L.L., 2002. Cyclic nucleotide research -- still expanding after half a century,  
361 *Nat Rev Mol Cell Biol.* 3, 710-8.
- 362 Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G. et al., 1986. Cloning of the gene  
363 and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin, *Nature.*  
364 321, 75-9.
- 365 Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S. et al., 2007. High-  
366 resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled  
367 receptor, *Science.* 318, 1258-65.
- 368 Rasmussen, S.G., Choi, H.J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S. et al., 2007. Crystal structure  
369 of the human beta2 adrenergic G-protein-coupled receptor, *Nature.* 450, 383-7.
- 370 Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y. et al., 2011. Crystal structure of the  
371 beta2 adrenergic receptor-Gs protein complex, *Nature.* 477, 549-55.
- 372 Koehl, A., Hu, H., Maeda, S., Zhang, Y., Qu, Q. et al., 2018. Structure of the  $\mu$ -opioid receptor-Gi  
373 protein complex, *Nature.* 558, 547-552.
- 374 Liang, Y.L., Khoshouei, M., Radjainia, M., Zhang, Y., Glukhova, A. et al., 2017. Phase-plate cryo-EM  
375 structure of a class B GPCR-G-protein complex, *Nature.* 546, 118-123.
- 376 Zhang, Y., Sun, B., Feng, D., Hu, H., Chu, M. et al., 2017. Cryo-EM structure of the activated GLP-1  
377 receptor in complex with a G protein, *Nature.* 546, 248-253.
- 378 Kang, Y., Zhou, X.E., Gao, X., He, Y., Liu, W. et al., 2015. Crystal structure of rhodopsin bound to  
379 arrestin by femtosecond X-ray laser, *Nature.* 523, 561-7.
- 380 Zhou, X.E., He, Y., de Waal, P.W., Gao, X., Kang, Y. et al., 2017. Identification of Phosphorylation  
381 Codes for Arrestin Recruitment by G Protein-Coupled Receptors, *Cell.* 170, 457-469 e13.
- 382 Steinberg, S.F. and Brunton, L.L., 2001. Compartmentation of G protein-coupled signaling pathways  
383 in cardiac myocytes, *Annu Rev Pharmacol Toxicol.* 41, 751-73.
- 384 Keely, S.L., 1979. Prostaglandin E1 activation of heart cAMP-dependent protein kinase: apparent  
385 dissociation of protein kinase activation from increases in phosphorylase activity and  
386 contractile force, *Mol Pharmacol.* 15, 235-45.
- 387 Brunton, L.L., Hayes, J.S. and Mayer, S.E., 1979. Hormonally specific phosphorylation of cardiac  
388 troponin I and activation of glycogen phosphorylase, *Nature.* 280, 78-80.
- 389 Hayes, J.S., Brunton, L.L. and Mayer, S.E., 1980. Selective activation of particulate cAMP-dependent  
390 protein kinase by isoproterenol and prostaglandin E1, *J Biol Chem.* 255, 5113-9.
- 391 Buxton, I.L. and Brunton, L.L., 1983. Compartments of cyclic AMP and protein kinase in mammalian  
392 cardiomyocytes, *J Biol Chem.* 258, 10233-9.
- 393 Tolkovsky, A.M. and Levitzki, A., 1978. Coupling of a single adenylate cyclase to two receptors:  
394 adenosine and catecholamine, *Biochemistry.* 17, 3811-7.
- 395 Tolkovsky, A.M. and Levitzki, A., 1978. Mode of coupling between the beta-adrenergic receptor and  
396 adenylate cyclase in turkey erythrocytes, *Biochemistry.* 17, 3795.
- 397 Insel, P.A., Head, B.P., Patel, H.H., Roth, D.M., Bunday, R.A. et al., 2005. Compartmentation of G-  
398 protein-coupled receptors and their signalling components in lipid rafts and caveolae,  
399 *Biochem Soc Trans.* 33, 1131-4.
- 400 Simons, K. and Ikonen, E., 1997. Functional rafts in cell membranes, *Nature.* 387, 569.
- 401 Simons, K. and Toomre, D., 2000. Lipid rafts and signal transduction, *Nat Rev Mol Cell Biol.* 1, 31-9.

402 Xiang, Y., Rybin, V.O., Steinberg, S.F. and Kobilka, B., 2002. Caveolar localization dictates physiologic  
403 signaling of beta 2-adrenoceptors in neonatal cardiac myocytes, *J Biol Chem.* 277, 34280-6.

404 Nikolaev, V.O., Moshkov, A., Lyon, A.R., Miragoli, M., Novak, P. et al., 2010. Beta2-adrenergic  
405 receptor redistribution in heart failure changes cAMP compartmentation, *Science.* 327,  
406 1653-7.

407 MacDougall, D.A., Agarwal, S.R., Stopford, E.A., Chu, H.J., Collins, J.A. et al., 2012. Caveolae  
408 compartmentalise beta 2-adrenoceptor signals by curtailing cAMP production and  
409 maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular  
410 myocyte, *Journal of Molecular and Cellular Cardiology.* 52, 388-400.

411 Wright, P.T., Nikolaev, V.O., O'Hara, T., Diakonov, I., Bhargava, A. et al., 2014. Caveolin-3 regulates  
412 compartmentation of cardiomyocyte beta2-adrenergic receptor-mediated cAMP signaling,  
413 *Journal of Molecular and Cellular Cardiology.* 67, 38-48.

414 Singer, S.J. and Nicolson, G.L., 1972. The fluid mosaic model of the structure of cell membranes,  
415 *Science.* 175, 720-31.

416 Milligan, G. and Bouvier, M., 2005. Methods to monitor the quaternary structure of G protein-  
417 coupled receptors, *FEBS J.* 272, 2914-25.

418 Lohse, M.J., Nuber, S. and Hoffmann, C., 2012. Fluorescence/bioluminescence resonance energy  
419 transfer techniques to study G-protein-coupled receptor activation and signaling, *Pharmacol*  
420 *Rev.* 64, 299-336.

421 Lefkimmatis, K. and Zaccolo, M., 2014. cAMP signaling in subcellular compartments, *Pharmacol*  
422 *Ther.*

423 Calebiro, D. and Maiellaro, I., 2014. cAMP signaling microdomains and their observation by optical  
424 methods, *Front Cell Neurosci.* 8, 350.

425 Calebiro, D., Nikolaev, V.O., Persani, L. and Lohse, M.J., 2010. Signaling by internalized G-protein-  
426 coupled receptors, *Trends Pharmacol Sci.* 31, 221-8.

427 Irannejad, R., Tomshine, J.C., Tomshine, J.R., Chevalier, M., Mahoney, J.P. et al., 2013.  
428 Conformational biosensors reveal GPCR signalling from endosomes, *Nature.* 495, 534-8.

429 Irannejad, R., Pessino, V., Mika, D., Huang, B., Wedegaertner, P.B. et al., 2017. Functional selectivity  
430 of GPCR-directed drug action through location bias, *Nat Chem Biol.* 13, 799-806.

431 Godbole, A., Lyga, S., Lohse, M.J. and Calebiro, D., 2017. Internalized TSH receptors en route to the  
432 TGN induce local Gs-protein signaling and gene transcription, *Nat Commun.* 8, 443.

433 Surdo, N.C., Berrera, M., Koschinski, A., Brescia, M., Machado, M.R. et al., 2017. FRET biosensor  
434 uncovers cAMP nano-domains at beta-adrenergic targets that dictate precise tuning of  
435 cardiac contractility, *Nat Commun.* 8, 15031.

436 Castro, L.R., Gervasi, N., Guiot, E., Cavellini, L., Nikolaev, V.O. et al., 2010. Type 4 phosphodiesterase  
437 plays different integrating roles in different cellular domains in pyramidal cortical neurons, *J*  
438 *Neurosci.* 30, 6143-51.

439 Maiellaro, I., Lohse, M.J., Kittel, R.J. and Calebiro, D., 2016. cAMP Signals in Drosophila Motor  
440 Neurons Are Confined to Single Synaptic Boutons, *Cell Rep.* 17, 1238-1246.

441 Calebiro, D. and Sungkaworn, T., 2017. Single-Molecule Imaging of GPCR Interactions, *Trends*  
442 *Pharmacol Sci.*

443 Morone, N., Fujiwara, T., Murase, K., Kasai, R.S., Ike, H. et al., 2006. Three-dimensional  
444 reconstruction of the membrane skeleton at the plasma membrane interface by electron  
445 tomography, *J Cell Biol.* 174, 851-62.

446 Sako, Y. and Kusumi, A., 1994. Compartmentalized structure of the plasma membrane for receptor  
447 movements as revealed by a nanometer-level motion analysis, *J Cell Biol.* 125, 1251-64.

448 Edidin, M., Kuo, S. and Sheetz, M., 1991. Lateral movements of membrane glycoproteins restricted  
449 by dynamic cytoplasmic barriers, *Science.* 254, 1379-1382.

450 Sako, Y. and Kusumi, A., 1995. Barriers for lateral diffusion of transferrin receptor in the plasma  
451 membrane as characterized by receptor dragging by laser tweezers: fence versus tether, *J*  
452 *Cell Biol.* 129, 1559-74.



453 Jacobson, K., Sheets, E.D. and Simson, R., 1995. Revisiting the fluid mosaic model of membranes,  
454 Science. 268, 1441-2.

455 Kusumi, A., Nakada, C., Ritchie, K., Murase, K., Suzuki, K. et al., 2005. Paradigm shift of the plasma  
456 membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-  
457 speed single-molecule tracking of membrane molecules, *Annu Rev Biophys Biomol Struct.*  
458 34, 351-78.

459 Murase, K., Fujiwara, T., Umemura, Y., Suzuki, K., Iino, R. et al., 2004. Ultrafine membrane  
460 compartments for molecular diffusion as revealed by single molecule techniques, *Biophys J.*  
461 86, 4075-93.

462 Suzuki, K., Ritchie, K., Kajikawa, E., Fujiwara, T. and Kusumi, A., 2005. Rapid hop diffusion of a G-  
463 protein-coupled receptor in the plasma membrane as revealed by single-molecule  
464 techniques, *Biophys J.* 88, 3659-80.

465 Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K. and Kusumi, A., 2002. Phospholipids undergo  
466 hop diffusion in compartmentalized cell membrane, *J Cell Biol.* 157, 1071-81.

467 Hiramoto-Yamaki, N., Tanaka, K.A., Suzuki, K.G., Hirose, K.M., Miyahara, M.S. et al., 2014.  
468 Ultrafast diffusion of a fluorescent cholesterol analog in compartmentalized plasma  
469 membranes, *Traffic.* 15, 583-612.

470 Kusumi, A., Suzuki, K.G., Kasai, R.S., Ritchie, K. and Fujiwara, T.K., 2011. Hierarchical mesoscale  
471 domain organization of the plasma membrane, *Trends Biochem Sci.* 36, 604-15.

472 Saxton, M.J., 2002. Chemically limited reactions on a percolation cluster, *The Journal of Chemical*  
473 *Physics.* 116.

474 Triller, A. and Choquet, D., 2003. Synaptic structure and diffusion dynamics of synaptic receptors,  
475 *Biol Cell.* 95, 465-76.

476 Sheng, M. and Sala, C., 2001. PDZ domains and the organization of supramolecular complexes, *Annu*  
477 *Rev Neurosci.* 24, 1-29.

478 Garner, C.C., Nash, J. and Hagan, R.L., 2000. PDZ domains in synapse assembly and signalling,  
479 *Trends Cell Biol.* 10, 274-80.

480 Compans, B., Choquet, D. and Hosy, E., 2016. Review on the role of AMPA receptor nano-  
481 organization and dynamic in the properties of synaptic transmission, *Neurophotonics.* 3,  
482 041811.

483 Matt, L., Kim, K., Hergarden, A.C., Patriarchi, T., Malik, Z.A. et al., 2018. alpha-Actinin Anchors PSD-95  
484 at Postsynaptic Sites, *Neuron.* 97, 1094-1109 e9.

485 Bats, C., Groc, L. and Choquet, D., 2007. The interaction between Stargazin and PSD-95 regulates  
486 AMPA receptor surface trafficking, *Neuron.* 53, 719-34.

487 Calebiro, D., Rieken, F., Wagner, J., Sungkaworn, T., Zabel, U. et al., 2013. Single-molecule analysis of  
488 fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics  
489 and organization, *Proc Natl Acad Sci U S A.* 110, 743-8.

490 Peverelli, E., Giardino, E., Treppiedi, D., Vitali, E., Cambiaghi, V. et al., 2014. Filamin A (FLNA) plays an  
491 essential role in somatostatin receptor 2 (SSTR2) signaling and stabilization after agonist  
492 stimulation in human and rat somatotroph tumor cells, *Endocrinology.* 155, 2932-41.

493 Treppiedi, D., Jobin, M.L., Peverelli, E., Giardino, E., Sungkaworn, T. et al., 2018. Single-Molecule  
494 Microscopy Reveals Dynamic FLNA Interactions Governing SSTR2 Clustering and  
495 Internalization, *Endocrinology.* 159, 2953-2965.

496 Mo, G.C., Ross, B., Hertel, F., Manna, P., Yang, X. et al., 2017. Genetically encoded biosensors for  
497 visualizing live-cell biochemical activity at super-resolution, *Nat Methods.*

498 Sungkaworn, T., Jobin, M.L., Burneck, K., Weron, A., Lohse, M.J. et al., 2017. Single-molecule  
499 imaging reveals receptor-G protein interactions at cell surface hot spots, *Nature.*

500 Ayoub, M.A., Trinquet, E., Pflieger, K.D. and Pin, J.P., 2010. Differential association modes of the  
501 thrombin receptor PAR1 with Galphai1, Galpha12, and beta-arrestin 1, *FASEB J.* 24, 3522-35.

502 Munro, S., 2003. Lipid Rafts, *Cell.* 115, 377-388.

503 Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K. et al., 2009. Direct  
504 observation of the nanoscale dynamics of membrane lipids in a living cell, *Nature*. 457, 1159-  
505 62.

506 Eggeling, C., 2015. Super-resolution optical microscopy of lipid plasma membrane dynamics, *Essays*  
507 *Biochem.* 57, 69-80.

508 Mayor, S. and Maxfield, F.R., 1995. Insolubility and redistribution of GPI-anchored proteins at the  
509 cell surface after detergent treatment, *Mol Biol Cell*. 6, 929-44.

510 Varma, R. and Mayor, S., 1998. GPI-anchored proteins are organized in submicron domains at the  
511 cell surface, *Nature*. 394, 798-801.

512 Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K. et al., 2004. Nanoscale organization of multiple  
513 GPI-anchored proteins in living cell membranes, *Cell*. 116, 577-89.

514 Lenne, P.F., Wawrezynieck, L., Conchonaud, F., Wurtz, O., Boned, A. et al., 2006. Dynamic molecular  
515 confinement in the plasma membrane by microdomains and the cytoskeleton meshwork,  
516 *EMBO J.* 25, 3245-56.

517 Honigsmann, A., Mueller, V., Ta, H., Schoenle, A., Sezgin, E. et al., 2014. Scanning STED-FCS reveals  
518 spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells, *Nat*  
519 *Commun.* 5, 5412.

520 Kusumi, A., Fujiwara, T.K., Morone, N., Yoshida, K.J., Chadda, R. et al., 2012. Membrane mechanisms  
521 for signal transduction: the coupling of the meso-scale raft domains to membrane-skeleton-  
522 induced compartments and dynamic protein complexes, *Semin Cell Dev Biol*. 23, 126-44.

523 Calebiro, D., Nikolaev, V.O., Gagliani, M.C., de Filippis, T., Dees, C. et al., 2009. Persistent cAMP-  
524 signals triggered by internalized G-protein-coupled receptors, *PLoS Biol.* 7, e1000172.

525 Ferrandon, S., Feinstein, T.N., Castro, M., Wang, B., Bouley, R. et al., 2009. Sustained cyclic AMP  
526 production by parathyroid hormone receptor endocytosis, *Nat Chem Biol*. 5, 734-42.

527 Kotowski, S.J., Hopf, F.W., Seif, T., Bonci, A. and von Zastrow, M., 2011. Endocytosis promotes rapid  
528 dopaminergic signaling, *Neuron*. 71, 278-90.

529 Feinstein, T.N., Yui, N., Webber, M.J., Wehbi, V.L., Stevenson, H.P. et al., 2013. Noncanonical control  
530 of vasopressin receptor type 2 signaling by retromer and arrestin, *J Biol Chem*. 288, 27849-  
531 60.

532 Kuna, R.S., Girada, S.B., Asalla, S., Vallentyne, J., Maddika, S. et al., 2013. Glucagon-like peptide-1  
533 receptor-mediated endosomal cAMP generation promotes glucose-stimulated insulin  
534 secretion in pancreatic beta-cells, *Am J Physiol Endocrinol Metab*. 305, E161-70.

535 Merriam, L.A., Baran, C.N., Girard, B.M., Hardwick, J.C., May, V. et al., 2013. Pituitary adenylate  
536 cyclase 1 receptor internalization and endosomal signaling mediate the pituitary adenylate  
537 cyclase activating polypeptide-induced increase in guinea pig cardiac neuron excitability, *J*  
538 *Neurosci.* 33, 4614-22.

539 Ismail, S., Gherardi, M.J., Froese, A., Zanon, M., Gigoux, V. et al., 2016. Internalized Receptor for  
540 Glucose-dependent Insulinotropic Peptide stimulates adenylyl cyclase on early endosomes,  
541 *Biochem Pharmacol.* 120, 33-45.

542 Lyga, S., Volpe, S., Werthmann, R.C., Gotz, K., Sungkaworn, T. et al., 2016. Persistent cAMP signaling  
543 by internalized LH receptors in ovarian follicles, *Endocrinology*. en20151945.

544 Tadevosyan, A., Vaniotis, G., Allen, B.G., Hebert, T.E. and Nattel, S., 2012. G protein-coupled receptor  
545 signalling in the cardiac nuclear membrane: evidence and possible roles in physiological and  
546 pathophysiological function, *J Physiol*. 590, 1313-30.

547 Hebert-Chatelain, E., Desprez, T., Serrat, R., Bellocchio, L., Soria-Gomez, E. et al., 2016. A  
548 cannabinoid link between mitochondria and memory, *Nature*. 539, 555-559.

549 Suofu, Y., Li, W., Jean-Alphonse, F.G., Jia, J., Khattar, N.K. et al., 2017. Dual role of mitochondria in  
550 producing melatonin and driving GPCR signaling to block cytochrome c release, *Proc Natl*  
551 *Acad Sci U S A*. 114, E7997-E8006.