Report from referee 2

> I am not a mathematician, so my comments are largely from biological perspective. The authors describe one possible approach to modeling GPCR signaling. While they talk about compartmentalization inside the cell, which is likely biologically important, their model does not contain this feature. Some features of the model appear to be unjustifiable oversimplifications.

We thank the reviewer for this general assessment. We would first like to reassure the reviewer that we are aware of the intricacies of GPCR-related signalling cascades, and we have also recently been involved in providing some insight into the downstream compartmentalization of second messengers (see refs. [1, 2]). Our reference to the compartmentalization concept is meant to set the stage to define the specific area on which we want to focus in our perspective, i.e. the role of modulation of the diffusion of membrane-bound actors in influencing downstream 2nd messenger production. We believe that local modulation of receptor diffusion may result in a local modulation of the cAMP produced, leading to the formation of a cAMP compartment; we agree with the reviewer that additional mechanisms would be needed to support a sustained second messenger compartment, such as cAMP buffered diffusion [2], that are not the focus of this perspective. We have clarified this aspect in the introduction.

We agree that our model is simple. We shall note here, however, that it does not deviate significantly from reported models and underlying ideas presented in the literature over the last 20+ years, which we actually review here. Our main goal is to provide an accessible and concise presentation of the concept and a qualitative prediction of what would happen to second messenger production if receptor diffusion speed was modulated; we think that this is an underappreciated aspect in current research, and in this respect we believe that the toy model we use achieves its objective while being analytically tractable throughout.

> For example, they don't take into account activation-dependent changes in G protein distribution within the cell. G proteins are in equilibrium between membrane-associated and cytoplasmic. Inactive heterotrimeric G proteins have two membrane anchors, whereas activated Gα and G$\beta\gamma$ have only one membrane anchor each. Thus, activation makes G proteins more likely not to be membrane-associated. The effector enzyme considered here is adenylyl cyclase. It is an integral membrane protein, like GPCRs, so activated Gα interactions with it would have the same complications as inactive heterotrimer interactions with GPCRs.

We agree with the referee that the possibility of cytoplasmic shuttling of the Gα and G$\beta\gamma$ subunits is an important point and is often overlooked. This has originally presented by Rasenik and coworkers [4] and more recently reviewed, for example,

in ref. [3]. We believe that a cytosolic pool would primarily act as a modulation to the steady state concentration of the membrane bound component, and thus would not alter our qualitative conclusions. Quantitatively, by introducing cytosolic active G protein G_c^* and rates h^+ (binding) and h^- (unbinding) for the transition between it and the membrane-bound G^*, one would expect equilibrium in the cytosolic-membrane flux to correspond to be given by $[G^*] = \frac{k_{\text{eff}}[RS]+h^+[G_c^*]}{h^-+k_{G^*G}}$ with the limit $h^{\pm} \to 0$ corresponding to the version in the manuscript. It seems plausible that (removed from all details) in equilibrium there would be a proportionality between the cytosolic and membrane-bound active G protein. Thus, the effect from coupling to a cytosolic pool acts to modulate the de-activation rate which we are agnostic about anyway. Essentially, all constants entering in our calculation are used in this spirit of being effective constants rather than molecular ones. Moreover, G protein concentration estimates, used in our work, are based on membrane concentration. We have clarified in the text that we only attempt to track plasma-membrane concentrations.

> In addition, editing to eliminate typos and correct the grammar is needed. A few examples: abstract, line 6, "separations" should be "separation"; line 20, "relative low" should be "relatively low"; lines 41-42, as is, the sentence "Eventually, if the reverse reaction is not carried over (by hydrolizing GTP to GDP) the pool of active G proteins would deplete" appears to mean the opposite of what the authors intend; line 44, correct term is "well stirred", not "well-mixed"; lines 442-443, "cooperation" should be "cooperativity".

We are thankful for pointing out typos and have changed these. We agree that well stirred probably is the more common nomenclature in biological context. The sentence that started on line 41 with "Eventually" was erroneously phrased as the active G protein would not deplete but the inactive would. This has been changed as well.

*References

[1] Selma E. Anton, Charlotte Kayser, Isabella Maiellaro, Katarina Nemec, Jan Möller, Andreas Koschinski, Manuela Zaccolo, Paolo Annibale, Martin Falcke, Martin J. Lohse, and Andreas Bock. Receptor-associated independent camp nanodomains mediate spatiotemporal specificity of gpcr signaling. Cell, 185(7):1130–1142.e11, 2022.

[2] Andreas Bock, Paolo Annibale, Charlotte Konrad, Annette Hannawacker, Selma E Anton, Isabella Maiellaro, Ulrike Zabel, Sivaraj Sivaramakrishnan, Martin Falcke, and Martin J Lohse. Optical mapping of camp signaling at the nanometer scale. Cell, 182(6):1519–1530, 2020.

[3] Shahriar M. Khan, Jennifer Y. Sung, and Terence E. Hébert. $G_{\beta\gamma}$ subunits—different spaces, different faces. Pharmacological Research, 111:434–441,

2016.

[4] Jiang-Zhou Yu and Mark M. Rasenick. Real-time visualization of a fluorescent gαs: Dissociation of the activated g protein from plasma membrane. Molecular Pharmacology, 61(2):352–359, 2002.