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Human Serotonin Receptor 5-HT$_{1A}$ Preferentially Segregates to the Liquid Disordered Phase in Synthetic Lipid Bilayers

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Supporting Information

ABSTRACT: We demonstrate successful incorporation of the G protein coupled receptor 5-HT$_{1A}$ into giant unilamellar vesicles using an agarose rehydration method. With direct observation using fluorescence techniques, we report preferential segregation of 5-HT$_{1A}$ into the cholesterol-poor liquid disordered phase of the membrane, contradicting previous reports of lipid raft segregation. Furthermore, altering the concentration of cholesterol and sphingomyelin in ternary mixtures does not alter 5-HT$_{1A}$ segregation into the liquid disordered phase.

G protein coupled receptors (GPCRs) represent one of the largest families of proteins in the human genome.[1] GPCRs mediate an extensive number of extracellular signals pertinent to many physiological responses[2] via activation of secondary messengers within the cell.[3] GPCRs are integral membrane proteins containing seven transmembrane helices. They are coupled via their cytoplasmic domains to heterotrimetric G proteins that shift between a guanosine triphosphate-bound state that induces intracellular downstream activity and an inactive guanosine diphosphate-bound state.[4]

The 5-hydroxytryptamine receptor subtype 1A (5-HT$_{1A}$) is a GPCR that is found throughout the central nervous system.[5] It binds the neurotransmitter serotonin (i.e., 5-hydroxytryptamine), which regulates mood, responses to stress, and emotion.[6] GPCRs in general and 5-HT$_{1A}$ in particular have been reported to partition into lipid rafts in mammalian cell plasma membranes.[7] Such rafts are suggested to be areas in the plasma membrane where sphingolipids, cholesterol, and proteins congregate. These raft domains have been suggested to play important roles in cell sorting and signaling.[8,9] It has been reported that mast cells[10] and T-cells[11] reduce their signaling activity when cholesterol is depleted, therefore suggesting that cellular lipid rafts facilitate signaling pathways. In 2009, Singh and Chattopadhyay reported that after treatment with sphingomyelinase, 5-HT$_{1A}$ displays a loss of agonist binding, suggesting a necessity for raft segregation for functionality.[12]

Detergent-resistant membrane (DRM) fragment separation is the traditional biochemical method used to determine the partitioning of proteins into lipid rafts.[13] DRM studies report that 5-HT$_{1A}$ is found in the cholesterol-rich layer of DRM separations, thus suggesting liquid ordered phase preference in mammalian cells.[14,15] Direct visualization of raft domains in mammalian cells, however, has been elusive.[16] Furthermore, while DRMs have been heavily used to associate proteins to lipid rafts, in 2005, Lichtenberg et al. reported that DRMs should not implicate lipid raft association due to congregating forces in the centrifugation process.[17] Liquid ordered and liquid disordered phase separation in model membranes is a prominent biomimetic platform for the assessment of lipid phase behavior which avoids artifacts like those associated with DRM fragment preparation.[18−20]

Phase separation in model membranes has been extensively observed,[21] though visualization of protein segregation into either of the phases has posed a challenge due to synthetic membrane fabrication processes. Incorporation of proteins into giant unilamellar vesicles (GUVs) is incompatible with traditional methods of fabrication, namely, electroformation and gentle hydration.[22,23] Less protein-disruptive methods of vesicle formation have been reported recently. Hydration methods utilizing both agarose[24] and cross-linked dextran[25] have been developed, and we have previously reported successful incorporation of proteins into GUVs using an agarose swelling method.[26]

While 5-HT$_{1A}$ has been incorporated into the membranes of nanoscale liposomes, this membrane format is inaccessible to fluorescent microscopy, making observations of phase segregation impossible. Giant unilamellar vesicles (10−100 μm in diameter) are more suitable for direct observations. In 2013, Kang et al. reported an application of hydrogel stamping followed by electroformation for GUV fabrication.[27] They were able to incorporate membrane fragments containing human acetylcholine receptor into GUVs. While the presence of protein was detected via antibody binding, phase segregation behavior was not reported.[27] Furthermore, May et al. in 2013 reported the insertion of *in vitro* expressed dopamine receptor D2 (DRD2) into polymersome membranes. While antibody and ligand binding was reported, the fabricated polymersomes were 100 nm in diameter,[28] were inaccessible to fluorescence microscopy, and deviated from the lipid cellular environment in which DRD2 is naturally found.

Here, we present for the first time the direct incorporation of 5-HT$_{1A}$ into GUVs and observe 5-HT$_{1A}$ in phase separated vesicles. Through fabrication of GUVs containing 5-HT$_{1A}$ membrane fragments, we are able to confirm the protein's incorporation and identify its location in the membrane through primary antibody labeling and ligand binding. We observe that 5-HT$_{1A}$ preferentially segregates into the

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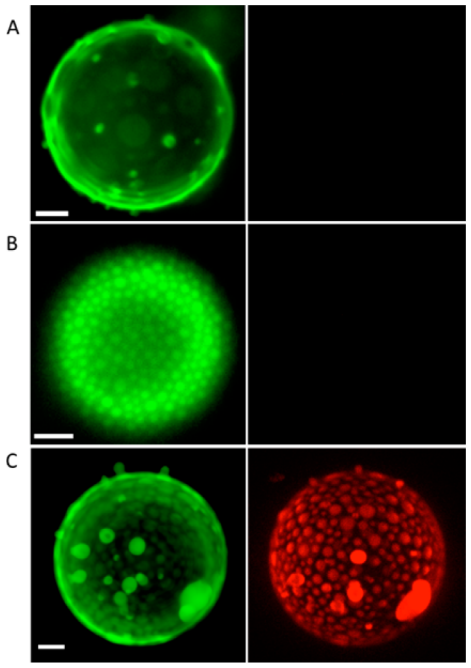

Figure 1. Phase separating vesicles with and without 5HT-$_{1A}$ incorporated. Lipid label fluorescence is on the left; 5HT-$_{1A}$ antibody fluorescence is on the right. (A) Phase separation observed on GUVs (1:1:3 POPC:Chol:BSM) without protein and incubated with antibody for 1 h. No signal is detected at 561 nm indicating that antibody does not bind to the lipid membrane. (B) Protein-incorporated GUV 1:1:3 POPC:Chol:BSM displaying phase separation prior to antibody binding (confocal slice). (C) GUV 1:1:3 POPC:Chol:BSM displaying phase separation after 1 h incubation with antibody. Signals from 491 and 561 nm excitation indicate successful specific binding of antibody to 5-HT$_{1A}$. All scale bars are 5 μm.

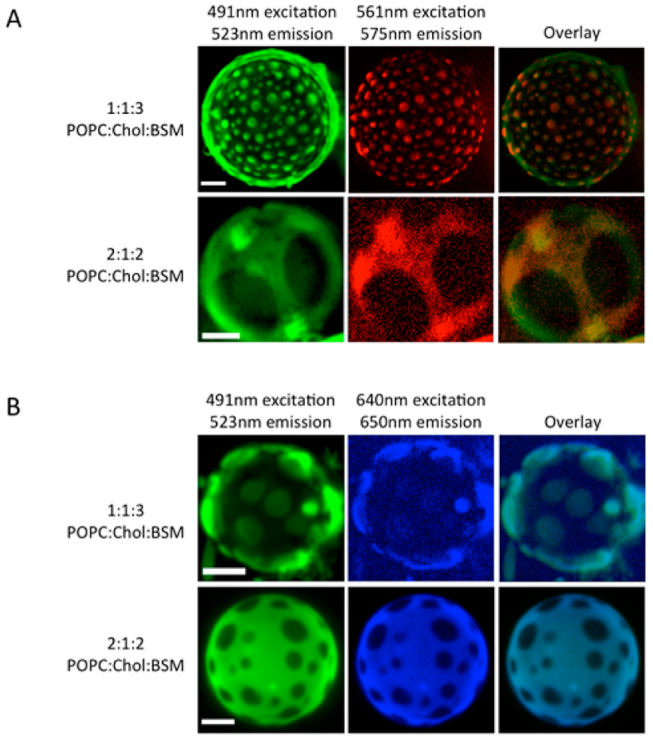

Figure 2. Antibody labeling and ligand binding results on protein incorporated GUVs at varying lipid compositions. (A) GUVs showing protein phase segregation via antibody binding. Excitations at 491 nm (left) and 561 nm (middle) are overlaid in the right image. Top is 1:1:3 and bottom is 2:1:2 POPC:Chol:BSM. This shows preferential segregation of 5-HT$_{1A}$ in the liquid disordered (bright) phase. (B) GUVs at 491 nm (left) and 640 nm (middle) excitation show 5-HT$_{1A}$ segregation into the liquid disordered phase after successful antagonist binding. Top is 1:1:3 and bottom is 2:1:2 POPC:Chol:BSM. Protein preferentially segregates to liquid disordered phase. All scale bars are 5 μm.

cholesterol-poor liquid disordered region. Furthermore, varying concentrations of cholesterol and brain sphingomyelin in the membrane do not affect the partitioning of 5-HT$_{1A}$.

GUVs were made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol (Chol), and brain sphingomyelin (BSM)—a simple tertiary mixture known to phase-separate at certain compositions and temperatures.[19] POPC is found in the outer leaflet of the plasma membrane[29] and is a major component of lipids extracted from biological sources.[30] Previous reports suggest that functionality of 5-HT$_{1A}$ is dependent on sphingomyelin.[12,31] To test the role of sphingomyelin in GPCR phase behavior, GUVs made from varying concentrations of BSM together with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and Chol were also investigated. The DOPC/DPPC-based system has been shown to separate into micrometer-scale liquid domains over a wide range of temperatures and is not subject to photooxidation-based artifacts that have been noted in POPC-based systems.[19,32,33]

Lipid bilayers were labeled with the fluorescent lipid ATTO-488-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (ATTO-488-DPPE), and vesicles were fabricated using the protein incorporation hydration method reported by Hansen et al. in 2013.[24] GUVs were swollen from a lipid film cast over a thin layer of agarose in which 5-HT$_{1A}$ membrane fragments had been dissolved (Figure S1). Protein-free control GUVs were fabricated by omitting the membrane fragments from the agarose. For the assessment of antibody binding, samples were

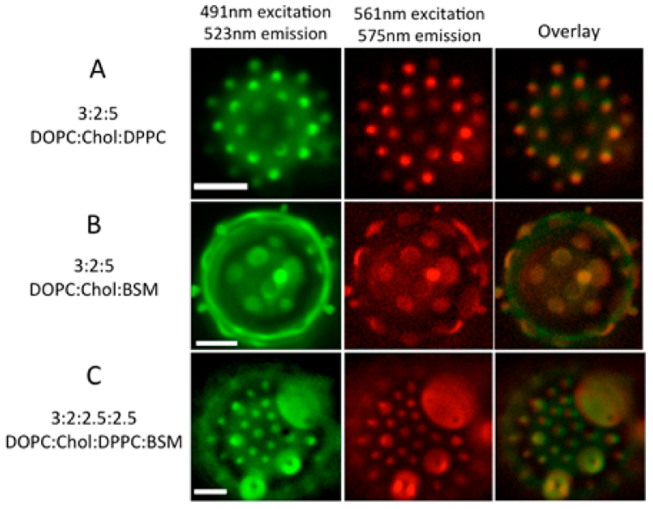

Figure 3. Results from varying BSM ratios in synthetic vesicles, showing no effect on preferential liquid disordered phase segregation of 5-HT$_{1A}$: (A) 3:2:5 DOPC:Chol:DPPC, (B) 3:2:5 DOPC:Chol:BSM, and (C) 3:2:2.5:2.5 DOPC:Chol:DPPC:BSM. All images show phase separation at 491 nm (left) and 561 nm (middle) excitation. 5-HT$_{1A}$ segregates into the liquid disordered phase regardless of sphingomyelin concentration. All scale bars are 5 μm.

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incubated at physiological temperature (37 °C) with 5-HT_{1A} antibodies labeled with rhodamine for 1 h. To confirm the proper folding of the protein, a fluorescent antagonist, a NAN-190 derivative, was used in a binding assay. Samples were exposed to 1 mM of the antagonist at 37 °C for 10 min.[34] Observation chambers were washed with 200 mM glucose in PBS (pH 7.4) to remove excess antibody or ligand. During observation GUVs were held at 30 °C to achieve microscale phase separation.[19] Fluorescence imaging was performed using spinning-disk confocal microscopy and images presented are standard deviation Z-stack projections unless otherwise stated.

Figure 1A shows a GUV without protein after incubation with labeled antibody; no antibody is seen to associate with the membrane. In 5-HT_{1A}-incorporated GUVs, antibody fluorescence can be seen to accumulate on the outer GUV surface during incubation (Figures S2 and S3). Figure 1B shows a protein-incorporated, phase-separated GUV prior to antibody incubation. After protein-containing GUVs were exposed to antibody for 1 h, binding of the labeled antibody to 5-HT_{1A} is clearly observed (Figure 1C). The protein co-segregates with the ATTO-488 fluorescent lipid tag.

Partitioning of ATTO-488-DPPE into the liquid disordered phase was confirmed by measuring domain size as a function of composition—dark domains occupy less vesicle surface area as the concentration of ordered phase-preferring lipids (BSM and Chol) is decreased (Figure S4). These dark regions can be identified as liquid ordered based on previous work showing that this lipid system exhibits liquid−liquid coexistence at the temperatures studied here.[35] Further, the ATTO-488-DPPE segregates in a manner identical to that of rhodamine-labeled DPPE, which has been previously shown to segregate preferentially to liquid disordered domains (Figure S5).[36]

5-HT_{1A} segregates to the liquid disordered phase over a range of compositions spanning the immiscible region of the POPC:Chol:BSM phase diagram. Figure 2A shows two compositions of POPC:Chol:BSM (1:1:3 and 2:1:2) yielding phase-separated GUVs with protein incorporated following antibody binding. 5-HT_{1A} preferentially segregates into liquid disordered phase regardless of sphingomyelin concentration, contradicting previous DRM-based reports.[14,15] This preference for the disordered phase remains the case as Chol concentration is varied across the immiscible region of the phase diagram (Figure S6).

We used a fluorescent antagonist to identify 5-HT_{1A} ligand binding on GUVs. The ligand only associates to GUVs when protein is present (negative control in Figure S7), indicating that the protein is properly folded with an available binding site. Furthermore, as shown in Figure 2B, overlap of fluorescence from the lipid and the ligand is observed, indicating that upon binding of the antagonist, 5-HT_{1A} remains in the liquid disordered phase.

To further understand the role of sphingomyelin on the phase separation of 5-HT_{1A} in GUVs, we prepared phase-separated GUVs in which BSM was replaced in part or completely with DPPC. Replacing POPC with DOPC in this system facilitates phase separation with DPPC (which we used as a saturated lipid to substitute for BSM) and eliminates potential photooxidation artifacts. Ternary compositions of 3:2:5 DOPC:Chol:DPPC and 3:2:5 DOPC:Chol:BSM and a quaternary composition of 3:2:2.5:2.5 DOPC:Chol:DPPC:BSM were investigated (Figure 3). In all compositions, 5-HT_{1A} is observed to partition into the liquid disordered phase suggesting that sphingomyelin has no particular effect on partitioning.

The membrane fragment-incorporated GUVs that we demonstrate here allow for the first direct observation of the phase behavior of GPCRs in model membranes. Our observations contradict conclusions from DRM-based studies and show that 5-HT_{1A} resides in the liquid disordered phase of membranes. Sphingomyelin does not have an effect on the preferential segregation of 5-HT_{1A}. This work provides a foundation for further investigations to characterize GPCRs through microscopic observation of model membranes.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental data, materials section, Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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