Biophysical Journal, Volume *110*

Supplemental Information

The Functional Activity of the Human Serotonin 5-HT_{1A} Receptor Is Controlled by Lipid Bilayer Composition

M. Gertrude Gutierrez, Kylee S. Mansfield, and Noah Malmstadt

The functional activity of the human serotonin 5-HT$_{1A}$ receptor is controlled by lipid bilayer composition

M. G. Gutierrez, K. Mansfield, and N. Malmstadt*

Supporting Material

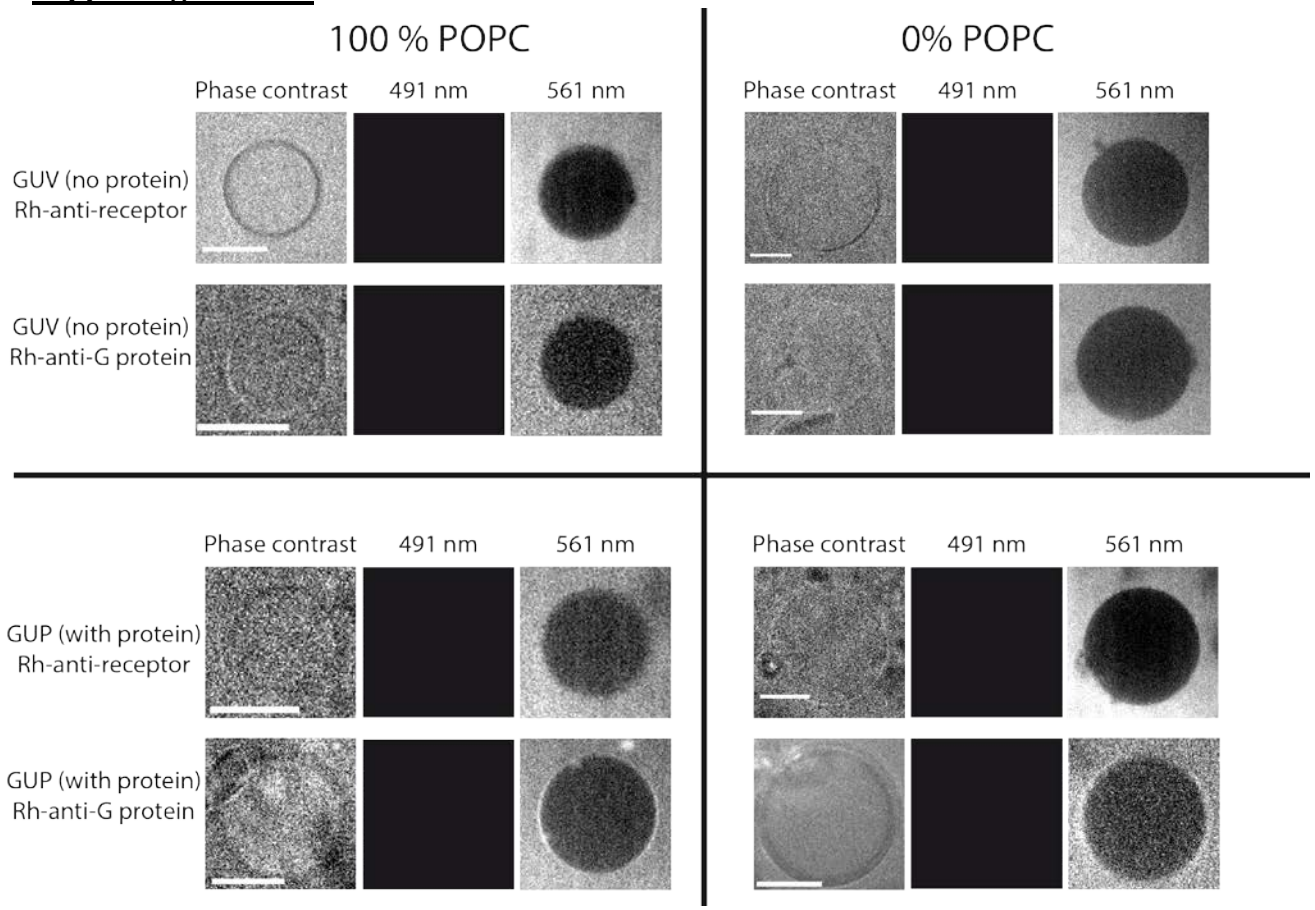


Figure S1. 100% POPC and 0% POPC (3:2 BSM:Chol) vesicles were formed without (GUV) and with (GUP) protein and then incubated with rhodamine-labelled antibody for either receptor (Rh-anti-receptor) or G protein (Rh-anti-G protein). Vesicles did not contain fluorescently labeled lipids and did not show any excitation under 491 nm. After incubation with rhodamine-antibodies, vesicles were imaged using phase contrast and 561 nm excitation for rhodamine visualization. In vesicles without protein (GUVs) neither antibody showed interaction with the surface, as indicated by a lack of fluorescence intensity accumulation at the surface of the vesicles. In protein-incorporated vesicles (GUPs), incubation with Rh-anti-receptor also showed no nonspecific interaction. Note that this is because the antibody is specific to the cytoplasmic face of the receptor, and the receptor is oriented such that this face is in the interior of the GUP. GUPs incubated with Rh-anti-G-protein showed fluorescence at the surface of the vesicles because G proteins are distributed on both leaflets (see Fig 2, S3). Scale bars are 10 μm.

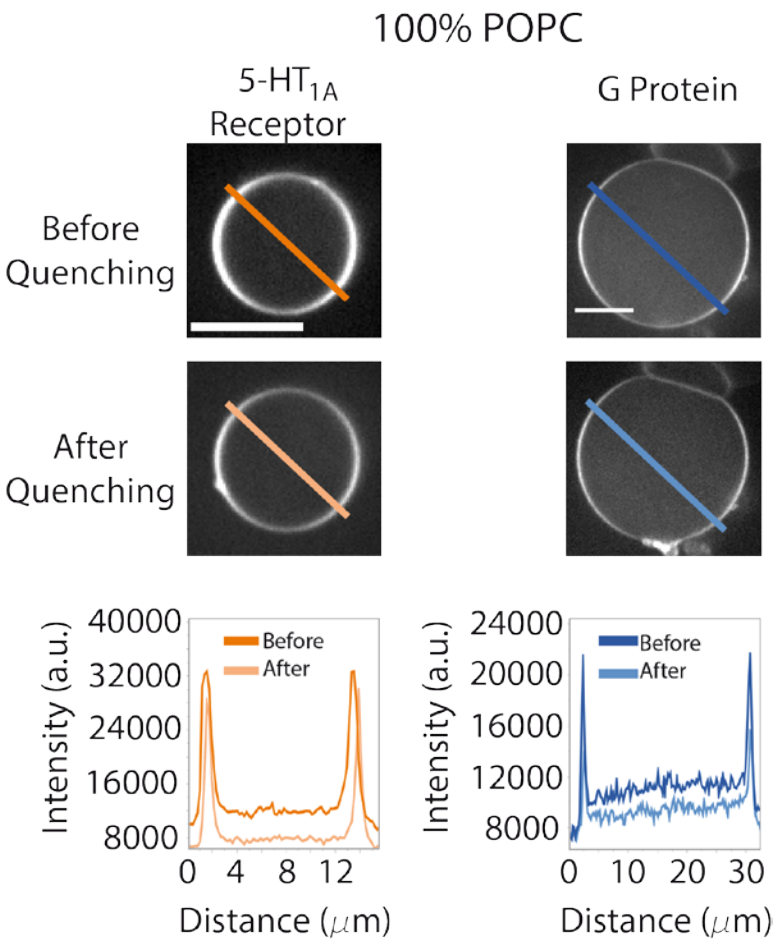


Fig S2. Examples of fluorescence quenching image analysis. All vesicles in these images were made of 100% POPC. The left micrographs show the 5-HT_{1A} receptor with rhodamine-antibody tagged on its cytosolic face. Note that for these experiments, antibody labeling was performed prior to vesicle formation so that the cytoplasmic face of the receptor was accessible. The right micrographs show GUPs with the G proteins tagged with rhodamine-antibody. The top row shows vesicles prior to incubation with QSY7 and bottom row shows GUPs after QSY7 quenching. The plots show the fluorescent intensities across the same line segment on the before and after images. Fluorescence intensity analysis was performed using values from the plots below the micrographs. The receptor antibody retained 90% of its intensity while the G protein retained ~60% of its intensitiy. This indicated a biased orientation of the receptor upon incorporation into GUPs.

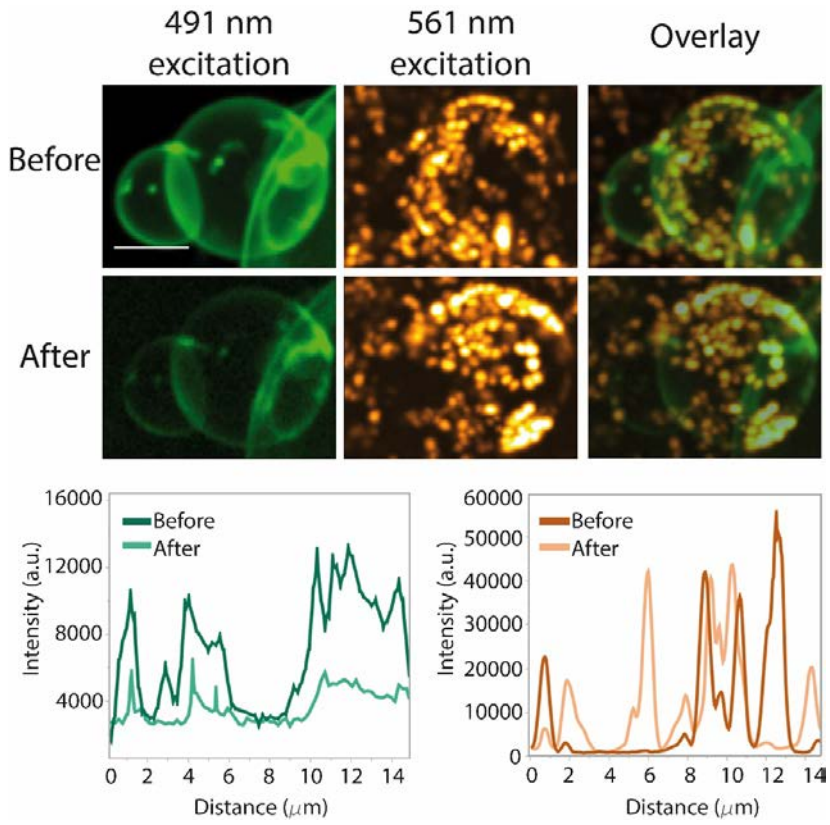


Figure S3. QSY7 does not cross GUP membranes. GUPs encapsulating 400 nm liposomes were imaged and quenched using QSY7. GUPs were tagged with ATTO-488-DPPE and imaged under 491 nm excitation. GUP composition is 15:3:2 POPC:BSM:Chol (75% POPC). 400 nm liposomes were prepared using ATTO-550-DPPE and fabricated in the same way as control GUPs without protein then subsequently extruded through a 400 nm filter. Liposomes were mixed with agarose during fabrication. GUPs were harvested and settled to remove excess liposomes in the surrounding buffer. After incubation with QSY7 for ten minutes at room temperature GUPs showed an intensity decrease of 53.2% ± 2.7% while encapsulated liposomes did not show any significant decrease in intensity (< 3% difference). QSY7 did not cross the bilayer membrane of our GUPs and the QSY7 effectively quenched fluorophore on the exterior of GUPs. Micrographs are Z-stack standard deviation projections using confocal microscopy. Scale bar is 5 μm.

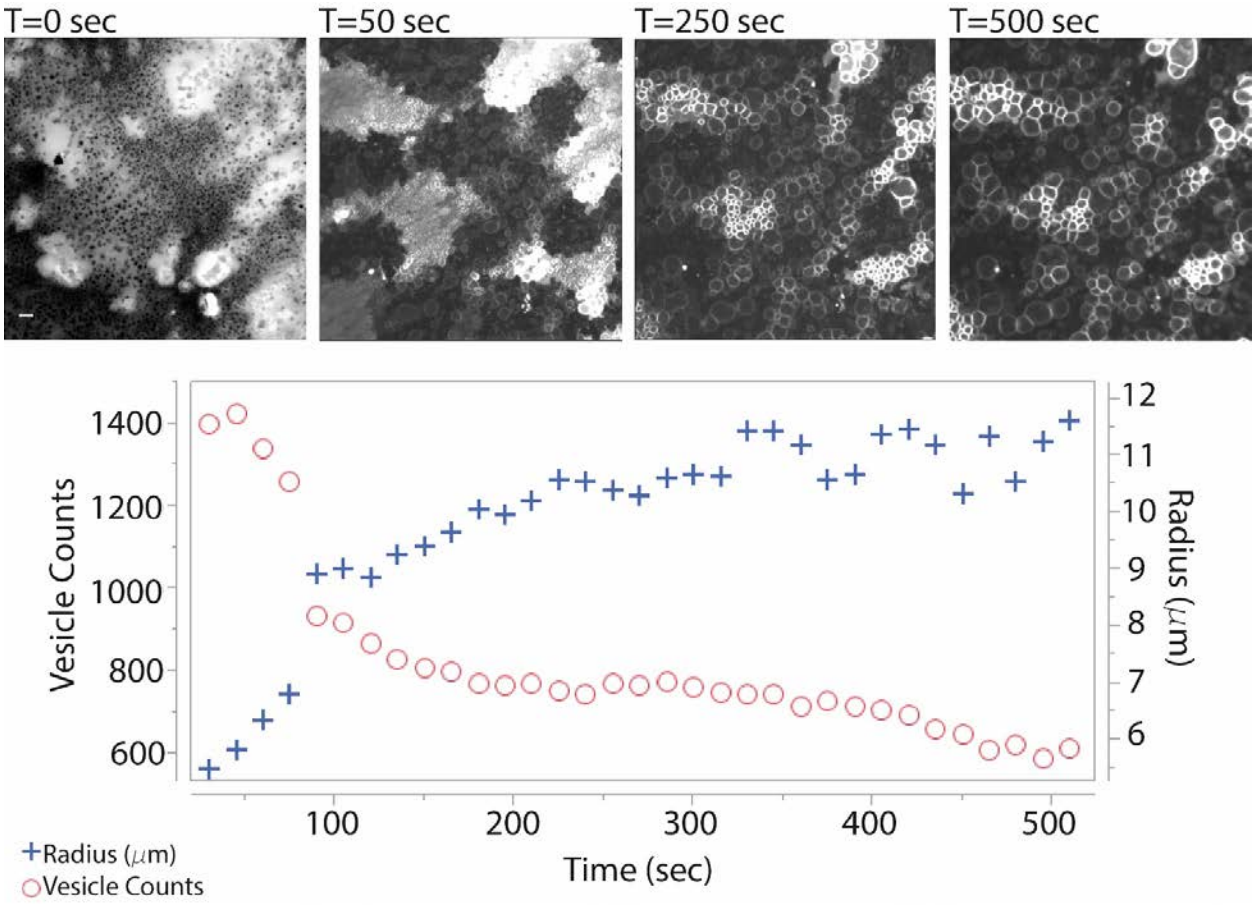


Fig S4. Vesicle growth from agarose lipid film. The top micrographs show example images of the agarose lipid film at the indicated time points during the hydration and vesicle swelling process. Vesicles form as small vesicles that coalesce over time to form giant unilamellar vesicles. A 100% POPC lipid mixture was used with 0.2%mol ATTO-488-DPPE as the fluorescent label. Micrographs are epifluorescent images. Scale bar is 20 μm. Systematic analysis of the film using ImageJ particle analyzer is plotted below the micrographs. As time progressed the number of vesicles decreased while the mean radius of the vesicle population increased, indicating vesicle fusion as a means of vesicle formation using the agarose hydration approach *(28)*.

Validation of activity assay

The measurement of receptor-catalyzed oligonucleotide exchange rates for GUPs was done through an activity assay as described in the Methods and Materials section in the main text. In order to validate this experimental approach, BODIPY-GTPγS autofluorescence, protein thermal stability, and GUP response to varying amounts of agonists were evaluated. Throughout the following discussion, 100% POPC refers to vesicles fabricated from pure POPC while 0% POPC refers to vesicles fabricated from a 3:2 molar ratio of BSM:Chol.

To determine the degree of non-specific interaction between BODIPY-GTPγS and lipids, GUVs of 100% POPC and 0% POPC (60%BSM:40%Chol) were made without protein. These GUVs were made using the same methods as GUP formation but omitting the protein from the agarose. GUVs contained BODIPY-GTPγS and were subjected to fluorescence intensity microplate reading at 37 ºC for 12 hours with and without the addition of agonist, 8-OH-DPAT. As shown in Figure S5, the percent intensity increase was roughly 5% for both GUV compositions and for both control (Ctl) and agonist exposed (+Ag) conditions. This amount of fluorescence intensity increase was significantly less than the typical fluorescence increase observed in GUPs with and without agonist. GUPs displayed a fluorescence intensity increase above 75% (normalized to 1 elsewhere in this work for ease of comparison of rates).

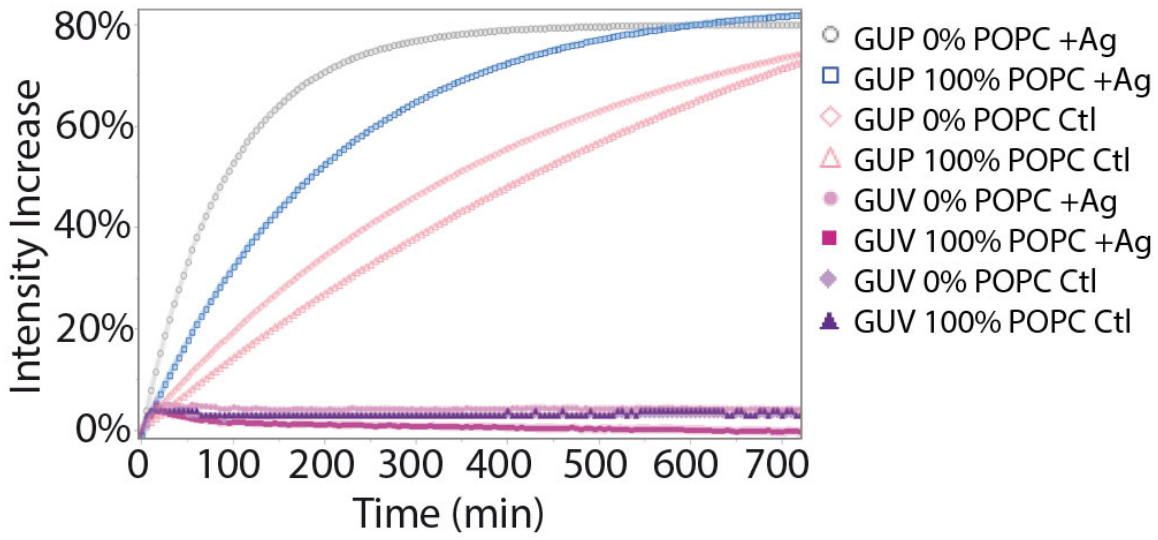


Fig S5. Nonspecific BODIPY-GTPγS fluorescence with GUVs. GUVs without protein were formed from 100% POPC and 0% POPC lipid compositions and incubated with (+Ag) and without agonist (Ctl). GUVs showed less than 5% fluorescence intensity increase while GUPs of the same composition showed over 75% fluorescence intensity increase. BODIPY-GTPγS interaction with GUVs yielded insignificant fluorescence.

To determine expected protein thermal stability over the 12-hour experimental course at 37 ºC, 5-HT_{1A}R membrane preparations were subjected to pre-incubation at 37 ºC for 0, 6, 18, and 24 hours prior to being used for GUP formation and subsequent evaluation of receptor-catalyzed oligonucleotide exchange via the activity assay. As seen in Figure S6 and Table S1, at 0hr, 6hr, and 18hr time points, the rates of receptor activity in GUPs of 100% POPC and 0% POPC were not statistically different. This indicated that the protein was stable up to 30 hours of incubation at 37 ºC. At 24 hours of pre-incubation however, the protein displayed no significant fluorescence increase and thus no rate was obtained from the activity assay. Thus, sometime between 30 hours (18 hours of pre-incubation) and 36 hours (24 hours of pre-incubation) the system failed to show protein activity.

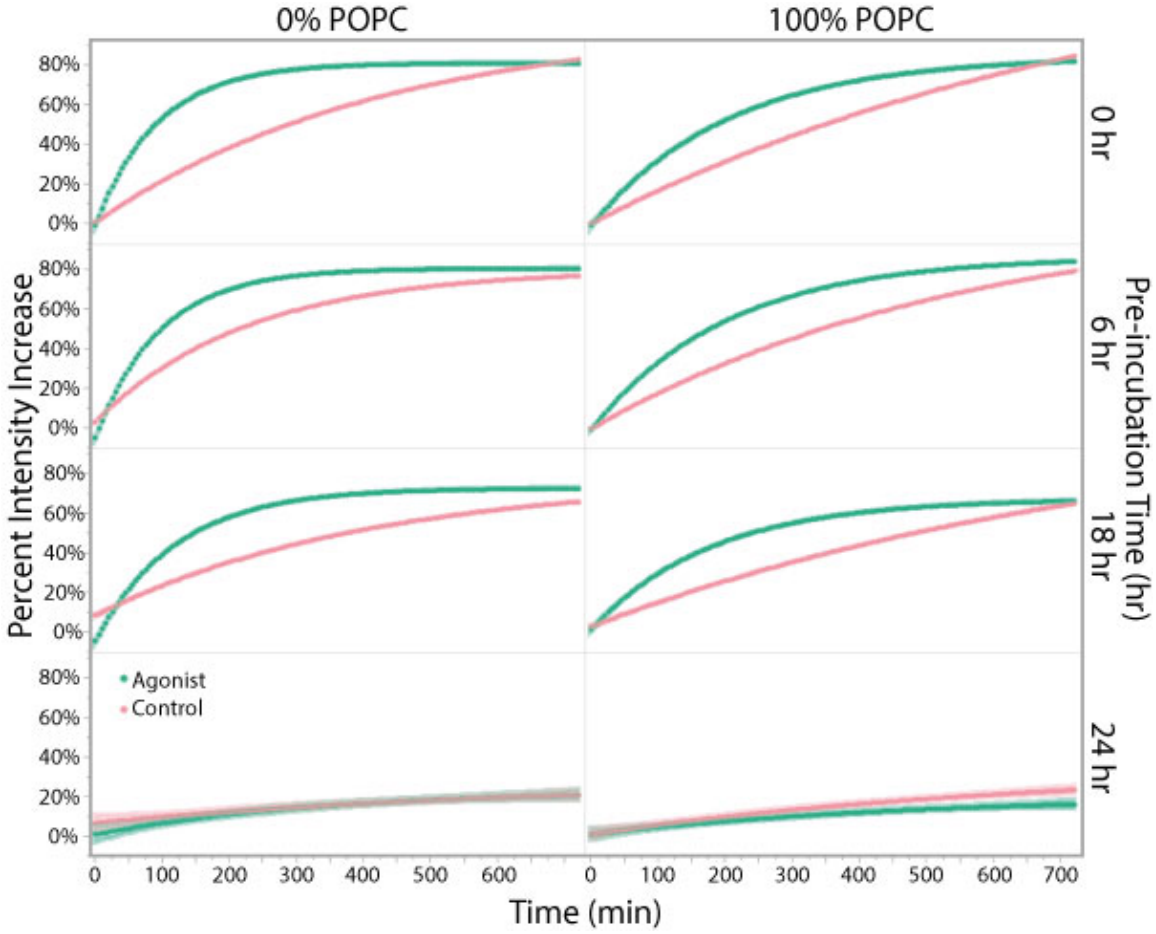


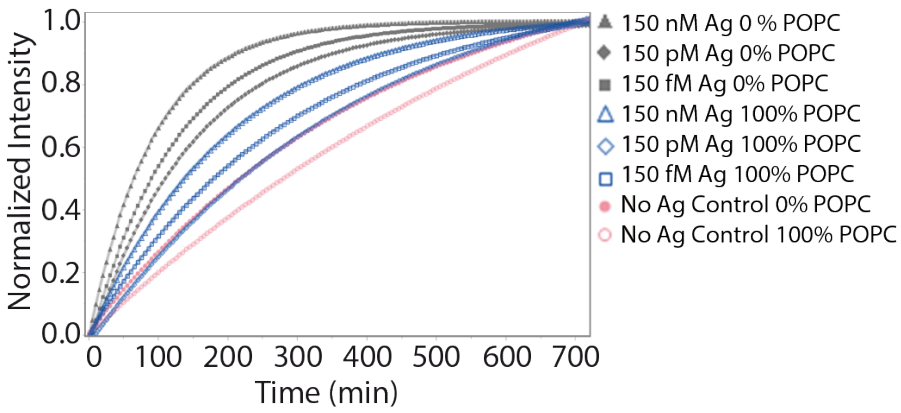
Figure S6. Protein thermal stability investigated by pre-incubating membrane fragments. 5-HT_{1A} membrane fragments were pre-incubated at 37 ºC for 0, 6, 18, and 24 hours and then incorporated in GUPs for the 12-hour activity assay. GUPs were made of 0% POPC or 100% POPC. Percent intensity increase was tracked over time and plotted. Plots are an average of 6 replicates and shaded areas around the points of the curve are the standard error of the mean. 0, 6, and 18 hr pre-incubated protein samples retained activity and fluorescence intensity increase. 24 hr pre-incubated GUPs did not display significant fluorescence intensity increase over time.

Table S1. 5-HT_{1A} thermal stability. 24 hr time point displayed no measurable protein activity, see Figure S5.

Pre - Incubation time	0% POPC +Ag Rate +/- Std Error (10^{-3} min^{-1})	0% POPC Ctl Rate +/- Std Error (10^{-3} min^{-1})	100% POPC +Ag Rate +/- Std Error (10^{-3} min^{-1})	100% POPC Ctl Rate +/- Std Error (10^{-3} min^{-1})
0 hr	10.83 +/- 0.26	3.24 +/- 0.05	4.26 +/- 0.09	1.23 +/-0.05
6 hr	10.47 +/- 0.10	4.41 +/- 0.04	4.96 +/- 0.54	1.49 +/- 0.49
18 hr	9.63 +/- 0.21	3.45 +/- 0.15	5.06 +/- 0.19	1.20 +/- 0.12
24 hr	N/A	N/A	N/A	N/A

To observe a pharmacological response to the addition of ligand, GUPs of 100% POPC and 0% POPC were exposed to increasing amount of agonist. GUPs were formed and assayed as previously described and 0 M, 150 fM, 150 pM, or 150 nM of agonist was added immediately prior to fluorescence intensity reading for 12 hours. As expected, increasing amounts of agonist resulted in increased rates of 5-HT_{1A}R catalyzed oligonucleotide exchange (Figure S7). The rates did not, however, display a logarithmic increase due to the presence of antagonist spiperone.

A



B

8-OH-DPAT Concentration	0% POPC Rate (10^{-3} min^{-1})	0% POPC Std Error (10^{-3} min^{-1})	100% POPC Rate (10^{-3} min^{-1})	100% POPC Std Error (10^{-3} min^{-1})
150 nM	▲ 10.83	0.26	△ 4.26	0.09
150 pM	◆ 8.14	0.13	◇ 3.43	0.76
150 fM	■ 6.75	0.15	□ 2.54	0.07
0 (control)	● 3.24	0.05	○ 1.23	0.05

Fig S7. Increasing agonist concentration. GUPs of 100% POPC and 0% POPC were formed and subjected to activity assay with increasing amounts of 8-OH-DPAT, 0 M, 150 fM, 150 pM, and 150 nM. A) Increasing the amount of agonist in the assay displayed increasing rates of intensity increase. B) Activity rates corresponding to curves in A.

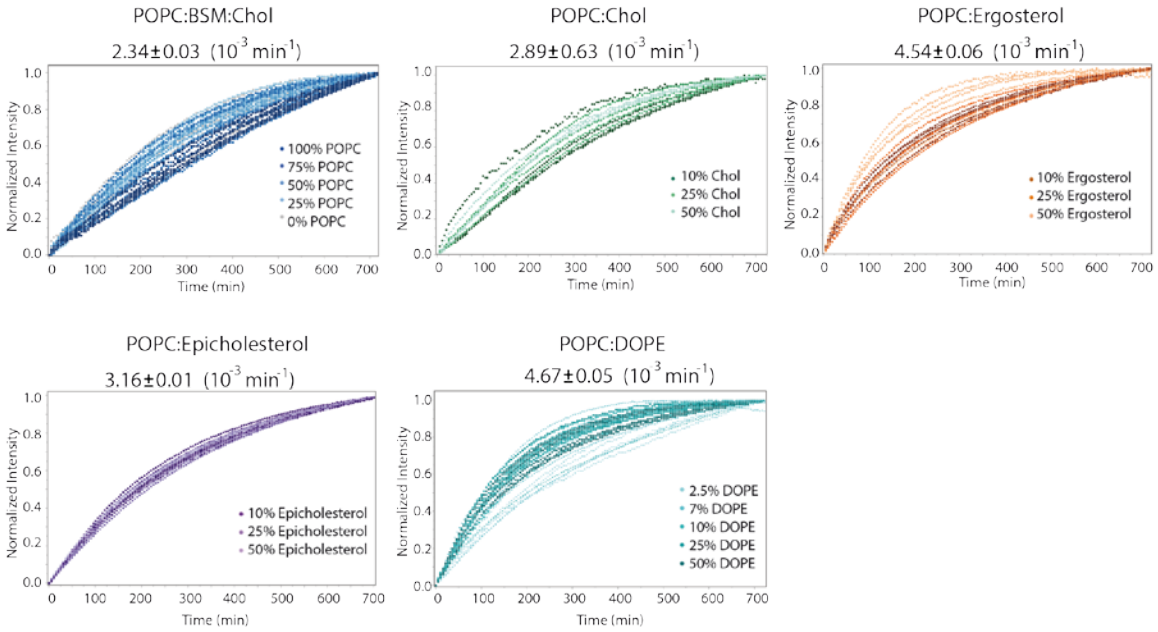
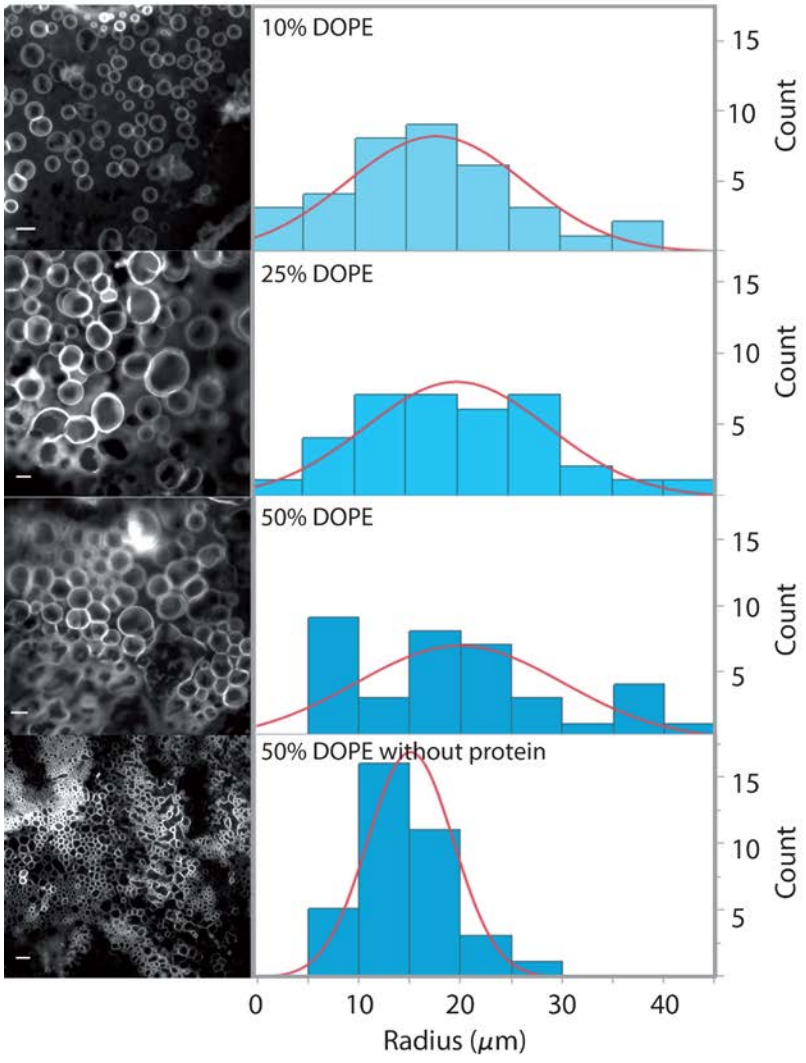


Fig S8. Control fluorescence intensity curves for all compositions investigated. These data represent protein activity with no agonist added; each curve represents a single sample. Each plot is indicated by the lipid system and the basal rate for that system as determined by a single exponential fit to the average of all curves shown in the plot. The control curves presented in the main text represent this average.



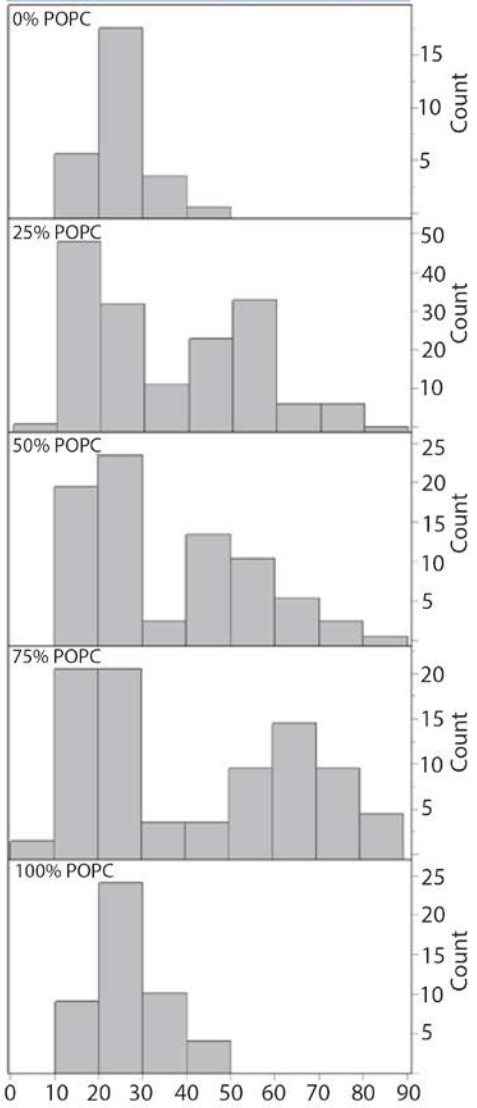
100% DOPE

Membrane Composition		Protein Incorporated	Average Radius	Standard Error Mean
% DOPE	% POPC		(μm)	(μm)
0	100	Yes	27.3	1.2
10	80	Yes	17.8	1.4
25	75	Yes	19.8	1.5
50	50	Yes	19.8	1.7
50	50	No	14.7	0.7
100	0	-	-	-

Fig S9. GUP formation of binary POPC:DOPE compositions. While DOPE is a nonlamellar forming lipid, GUPs were successfully formed in binary mixtures of POPC:DOPE with DOPE at 10%, 25%, 50%. In comparison with forming vesicles without proteins, GUPs of POPC:DOPE were larger in radius. No vesicles or bilayer structures were formed when the lipid film consisted of pure DOPE. The table shows average radii for different POPC:DOPE compositions. These binary compositions were smaller than 100% POPC GUPs (**Fig S10**).

A

Membrane Composition % POPC	Average Radius (μm)	Standard Error Mean (μm)
0	25.0	1.3
25	35.3	1.4
50	36.4	2.1
75	41.7	2.6
100	27.3	1.2



B

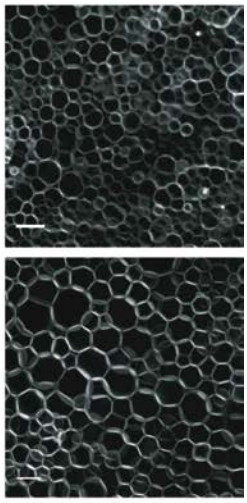


Figure S10. Size distribution of GUPs of POPC:BSM:Chol. GUPs were made as described and settled for imaging. GUPs with rhodamine labeled antibody tagged serotonin receptor were imaged using epifluorescence and size distribution analysis was performed using Image J particle analyzer. (**A**) Shows average radius in μm for each of the different compositions of GUPs, followed by respective histograms. (**B**) Two example confocal micrographs of typical GUP yield using agarose hydration method prior to GUP settling. Scale bar is 10 μm.

Supplementary Videos

Video S1. Intensity increase due to 5-HT_{1A} receptor-catalyzed oligonucleotide exchange in GUPs. Two hour time series of confocal images (8 frames per second, frames were taken at 5 minute intervals).

Video S2. GUP formation on agarose patch. 20 minute time series of epifluorescent images.