A GFP fluorescence-based approach to determine detergent insolubility of the human serotonin$_{1A}$ receptor

Shanti Kalipatnapu, Amitabha Chattopadhyay*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

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Abstract Insolubility in non-ionic detergents such as Triton X-100 is a widely used biochemical criterion for characterization of membrane domains. We report here a novel green fluorescent protein fluorescence-based approach to directly determine detergent insolubility of specific membrane proteins. We have applied this method to explore the detergent resistance of an important G-protein coupled receptor, the serotonin$_{1A}$ (5-HT$_{1A}$) receptor. Our results show, for the first time, that a small yet significant fraction of the 5-HT$_{1A}$ receptor exhibits detergent insolubility. These results are validated by control experiments involving fluorescent lipid probes and protein markers. Our results assume relevance in the context of localization of the 5-HT$_{1A}$ receptor in membrane domains and its significance in receptor function and signaling.

Keywords: Detergent insolubility; Enhanced yellow fluorescent protein fluorescence; DiI; Membrane domain; Triton X-100; Serotonin$_{1A}$ receptor

1. Introduction

The crucial role of organization of lipids and proteins in membranes and its relevance in membrane function is being increasingly recognized [1–3]. There is growing evidence for the concept of membranes being organized into domains with defined lipid and protein compositions. These domains, sometimes referred to as 'rafts', are believed to serve as platforms for signaling by concentrating certain lipids (such as cholesterol and sphingolipids) and proteins while excluding others [2–5]. Work from several laboratories has suggested that organization of membranes into domains could play a key role in a number of cellular functions such as membrane trafficking, sorting, signal transduction, and pathogen entry [2–8]. The presence of a diverse variety of lipids in biological membranes presents an interesting possibility of phase separation among lipids with different phase properties, thereby resulting in segregation of the membrane into domains [2,9]. Insolubility in non-ionic detergents such as Triton X-100 is a widely used biochemical criterion for characterization of certain membrane domains [10–13]. Early work which demonstrated the phenomenon of insolubility of membrane components in cold non-ionic detergents such as Triton X-100 [10,14,15] has been recently explained on the basis of phase separation in membranes [2]. This is reinforced by results from model membrane studies which show that enrichment with lipids such as sphingolipids (with high melting temperature) and cholesterol serves as an important determinant for the phenomenon of detergent resistance [16,17]. The tight acyl chain packing of sphingolipids and saturated lipids is thought to confer detergent resistance to membrane regions enriched in these lipids and to the proteins which reside in them.

The concept of membrane domains has evolved over the last few years with the increasing application of sensitive fluorescence-based approaches, and advanced microscopic and imaging techniques for the detection and visualization of domains in biological and model membranes [1,18–20]. It may not, however, be convenient to utilize these approaches either due to the technical and conceptual difficulty encountered for application to cellular systems, or due to the requirement of specialized equipment and expertise not always readily available in biochemical laboratories. The need for relatively simple and straightforward biochemical methods for detecting membrane domains therefore persists. Resistance to solubilization by mild non-ionic detergents such as Triton X-100 at low temperature has emerged as an extensively used biochemical tool to identify, isolate and characterize certain types of membrane domains [10–13,21]. Thus, insolubility in cold Triton X-100 has been increasingly used as a hallmark of the presence of 'rafts', a class of membrane domains enriched in sphingolipids and cholesterol [4]. Several GPI-anchored proteins, few transmembrane proteins and certain G-proteins have been found to reside in detergent resistant membrane domains, popularly referred to as DRMs [10–13,21].

Conventionally, detection of proteins in DRMs is performed either by immunoblotting or ligand binding. However, these methods are not suitable in cases where ligand binding of the protein in question is compromised in the presence of the detergent [22] and/or is limited by availability of antibodies with high specificity [23]. In order to overcome such difficulties, we report here a novel green fluorescent

* Corresponding author. Fax: +91-40-2716-0311.
E-mail address: amit@ccmb.res.in (A. Chattopadhyay).

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 5-HT$_{1A}$R-EYFP, 5-hydroxytryptamine-1A receptor tagged to EYFP; DiI, dialkylindocarbocyanine; DiIC$_{16}$, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; EYFP, enhanced yellow fluorescent protein; *FAST* DiI, 1,1′-dilinoleyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate; GFP, green fluorescent protein; GPCR, G-protein coupled receptor

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protein (GFP) fluorescence-based approach to directly determine detergent insolubility of specific membrane proteins expressed in cells in culture. We have applied this method to explore the detergent resistance of an important G-protein coupled neurotransmitter receptor, the serotonin$_{1A}$ (5-HT$_{1A}$) receptor. We show here, for the first time, that a small fraction of the 5-HT$_{1A}$ receptor is insoluble in the non-ionic detergent Triton X-100. This approach is validated through control experiments performed with membrane domain-specific fluorescent lipid probes and protein markers.

The 5-HT$_{1A}$ receptor belongs to the family of G-protein coupled receptors (GPCRs) and is the best characterized among the serotonin receptors [24,25]. It is involved in a variety of cognitive and behavioral functions including memory, anxiety and depression and in neural development. The 5-HT$_{1A}$ receptor is implicated in several anxiety-related disorders and the 5-HT$_{1A}$ receptor knockout mouse serves as an excellent model system to understand anxiety-related behavior in higher animals [26]. We have earlier solubilized and characterized the 5-HT$_{1A}$ receptor in native hippocampal membranes as well as in cellular systems [27–29]. Work from our laboratory has shown that membrane cholesterol modulates ligand-binding properties and G-protein coupling of the 5-HT$_{1A}$ receptor [30,31]. This report provides novel information on the detergent insolubility of the 5-HT$_{1A}$ receptor. In addition, it presents a GFP fluorescence-based approach which can be utilized in exploring membrane organization of other GPCRs.

2. Materials and methods

2.1. Materials

Gentamycin sulfate, penicillin, sodium bicarbonate and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum, DMEM/F-12 (Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1)) and geneticin (G 418) were from Invitrogen Life Technologies (Grand Island, NY). Texas-red labeled transferrin, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC$_{16}$) and 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (*FAST* DiI) were obtained from Molecular Probes (Eugene, OR). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Photoetched grid coverslips were purchased from Bellco (Vineland, NJ).

2.2. Cells and cell culture

CHO-K1 cells or CHO-K1 cells stably expressing the serotonin$_{1A}$ receptor, tagged to enhanced yellow fluorescent protein (referred to as CHO-5-HT$_{1A}$R-EYFP), were used. Cells were grown in DMEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamycin sulfate in a humidified atmosphere with 5% CO_2 at 37 °C. CHO-5-HT$_{1A}$R-EYFP cells were maintained in the above-mentioned conditions with 300 μg/ml geneticin. These cells, when used for detergent extraction and fluorescence microscopy experiments, were grown in glass bottom dishes made by replacing the bottoms of 35-mm plastic tissue culture dishes with photoetched grid coverslips as previously described [32].

2.3. Cell labeling

CHO-K1 cells were plated on glass coverslips and grown for 2 days under conditions described above. Cells were washed twice in cold HEPES–Hanks buffer (pH 7.4) before labeling them with either membrane domain-specific fluorescent lipid probes, DiIC$_{16}$ and *FAST* DiI, or fluorescently labeled transferrin. Stock solutions of the dialkylindocarbocyanine (DiI) probes were made in ethanol and diluted in HEPES–Hanks buffer to prepare the labeling solutions making sure that the residual ethanol concentration was always <1% (v/v). Cells were labeled with either 8 μM DiIC$_{16}$ for 75 min or 14 μM *FAST* DiI for 35 min at 4 °C. Stock solution of Texas-red labeled transferrin was made in PBS. Cells were labeled with 100 μg/ml Texas-red labeled transferrin in HEPES–Hanks buffer for 30 min at 37 °C. Labeled cells were washed three times in cold HEPES–Hanks buffer before detergent extraction.

2.4. Extraction with Triton X-100

CHO-5-HT$_{1A}$R-EYFP cells were plated in glass bottom dishes and grown in DMEM/F-12 medium for 2 days. The medium was washed off and cells were imaged in HEPES–Hanks buffer to record the fluorescence intensity before detergent extraction. Cells were then incubated with 0.05% (w/v) cold Triton X-100 for 10 min on ice. The detergent solution was then removed and cells were carefully washed in cold HEPES–Hanks buffer before imaging the same group of cells whose fluorescence intensity was recorded before detergent extraction. CHO-K1 cells grown on glass coverslips were labeled with fluorescent lipid probes or with Texas-red labeled transferrin as described above and extracted with cold Triton X-100 under identical conditions. Control experiments, where cells were processed through all steps of the extraction procedure using cold HEPES–Hanks buffer without any detergent, were carried out in parallel. These cells were imaged after mounting the coverslips on a glass slide.

2.5. Fluorescence microscopy and image analysis

CHO-5-HT$_{1A}$R-EYFP cells were imaged using a Meridian Ultima 570 confocal laser scanning microscope system attached to an inverted Olympus fluorescence microscope. The same group of cells was imaged before and after detergent extraction. Optical sections of the cells were recorded using a 60×, 1.4 NA oil-immersion objective using the 514-nm line of an Ar laser at a *z*-slice thickness of 0.5 μm. Fluorescence emission was collected using the 505–535 nm bandpass filter. Image analysis was carried out using the Meridian DASY Master Program v4.19. Sections of the cells largely representing the cell plasma membrane were selected and projected together resulting in a single combined image of the chosen sections. Outlines of each cell (or a small group of cells) were drawn out and integrated fluorescence intensities were determined using the Meridian DASY Master program. Integrated fluorescence intensities of each cell or a group of cells before and after detergent extraction were monitored. Detergent insolubility of the receptor was estimated by determining the residual fluorescence of cells upon detergent treatment. In the case of cells labeled with membrane domain-specific fluorescent lipid probes or Texas-red labeled transferrin, imaging was carried out using an inverted Zeiss LSM 510 Meta confocal microscope with a 63×, 1.2 NA water-immersion objective using the 543-nm line of a He–Ne laser. Fluorescence emission was collected using a 560 nm longpass filter for DiI probes and a 565–615 nm bandpass filter for Texas-red labeled transferrin. Midplane confocal sections of detergent extracted cells and control cells were acquired.

3. Results

CHO-K1 cells stably expressing the 5-HT$_{1A}$ receptor tagged to EYFP (previously known as GFP-10C), a red-shifted emission variant of GFP [33], were used to assess detergent insolubility of the 5-HT$_{1A}$ receptor. These cells display typical plasma membrane localization of 5-HT$_{1A}$R-EYFP characterized by greater fluorescence intensity at the cell periphery (Fig. 1A). The ligand-binding properties, G-protein coupling and signaling functions of the 5-HT$_{1A}$R-EYFP, were found to be similar to the native receptor (Pucadyil et al., submitted for publication). CHO-K1 cells expressing 5-HT$_{1A}$R-EYFP, therefore, represent a reliable model system to explore the membrane organization of the 5-HT$_{1A}$ receptor. CHO-K1 cells stably expressing 5-HT$_{1A}$R-EYFP were treated with the non-ionic detergent Triton X-100 and imaged (Fig. 1B) as described in Section 2. Fluorescence intensity of the same group of cells

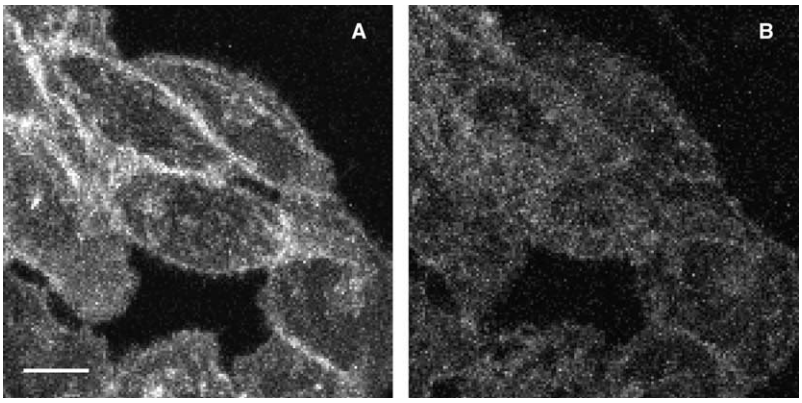

Fig. 1. Visualization of detergent insolubility of the 5-HT_{1A}R-EYFP: (A) CHO-K1 cells stably expressing 5-HT_{1A}R-EYFP and (B) after treatment with 0.05% (w/v) cold Triton X-100 for 10 min. Cells were then washed and imaged as described in Section 2. The images represent combined mid-plane confocal sections of the same group of cells before and after detergent extraction. Scale bar represents 10 μm. See Section 2 for other details.

before and after detergent treatment was quantitated using the Meridian DASY Master program. This analysis showed that ~26% of 5-HT_{1A}R-EYFP fluorescence is retained upon detergent extraction. This represents the fraction of the 5-HT_{1A} receptor which is resistant to detergent treatment under these conditions. Control experiments were performed where the cells were carried through the incubation and washing steps using cold HEPES–Hanks buffer without any detergent. Quantitation of fluorescence intensity for the control cells did not show any significant change in fluorescence.

In order to validate this fluorescence microscopic approach toward determination of detergent insolubility of membrane components, we utilized specific lipid and protein markers whose organization in membranes and ability to be extracted by cold non-ionic detergents have been well documented. Incorporation of fluorescent lipid analogs into cellular membranes has proved to be a useful and convenient approach to study lipid metabolism and transport and to explore domain organization in membranes [34,35]. The DiI series of lipid analogs serve as well characterized membrane domain-specific fluorescent probes [36–38]. The DiI analogs are composed of an indocarbocyanine headgroup and two hydrophobic alkyl chains (see Fig. 2), which impart an overall amphiphilic character. They have been shown to exhibit preferential phase partitioning into biological and model membranes of varying degrees of order (fluidity) depending on the relative headgroup to tail cross-sectional areas and the chain length [36–38]. In systems with coexisting ordered and disordered phases, an approximate match of the probe alkyl chain length with those of the host lipid acyl chains leads to a preferential partitioning of the probe into the ordered phase [36,37]. The alkyl chain length of $DiIC_{16}$ (shown in Fig. 2A) approximately matches that of most prevalent lipids of various CHO cell lines [39]. In CHO cell membranes, $DiIC_{16}$ would therefore be expected to preferentially partition into relatively rigid (highly ordered) domains. On the other hand, *FAST* DiI, the other DiI probe used (Fig. 2B), has two 18-carbon chains with two cis double bonds in each chain. This makes the ratio of headgroup to tail cross-sectional area such that this probe preferentially partitions into fluid domains in membranes [38], since packing of this probe would not be very efficient in an ordered phase.

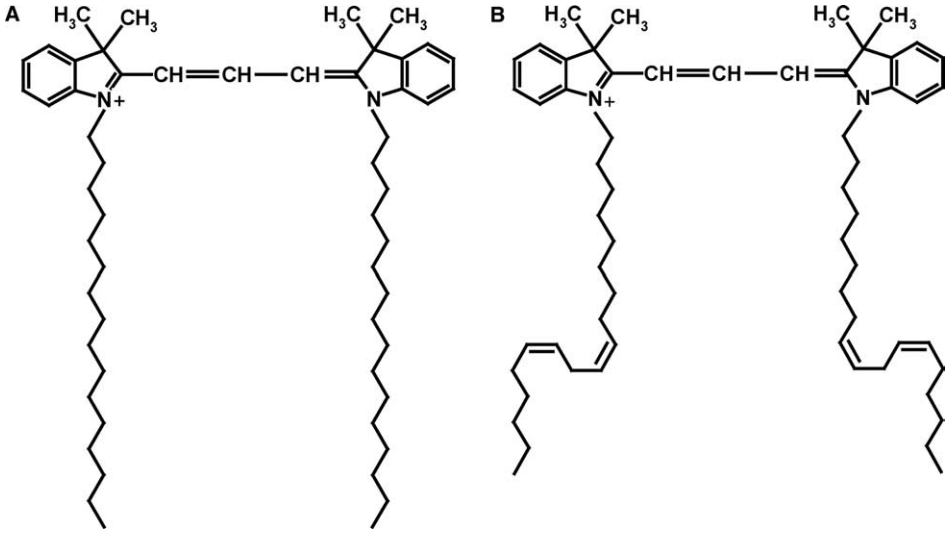

Fig. 2. Chemical structures of membrane domain-specific fluorescent lipid probes used: (A) $DiIC_{16}$ (B) *FAST* DiI

Ordered membrane domains and the proteins that partition into them are known to be resistant to detergents [2]. Accordingly, $DiIC_{16}$ would be insoluble in detergent to a greater extent than *FAST* DiI. In order to validate our method of detection of detergent resistant membrane domains using residual GFP fluorescence, CHO-K1 cells were labeled with $DiIC_{16}$ (Fig. 3A and B) or *FAST* DiI (Fig. 3C and D) and extracted with cold Triton X-100. Fluorescence images of the cells in each of these cases were acquired under the same conditions. Control cells (Fig. 3A and C), which were carried through all the steps of extraction in the presence of HEPES–Hanks buffer without any detergent, showed no significant change either in fluorescence distribution or intensity. Upon extraction with detergent, extensive loss in fluorescence was observed in case of cells labeled with *FAST* DiI (Fig. 3D) in contrast to cells labeled with $DiIC_{16}$, which largely retained their fluorescence (Fig. 3B). This shows that the present approach is able to detect ordered domains labeled by $DiIC_{16}$, which preferentially partitions into such regions of the membrane. Further, this approach is capable of distinguishing these ordered regions from the fluid regions of the membrane characterized by *FAST* DiI labeling.

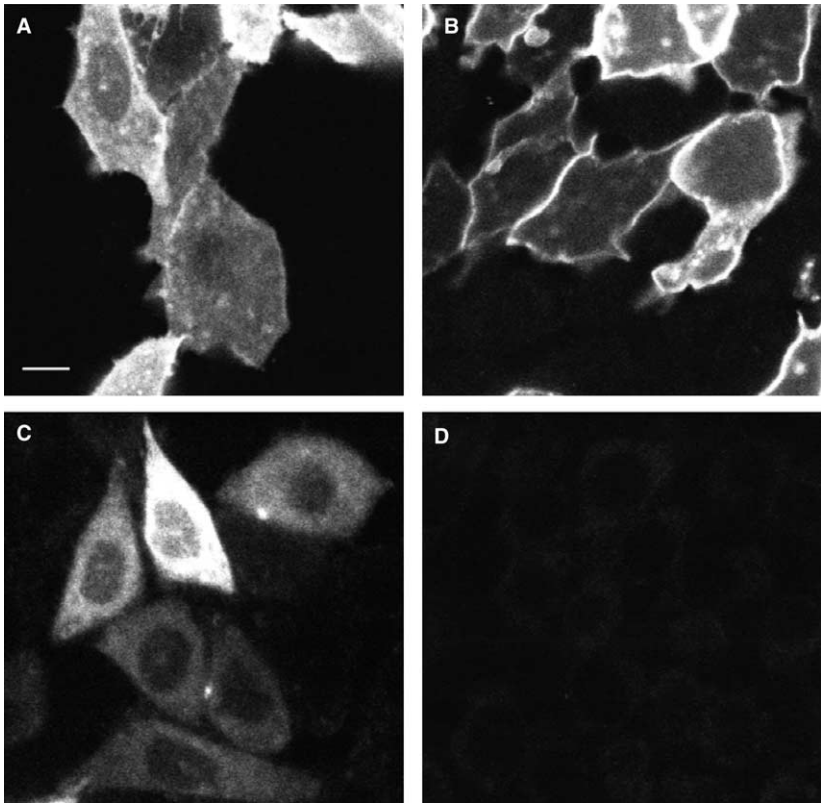

Fig. 3. CHO-K1 cells were labeled with the fluorescent lipid probes $DiIC_{16}$ (panel A) or *FAST* DiI (panel C) and were treated with 0.05% (w/v) cold Triton X-100 for 10 min (panels B and D, respectively). Cells were imaged as described in Section 2. Representative images of control (A and C) and detergent treated cells (B and D) are shown. Scale bar represents 10 μm. See Section 2 for other details.

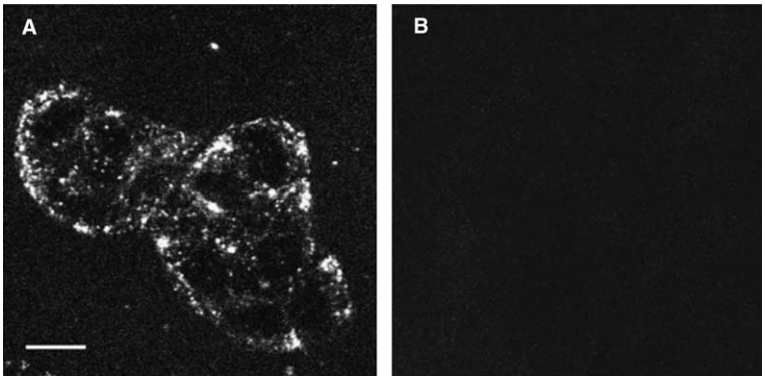

Fig. 4. CHO-K1 cells labeled with Texas-red labeled transferrin were treated with 0.05% (w/v) cold Triton X-100 for 10 min. Cells were imaged as described in Section 2. Representative images of (A) control and (B) detergent treated cells are shown. Scale bar represents 10 μm. See Section 2 for other details.

In order to confirm the validity of this approach using a protein marker, we monitored the insolubility of the transmembrane protein transferrin receptor in Triton X-100. Several earlier reports have shown this receptor to be soluble in Triton X-100 and, therefore, it is often used as one of the controls for detergent insoluble membrane components [12,32]. CHO-K1 cells which endogenously express the transferrin receptor were labeled with Texas-red labeled transferrin. As shown in Fig. 4A, the control cells display typical fluorescence distribution of the transferrin receptor. Interestingly, there is a tremendous loss in fluorescence upon detergent extraction (Fig. 4B), which faithfully reflects the previously characterized phenomenon of solubility of transferrin receptor in Triton X-100. This demonstration lends further support for the fluorescence-based approach we propose to monitor detergent insolubility of the 5-HT_{1A} receptor. Taken together, the combined results using the lipid (DiI) and protein (transferrin receptor) markers validate the GFP fluorescence-based approach in general, and the novel observation of detergent insolubility of the 5-HT_{1A} receptor, in particular.

4. Discussion

As mentioned earlier, detection of a protein in detergent insoluble fractions is usually carried out either by immunoblotting, or ligand binding using radiolabeled or fluorescently labeled ligands. Results from our (Kalipatnapu, S. and Chattopadhyay, A., unpublished observations) and other laboratories [22] have shown that ligand binding to the 5-HT_{1A} receptor is inhibited in the presence of Triton X-100. Moreover, this effect appears to be irreversible since removal of the detergent does not restore ligand binding, thereby ruling out the possibility of detection of the receptor in detergent treated membranes using radioligand binding. The immunoblotting approach may not be suitable for the 5-HT_{1A} receptor, since no monoclonal antibodies for this receptor are available yet, and the polyclonal antibodies have been reported to give variable results in immunoblotting experiments [23]. It is therefore difficult to assess detergent insolubility of the 5-HT_{1A} receptor by immunoblotting. This prompted us to explore detergent insolubility of this receptor using GFP fluorescence. We show here for the first time, using this novel approach, that a small yet significant fraction of the 5-HT_{1A} receptor exhibits detergent insolubility.

It is important to mention here that detergent insolubility does not necessarily ensure localization of a certain membrane component in membrane domains [12,19,40,41]. In addition, some of the known important criteria for detergent insolubility such as requirement of cholesterol have been critically assessed [42]. Nonetheless, the phenomenon of detergent insolubility continues to represent one of the principal tools available to probe membrane domain organization. In addition, information obtained utilizing this extensively used biochemical approach can form the basis for a more detailed analysis of membrane domains. When viewed from this perspective and in the light of the lack of literature reports on detergent insolubility of the 5-HT_{1A} receptor, these results represent a significant step in understanding the membrane organization of this receptor. Interestingly, the efficiency of signal transduction processes carried out by GPCRs appears to be influenced by the local composition and organization of lipids within the plasma membrane [43–45]. Therefore, the organization of GPCRs such as the 5-HT_{1A} receptor in membranes and their interaction with the membrane lipid environment could modulate their function [30]. More importantly, the fact that 50% of clinically prescribed drugs act through GPCRs [46] highlights their crucial role in human health and brings to light the need to understand the role of the membrane environment in their function.

Our results, therefore, assume relevance in the context of localization of the 5-HT_{1A} receptor in membrane domains and its significance in signaling by the receptor. To the best of our knowledge, this report represents one of the first attempts toward exploring the organization of this important neurotransmitter receptor in membrane domains. Importantly, the method of analysis of detergent insolubility reported here could be potentially useful in exploring membrane localization of other GPCRs.

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