



Metabolic depletion of sphingolipids enhances the mobility of the human serotonin_{1A} receptor

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ABSTRACT

Sphingolipids are essential components of eukaryotic cell membranes. We recently showed that the function of the serotonin_{1A} receptor is impaired upon metabolic depletion of sphingolipids using fumonisin B₁ (FB₁), a specific inhibitor of ceramide synthase. Serotonin_{1A} receptors belong to the family of G-protein coupled receptors and are implicated in the generation and modulation of various cognitive, behavioral and developmental functions. Since function and dynamics of membrane receptors are often coupled, we monitored the lateral dynamics of the serotonin_{1A} receptor utilizing fluorescence recovery after photobleaching (FRAP) under these conditions. Our results show an increase in mobile fraction of the receptor upon sphingolipid depletion, while the diffusion coefficient of the receptor did not exhibit any significant change. These novel results constitute the first report on the effect of sphingolipid depletion on the mobility of the serotonin_{1A} receptor. Our results assume greater relevance in the broader context of the emerging role of receptor mobility in understanding cellular signaling.

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1. Introduction

Sphingolipids are essential components of eukaryotic cell membranes and constitute 10–20% of the total membrane lipids [1]. They are recognized as diverse and dynamic regulators of a multitude of cellular processes such as cell signaling, growth, differentiation and neoplastic transformation. The distribution of sphingolipids in cellular plasma membranes appears heterogeneous and patchy, and it has been postulated that sphingolipids and cholesterol occur in laterally segregated lipid domains (sometimes termed as 'lipid rafts') [2,3]. Many of these domains are believed to be important for the maintenance of membrane structure and function, although analyzing the spatiotemporal resolution of these domains is proving to be challenging [4,5]. The idea of such membrane domains gains significance since physiologically important functions such as cellular membrane sorting, trafficking [6], signal transduction [7] and the entry of pathogens into cells [8,9] have been attributed to these domains.

Abbreviations: 5-HT_{1A} receptor, 5-hydroxytryptamine-1A receptor; 5-HT_{1A}R-EYFP, 5-hydroxytryptamine_{1A} receptor tagged to enhanced yellow fluorescent protein; EYFP, enhanced yellow fluorescent protein; FB₁, fumonisin B₁; GPCR, G-protein coupled receptor.

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The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [10,11]. GPCRs regulate physiological responses to a diverse array of stimuli, and mediate multiple physiological processes. As a consequence of this, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas [12]. The serotonin_{1A} (5-HT_{1A}) receptor is an important member of the GPCR superfamily and is crucial in a multitude of physiological processes [13,14]. It serves as an important target in the development of therapeutic agents for neuropsychiatric disorders.

Since GPCRs are integral membrane proteins with a considerable portion embedded in the membrane interior, the membrane lipid environment represents an important determinant of receptor structure and function [15]. In the context of the serotonin_{1A} receptor, its interaction with surrounding membrane lipids assumes greater significance with increasing pharmacological relevance of the receptor. Seminal work from our laboratory has comprehensively demonstrated the requirement of membrane cholesterol in the function of the serotonin_{1A} receptor (recently reviewed in [15]). Interestingly, we previously reported that sphingolipids are necessary for ligand binding and cellular signaling of the human serotonin_{1A} receptor [16,17]. For example, we recently showed that the function of the serotonin_{1A} receptor is impaired upon metabolic depletion of sphingolipids using fumonisin B₁ (FB₁), a specific inhibitor of ceramide synthase [17]. Fumonisin is a group of naturally occurring mycotoxins, which are

ubiquitous contaminants of corn and other grain products, produced by *Fusarium verticelloides* and several other *Fusarium* species [18,19]. The most abundant among the fumonisin family is fumonisin B₁ (FB₁) [20], which is structurally similar to sphingoid bases such as sphinganine and sphingosine. FB₁ is a specific inhibitor of the reaction catalyzed by sphinganine *N*-acetyltransferase (ceramide synthase) [19,21].

Changes in functionality of membrane receptors and cellular signaling are often associated with changes in lateral dynamics of the receptor [22–24]. In order to explore whether the reported change in serotonin_{1A} receptor function upon metabolic depletion of sphingolipids by FB₁ treatment [17] is accompanied by any change in lateral dynamics of the receptor, we monitored the lateral dynamics of the serotonin_{1A} receptor utilizing fluorescence recovery after photobleaching (FRAP). Our results show an increase in mobile fraction of the receptor upon sphingolipid depletion, although the diffusion coefficient of the receptor did not exhibit any significant change. To the best of our knowledge, our results constitute the first report on the effect of sphingolipid depletion on receptor mobility.

2. Materials and methods

2.1. Materials

FB₁, CaCl₂, MgCl₂, penicillin, streptomycin, gentamicin sulfate, and sodium bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). D-MEM/F-12 [Dulbecco's modified Eagle medium:nutrient mixture F-12 (Ham) (1:1)], fetal calf serum, and geneticin (G 418) were from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals and solvents used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Cell culture and FB₁ treatment

CHO-K1 cells stably expressing the human serotonin_{1A} receptor tagged to enhanced yellow fluorescent protein (EYFP) (termed as CHO-5-HT_{1A}R-EYFP) were maintained in D-MEM/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, 50 µg/ml gentamicin sulfate, and 300 µg/ml geneticin in a humidified atmosphere with 5% CO₂ at 37 °C. Stock solutions (1 mM) of FB₁ were prepared in water and added to cells grown for 24 h (final concentration of FB₁ was 6 µM) and incubated in 5% serum for ~66 h. Control cells were grown under similar conditions without FB₁ treatment.

2.3. Fluorescence recovery after photobleaching measurements and analysis

FRAP experiments were carried out at room temperature (~23 °C) on CHO-5-HT_{1A}R-EYFP cells that were grown in D-MEM/F-12 medium containing 5% serum with or without FB₁ treatment on Lab-Tek chambered coverglass (Nunc, Denmark). Fluorescence images of cells grown on Lab-Tek chambers were acquired in the presence of PBS buffer pH 7.4, containing 0.5 mM MgCl₂ and 1 mM CaCl₂. Images were acquired at room temperature (~23 °C), on an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany), with a 63×, 1.2 NA water immersion objective using the 514 nm line of an argon laser for excitation and 535–590 nm filter for the collection of EYFP fluorescence. Images were recorded with a pinhole of 225 µm, giving a z-slice of 1.7 µm. A circular region of interest (ROI), with a radius of 1.4 µm was chosen as the bleach ROI. The time interval between successive scans was ~0.53 s. The distinct membrane fluorescence of the cell periphery

was targeted for bleaching and monitoring of fluorescence recovery [25,26]. Analysis with a control ROI drawn a certain distance away from the bleach ROI indicated no significant bleach while fluorescence recovery was monitored. Data representing the mean fluorescence intensity of the bleached ROI were background subtracted using an ROI placed outside the cell. Fluorescence recovery plots with fluorescence intensities normalized to pre-bleach intensities were analyzed according to the uniform disc illumination condition [27]:

$$F(t) = [F(\infty) - F(0)][\exp(-2\tau_d/t)(I_0(2\tau_d/t) + I_1(2\tau_d/t))] + F(0) \quad (1)$$

where $F(t)$ is the mean background corrected and normalized fluorescence intensity at time t in the bleached ROI, $F(\infty)$ is the recovered fluorescence at time $t = \infty$, $F(0)$ is the bleached fluorescence intensity set at time $t = 0$, and τ_d is the characteristic diffusion time. I_0 and I_1 are modified Bessel functions. Diffusion coefficient (D) is determined from the equation:

$$D = \omega^2/4\tau_d \quad (2)$$

where ω is the actual radius of the bleached ROI. Mobile fraction (R) estimates of the fluorescence recovery were obtained from the equation:

$$R = [F(\infty) - F(0)]/[1 - F(0)] \quad (3)$$

where the mean background corrected and normalized prebleach fluorescence intensity is equal to unity. Normalized intensities of each data set were fitted individually to Eq. (1), and parameters derived were used in Eqs. (2) and (3). Statistical analysis was performed on the entire set of derived parameters for all given conditions.

2.4. Nonlinear curve fitting and statistical analysis

Nonlinear curve fitting of the fluorescence recovery data to Eq. (1) was carried out using the Graphpad Prism software version 4.00 (San Diego, CA, USA). Significance levels were estimated by Student's two-tailed unpaired *t*-test using the same software. Frequency distribution plot and analysis was performed using Origin software version 5.0 (OriginLab Corp., Northampton, MA, USA).

3. Results and discussion

We have earlier shown that EYFP fusion to the serotonin_{1A} receptor does not affect the ligand binding properties, G-protein coupling and signaling functions of the receptor [23]. It is advantageous to use EYFP as a fluorophore since it avoids cellular autofluorescence, is relatively photostable and has a high quantum yield [28]. CHO cells stably expressing the serotonin_{1A} receptor tagged to enhanced yellow fluorescent protein (5-HT_{1A}R-EYFP) therefore represent a reliable cellular system to explore the membrane organization and dynamics of the serotonin_{1A} receptor [29]. In order to achieve metabolic depletion of sphingolipids, we treated CHO cells stably expressing the human serotonin_{1A} receptor with FB₁. The concentration of FB₁ (6 µM) was carefully chosen so that cytotoxic effects of FB₁ [30] are avoided. We have previously shown that sphingomyelin content is considerably reduced upon treatment of cells with 6 µM FB₁ [17]. Analysis of the fluorescence distribution of 5-HT_{1A}R-EYFP in control cells and upon treatment with 6 µM FB₁ did not show significant redistribution of fluorescence (see Fig. 1A and B). These results set up the background for the FRAP experiments described below to assess diffusion characteristics of the receptor as they indicate that the analysis of fluorescence recovery is not complicated by any significant alteration in the distribution of receptors due to FB₁ treatment during these

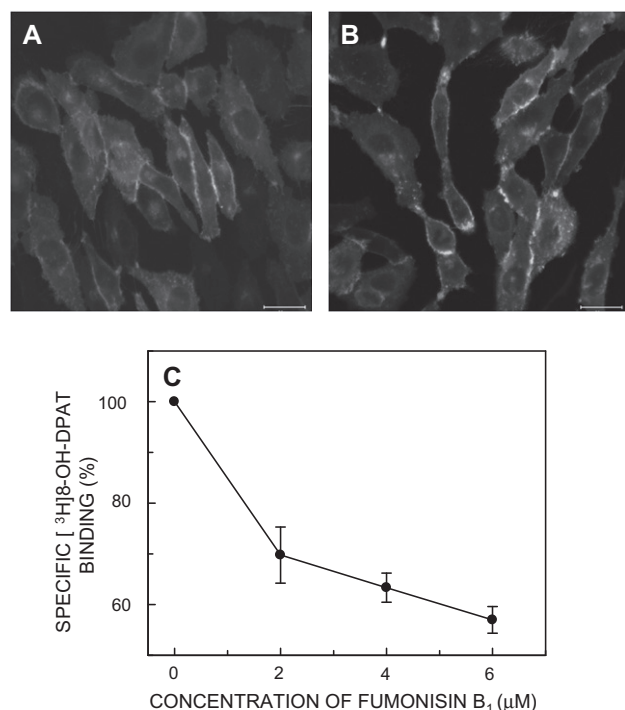


Fig. 1. Cellular morphology and the overall distribution of 5-HT_{1A}R-EYFP remain unaltered in control and cells treated with 6 μM FB₁. Typical fluorescence distribution of 5-HT_{1A}R-EYFP in CHO-5-HT_{1A}R-EYFP cells is shown under (A) control and (B) FB₁-treated conditions. Fluorescence images of cells grown on coverslips and placed in Biopetechs FCS2 closed chamber system were acquired at 37 °C in presence of HEPES-Hanks buffer. The scale bar represents 20 μm. Panel C shows the loss of specific agonist binding of the human serotonin_{1A} receptor upon FB₁ treatment. Adapted and modified from Ref. [17].

measurements. Importantly, the specific agonist ([³H]8-OH-DPAT) binding exhibits considerable reduction upon metabolic depletion of sphingolipids under these conditions (see Fig. 1C; [17]).

FRAP involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the sample region [23,31]. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from the unbleached regions in the membrane is an indicator of the mobility of the fluorophores in the membrane. A representative panel of images demonstrating the recovery of fluorescence after photobleaching is shown in Fig. 2. The fit to the recovery of fluorescence into the bleached region in FRAP experiments provides two parameters, an apparent diffusion coefficient (*D*) and mobile fraction (*R*) (see Section 2). The rate of fluorescence recovery provides an estimate of the *D* of molecules, while the extent to which fluorescence recovers provides an estimate of *R* of molecules.

Due to the inherent variation in biological samples, parameters derived from a large dataset of independent measurements were subjected to statistical and histogram analysis. The histogram representation of mobility of the 5-HT_{1A}R-EYFP in control and FB₁-treated cells are shown in Fig. 3. As shown in panels A and B of Fig. 3, the diffusion coefficient of the receptor does not exhibit significant difference upon FB₁ treatment. In addition, the distribution of diffusion coefficients remains unimodal with comparable standard deviations indicating that the population of the mobile receptors does not undergo any appreciable change in terms of diffusion coefficient. Importantly, the mobile fraction (shown in Fig. 3C and D) shows a significant change upon FB₁ treatment. While the population remains unimodal, thereby indicating the presence of a single mobile population, the mean mobile fraction displays a significant increase (~8%, *p* < 0.001) upon FB₁ treatment. The in-

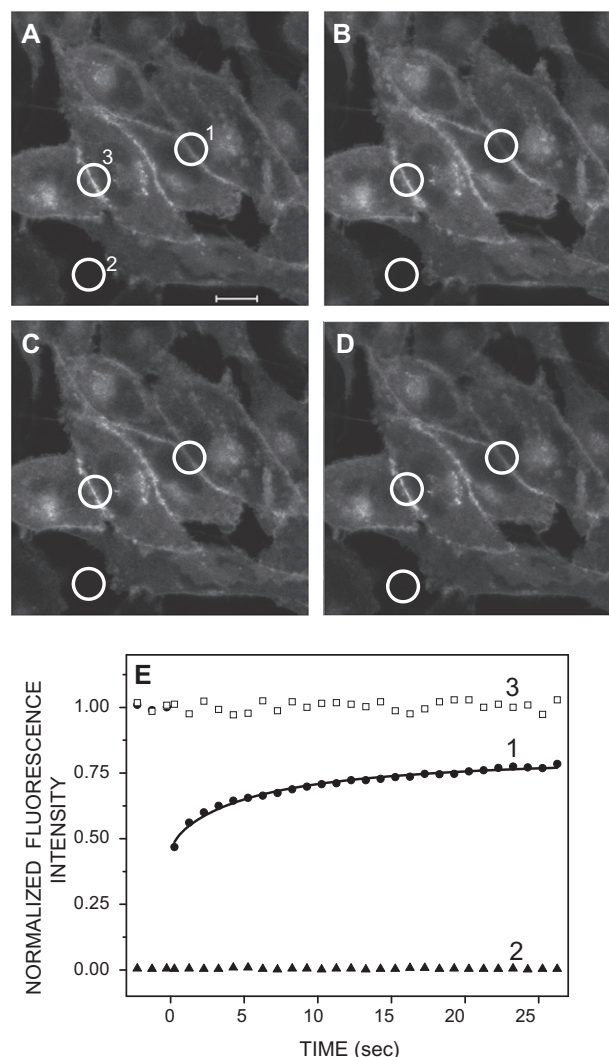


Fig. 2. Representative images of fluorescence recovery after photobleaching of 5-HT_{1A}R-EYFP. Fluorescence images of CHO-5-HT_{1A}R-EYFP cells represent confocal sections of the cell periphery acquired at room temperature (~23 °C). Panels A–D show fluorescence intensity monitored at various time points corresponding to pre-bleach, bleach, immediate post-bleach and complete recovery, respectively. Panel E shows a representative plot for the normalized fluorescence intensity of 5-HT_{1A}R-EYFP (●) corresponding to region 1, and the normalized background intensity (▲), corresponding to region 2. The normalized fluorescence intensity in a control region 3 (□) monitored for the same time period, indicates no significant photobleaching due to repeated scanning. The dimensions of the regions are only representative. The scale bar represents 10 μm. See Section 2 for other details. Taken from Ref. [24].

crease in the mobile fraction can also be appreciated from the frequency distribution, where the histogram exhibits a right shift on the abscissa scale. Interestingly, change in mobile fraction of comparable magnitude has earlier been shown to be associated with considerable change in downstream signaling [24,32].

In summary, we show here that the impaired ligand binding and signaling of the human serotonin_{1A} receptor upon metabolic depletion of sphingolipids is accompanied by a change in receptor mobility in the plasma membrane, as assessed by the change in mobile fraction of the receptor by FRAP measurements. Interestingly, our results show that the diffusion coefficient of the receptor exhibits no significant change with sphingolipid depletion, while the mobile fraction of the receptor increases. Such a change in the mobile fraction could be attributed to the perturbation of receptor–sphingolipid assembly (domain) that existed in the membrane prior to sphingolipid depletion. The population of receptor in

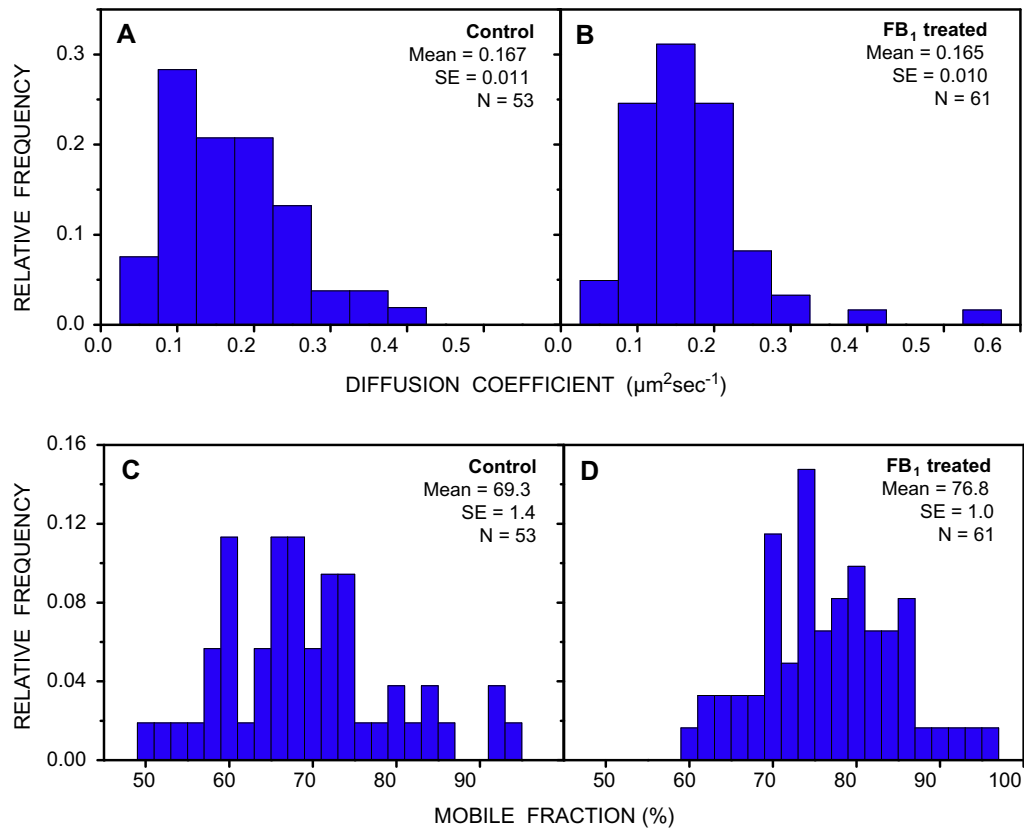


Fig. 3. Frequency distribution histograms of diffusion coefficient and mobile fraction of 5-HT_{1A}R-EYFP. The frequency distribution histograms are obtained by fitting the normalized recovery of individual experiments. The histograms for diffusion coefficients (panels A and B) and mobile fraction (panels C and D) for control (untreated) cells, and FB₁-treated cells, respectively, are shown. The means $\pm$ standard errors are shown in all cases. *N* represents the number of independent experiments performed in each case. See Section 2 and Table 1 for other details.

Table 1
Estimated diffusion coefficients and mobile fractions.^a

Condition	<i>D</i> ($\mu\text{m}^2 \text{s}^{-1}$)	<i>R</i> (%)	<i>N</i>
Control	0.167 (± 0.011)	69.3 (± 1.4)	53
FB ₁ -treated	0.165 (± 0.010)	76.8 (± 1.0)	61

^a *N* represents the number of independent measurements. Individual data sets were fitted to Eq. (1) and the derived parameters were used to calculate corresponding *D* and *R* using Eqs. (2) and (3), respectively. Means $\pm$ standard errors are shown. See Section 2 for other details.

such an assembly could appear immobile in the time scale of FRAP measurements, perhaps limited by the rate of diffusion of the entire assembly. Upon sphingolipid depletion, some of these receptors may be released resulting in an increase in mobile fraction. These results assume significance in the overall context of the emerging role of receptor mobility in cellular signaling [24,33].

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