Specific oligomerization of the 5-HT1A receptor in the plasma membrane

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Abstract In the present study we analyze the oligomerization of the 5-HT1A receptor within living cells at the subcellular level. Using a 2-excitation Förster Resonance Energy Transfer (FRET) method combined with spectral microscopy we are able to estimate the efficiency of energy transfer based on donor quenching as well as acceptor sensitization between CFP-and YFP-tagged 5-HT1A receptors at the plasma membrane. Through the analysis of the level of apparent FRET efficiency over the various relative amounts of donor and acceptor, as well as over a range of total surface expressions of the receptor, we verify the specific interaction of these receptors. Furthermore we study the role of acylation in this interaction through measurements of a palmitoylation-deficient 5-HT1A receptor mutant. Palmitoylation increases the tendency of a receptor to localize in lipid rich microdomains of the plasma membrane. This increases the effective surface density of the receptor and provides for a higher level of stochastic interaction.

Keywords Palmitoylation · Serotonin · 5-HT1A receptor · Lipid rafts · FRET · Oligomerization

Abbreviations
GPCR G-protein coupled receptor
$E_{fD}$ Donor dependent apparent FRET efficiency
$E_{fA}$ Acceptor dependent apparent FRET efficiency
$x_D$ Donor mole fraction
$T_D$ Relative total Concentration

Introduction

G-protein coupled receptors (GPCRs) are a superfamily of proteins that govern the reactions to a wide range of signals via activation of the heterotrimeric G-proteins. Structurally, GPCRs possess seven transmembrane domains linked by alternating intracellular ($i1$–$i3$) and extracellular ($e1$–$e4$) loops. The extracellular receptor surface, including the N-terminus, is known to be critically involved in ligand binding. The intracellular receptor surface, including C-terminal domain and intracellular loops, is known to be important for G-protein recognition and activation [1]. Members of GPCR family are usually glycosylated and possess at least one glycosylation site on extracellular N-terminus. Functionally, N-linked glycosylation of GPCRs have been shown to be evolved in the regulation of the receptor’s expression and functions (for review see [2]). Another common post-translational modification of GPCRs...
is the covalent attachment of palmitic acid to cysteine residues located within their cytoplasmic C-terminus [3]. Palmitoylation of several GPCRs has been shown to play a central role in the regulation of the receptor’s functions [4]. Previously, our group has demonstrated that the 5-HT1A receptor is stably palmitoylated at its C-terminal cysteine residues Cys417 and Cys420. Furthermore, we have shown that palmitoylation of 5-HT1A receptor is necessary for Gi-protein coupling and effector signaling [5].

In recent years there has been a shift in the understanding of distribution of GPCRs in the plasma membrane. Originally, it was believed that GPCRs existed and functioned as monomeric units. However, it is now widely accepted that in many cases oligomerization of these receptors occurs [6, 7]. Interestingly, receptor oligomerization seems to be independent of glycosylation, as has been shown for human hisamine H(4) and alpha-1b adrenoreceptors [8, 9]. In some cases this specific interaction occurs between the same receptor type (homo-oligomerization) and in others between different receptors (hetero-oligomerization). For several GPCRs, oligomerization has been found to occur early in the receptors lifetime, shortly after translation. On the other hand, oligomerization has also been shown to occur at the plasma membrane both in ligand-dependent and independent manner [10, 11]. Functionally, homo-and hetero-oligomerization have been found to play an important role in receptor trafficking, ligand specificity and coupling to specific signaling pathways [12–17]. Thus, oligomerization may provide an additional level of control for signal transduction and for the fine tuning of cellular processes.

Another area of interest in the study of GPCRs is their lateral distribution within the plasma membrane. Rather than being uniformly distributed throughout the plasma membrane, many studies suggest that GPCRs may localize in cholesterol-rich membrane microdomains or lipid rafts [18–20]. Many of these reports, often based on biochemical assay as well as fluorescence techniques, propose that compartmentalization is necessary for the efficient interaction between signaling partners with low surface concentration. Generally, it has been suggested that lipid rafts may represent “hot spots” for signaling, working as a platform for co-localization of GPCRs with corresponding G-proteins. In addition, it has been proposed that compartmentalization in cholesterol rich microdomains occurs due to hydrophobic mismatch of transmembrane domains as well as by post-translational acylation of both the receptor and G-proteins [21–23].

In addition to the role of palmitoylation in the coupling of the receptor with G-protein [5], we have recently demonstrated the importance of this modification for the localization of the 5-HT1A receptor in the lipid rafts [24], implying that palmitoylation plays a central role in the 5-HT1A function. We have also recently demonstrated specific homo-oligomerization of the 5-HT1A receptors [25]. In this previous investigation we applied both classical biochemical assays (e.g. co-immunoprecipitation and cross-linking) as well as biophysical approaches, including acceptor-photobleaching FRET, time correlated single-photon counting (TCSPC) FRET, as well as, a novel FRET approach, lux-FRET, allowing for measurements of the donor-acceptor ratio and apparent FRET efficiencies from populations of living cells [26]. In the current study we applied this method to spectral images acquired using confocal microscopy, which allows for the investigation of localization and interaction of 5-HT1A receptors in living cells with high spatial resolution. Moreover, by defining a confocal slice which encompasses only the plasma membrane immediately adjacent to the coverslip, we can measure FRET signal resulting from oligomerization of 5-HT1A receptors specifically at the membrane without any contamination from fluorescence from receptors localized in other intercellular compartments.

Materials and methods

Adherent cell culture and transfection

Mouse N1E-115 neuroblastoma cells from the American Type Culture collection (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C under 5% CO2. For transient transfection, cells were seeded at low-density in on 35-mm cover-slips and transfected with vectors encoding CFP-and YFP-tagged wild-type or acylation-deficient 5-HT1A receptors using Lipofectemine2000 Reagent (Invitrogen) according to the manufacture’s instruction. Four hours after transfection, cells were serum starved overnight before analysis.

Live cell confocal microscopy and spectral imaging

Coverslips with N1E-115 cells co-expressing 5-HT1A-CFP and 5-HT1A-YFP fusion proteins were placed in a custom made image acquisition chamber in 2 ml of D-PBS at room temperature. Images were acquired with an LSM 510-Meta confocal microscope (Carl Zeiss Jena) using a 40×/1.3 NA oil-immersion objective with a pixel count of 256×256, a pixel dwell time of 1.92 µs, and 4× frame averaging, resulting in a total image acquisition time of approximately 1 s. The 458 and 488 nm lines of a 40 mW argon laser were used for the two excitation FRET method as described in [26]. Fluorescence emission was collected in eight channels of the Zeiss Meta detector at 21.4 nm steps. All images were digitized/collected with 12 bit resolution.
FRET analysis

Prior to FRET analysis, images collected at two excitation wavelengths were spatially aligned with respect to each other, in order to eliminate artifacts resulting from misalignment. Image registration usually required less than a 2 pixel shift in the x–y plane. Next the spectral data from each pixel of the two images were analyzed according to the method described by Włodarczyk et al. [26]. Briefly, for each pixel of the spectral images of the FRET samples, linear unmixing was performed using CFP, YFP and background reference spectra to separate the respective contributions of these species to total fluorescence. Using the apparent concentration of the donor and acceptor fluorophores along with calibration information about the relative level of excitation of our donor and acceptor at the two different wavelengths, we are able to calculate donor apparent FRET efficiency, $Ef_D$, acceptor apparent FRET efficiency, $Ef_A$, the FRET corrected acceptor to donor ratio and FRET corrected total concentration with respect to either reference measurement. All images and procedures were analyzed using Matlab 7.2 (The MathWorks, Natick, MA, USA).

Model of FRET in oligomers

A model characterizing apparent FRET efficiency, $Ef_D$, as a function of donor mole fraction, $x_D$, for oligomeric structures has been developed previously [27].

$$Ef_D = E (1 - x_D^{n-1}).$$  

Fitting this model to experimental data allows for the estimation of the true transfer efficiency, $E$, and also provides information about the number of units, $n$, interacting in the oligomeric complex. Recently, this model has been slightly augmented for use with $Ef_A$ [28].

$$Ef_A = E \frac{x_D}{1 - x_D} (1 - x_D^{n-1}).$$  

Results and discussion

Measurements of FRET signal from the plasma membrane of living cells

We have recently verified the oligomerization state of the 5-HT1A receptor by using a co-immunoprecipitation and cross-linker assays performed in neuroblastoma N1E-115 cells [25]. These biochemical methods represent the classical approaches used for the detection of GPCR oligomerization. However, these methods often result in artificial aggregation of receptors and do not allow for the analysis of GPCR oligomerization in living cells [29]. To overcome these limitations, we have implemented a novel lux-FRET approach allowing for oligomerization analysis in living cells [26]. Despite the advantages of this method, it has only been applied to the populations of cells and therefore has not allowed for the differentiation between receptors localized at the plasma membrane and those residing in the endoplasmic reticulum, Golgi network or in transport vesicles.

In the present study we are primarily interested in the oligomerization of 5-HT1A receptors at the plasma membrane due to its possible influence on ligand binding and selectivity, G-protein coupling and activation, and receptor internalization. For these reasons it is important to verify receptor oligomerization specifically at the plasma membrane and exclude artifacts from interaction in other intercellular compartments. Therefore, we adapted the lux-FRET approach to the application at confocal microscopy, allowing for the analysis of receptor oligomerization with sub-cellular resolution. Figure 1A shows the 3-D reconstruction of a series of confocal slices through an N1E-115 cell co-expressing 5-HT1A-CFP and 5-HT1A-YFP receptor constructs at a 1:1 ratio. The center panel shows the confocal slice where fluorescence was detected specifically at the plasma membrane immediately adjacent to the coverslip on which the cell is growing. In addition to the clear membrane localization of the fluorescently-tagged receptors, some intercellular fluorescence is also detectable (particularly apparent in the y-z section). In the following FRET measurements similar confocal slices were used for analysis. To avoid artefacts resulting from overexpression, we adjusted the total expression level for the CFP- and YFP-tagged receptor to 1.000–1.200 fmol/mg proteins in all following FRET experiments, which allows for quantitative analysis of results obtained in different experiments [25]. Moreover, similar amounts of endogenous 5-HT1A receptors has been obtained in hippocampus under physiological conditions [30, 31]. The lux-FRET method implemented in this investigation requires the acquisition of fluorescent emission spectra resulting from two separate excitation wavelengths. To apply this method to microscopy, two images were acquired, one with excitation using the 458 nm laser line, the other with excitation at 488 nm. Fluorescence emission was collected simultaneously over eight channels for each acquisition, allowing for reconstruction of emission spectra on a per pixel basis. Figure 1B shows the measured emission intensities of a single pixel over the eight channels used in the image acquisition for a cell co-expressing 5-HT1A-CFP and 5-HT1A-YFP. This figure also shows the weighted contributions of CFP, YFP and background signal which combine to provide a fit to the acquired fluorescent spectra of the FRET sample. Using the per pixel weights of CFP and YFP from the fitting of two
emission spectra, we can apply the lux-FRET methods to define donor-and acceptor-based apparent FRET efficiencies \(E_{fD}\) and \(E_{fA}\), a FRET-corrected donor molar fraction \(x_{DP}\), as well as a FRET-corrected total receptor concentration \(T_D\) for each pixel of the image. This allows us to not only quantify apparent FRET as a function of donor/acceptor ratio, but also to investigate the effect of local concentration differences on oligomerization specificity at the single-cell level. It is also notable, that although the GFP-variants used in this study do not have the monomeric mutation, this does not result in artificial FRET signal. We have previously investigated FRET resulting from the assumed spontaneous aggregation of the cytosolic non-monomeric CFP and YFP coexpressed in neuroblastoma N1E-115 cells [26]. In this study we reported that the level of FRET in control measurements showed a strong dependence on concentration, with the maximum \(E_{fD}\) value of 0.05 only occurring at a much higher concentration than was used in the current investigation.

Oligomerization of the 5-HT1A receptor in the plasma membrane is independent of its palmitoylation state

The apparent FRET efficiencies, \(E_{fD}\) and \(E_{fA}\), measured by the lux-FRET technique are estimates for the true energy transfer efficiency that are scaled by donor or acceptor fractional labeling. The fractional labeling terms, \(fD\) and \(fA\), are defined as the concentration of donor and acceptor in the FRET complexes, \([DA]\), divided by total respective fluorophore concentration, \([D']\) and \([A']\). The fractional labeling terms are inherently dependent on the acceptor to donor ratio. Based on this dependency, a model has been previously developed allowing for the estimation of the true energy transfer efficiency as well as the number of units interacting in an oligomeric complex [27, 28]. This model can correctly identify case of dimerization \((n=2)\) and, although it cannot accurately quantify the number of units reacting if above two, it still can be used to identify such cases.

Application of this model requires not only the measurement of apparent FRET efficiency, but also of an acceptor to donor ratio that is corrected for donor quenching as well as for acceptor sensitization. The lux-FRET provides these values and allows us to correctly apply this model to characterize the homo-oligomerization between wild-type and acylation-deficient 5-HT1A receptors. To acquire the data required to implement this model, cells grown on coverslips were co-transfected with different amounts of CFP-and YFP-tagged receptors. It is noteworthy that the total DNA concentration used for each transfection was held constant, while the ratios between donor (CFP-tagged receptors) and acceptor (YFP-tagged receptor) was varied between 1:7 and 7:1. In the image acquisition for FRET measurements, the confocal plane was adjusted so that only fluorescence from proteins localized in the plasma membrane immediately adjacent to the coverslip was collected. Initial images were acquired with the 458 nm laser line and immediately followed by acquisitions with the 488 nm laser line. Finally, FRET calculations were performed on a per-pixel basis as described in the “Materials and methods” section. For the determination of the relationship between apparent FRET efficiency and acceptor to donor ratio, 115
Fig 2 Oligomerization of the wild-type 5-HT1A receptor measured by lux-FRET. (A) Application of the two excitation FRET method described in “Materials and methods” and outlined in Fig. 1, allows us to create images of $E_{fD}$, $E_{fA}$, $x_d$, as well as FRET corrected total concentration $T_d$. (B) Mean values of 10×10 μm regions of interest from cells expressing different ratios of 5-HT1A-CFP and 5-HT1A-YFP receptors allows to characterize the apparent FRET efficiency over the donor mole fraction. The data were collected from 115 cells and binned along donor mole fraction in 0.05 increments. Data points represent the mean ±SD of the apparent FRET efficiency values from each bin. The fits to the data are based on the models described in Eqs. 1 and 2. The fitted parameters from the $E_{fD}$ model were found to be $E=0.11$ and $n=2.96$ ($R^2=0.69$) and from the $E_{fA}$ model, $E=0.16$ $n=2.44$ ($R^2=0.83$). (C) To address the effect of surface concentration on measured FRET a measured $E_{fA}$ is shown plotted as a function of corresponding relative total concentration $T_D$. The plot shows that $E_{fA}$ is fairly independent from the total concentration at the region of membrane at which it was measured. The data points shown represent mean ±SE of $E_{fA}$ values separated by corresponding $T_D$ value into 15 bins. The relative total surface concentration, $T_D$, was calculated by the lux-FRET method and corrected for both donor quenching and acceptor sensitization (independent of FRET) and is given in units of donor reference concentration.
Fig 3 Oligomerization of the palmitoylation-deficient 5-HT1A receptor measured by lux-FRET. (A) lux-FRET images of N1E-115 cells expressing palmitoylation-deficient mutant receptors show that the membrane distribution of the calculated values appear no different than for the wild-type receptors shown in Fig. 2A. (B) The characterization of FRET as a function of donor mole fraction further shows that there is little difference between the interaction of the wild type receptors and the interaction of the mutant receptors. Application of the previously mentioned models suggests that for the $E_{D}$ data $E=0.21$ and $n=1.70$ ($R^2=0.86$) and for the $E_{A}$ data $E=0.17$ and $n=2.10$ ($R^2=0.89$). (C) Similarly to the wild-type receptor, plotting $E_{A}$ values against spatially corresponding concentration values shows that there is no dependence of measured FRET on surface concentration of the palmitoylation-deficient 5-HT1A receptor.
cells were measured for both wild-type and mutant receptors. Results obtained with a donor mole fraction below 0.2 and above 0.8 were excluded from the analysis due to error resulting from excessive differential bleaching between CFP and YFP.

Figure 2A shows the spatial distribution of the apparent FRET efficiencies (EfD and EfA), the donor mole fraction, as well as the relative total concentration at the plasma membrane of the cell co-transfected with the CFP-and YFP-tagged wild-type receptors. Based on these results we were able to build the relationship between the apparent FRET efficiencies and the donor mole fraction, xD. Analysis of the data according to the aforementioned model reveals that the best fit for the EfD data was achieved with values of E=0.11 and n=2.95 (R²=0.69). The best fit for the EfA data was achieved with E=0.16 and n=2.44 (R²=0.83). Taken together these data suggest that in the case of the wild-type 5-HT1A receptor, more than two units participate in the formation of oligomers. However, due to the limitations of the model, we cannot exactly define the n-value.

To analyze the role of receptor palmitoylation for the oligomerization at the plasma membrane, we performed similar single-cell FRET analysis for the cells expressing CFP-and YFP-tagged acylation-deficient mutants (Fig. 3A). Comparison of the panel A in Figs. 2 and 3 does not reveal any significant differences in receptor or FRET distribution at the plasma membrane between wild-type and mutated receptors. These figures also demonstrate that the overall levels of the FRET for the cases of wild-type and non-palmitoylated receptor expression were comparable. However, we obtained a slight difference in the relationship of FRET to the donor mole fraction. In the case of the mutant receptor, the limiting value of EfD approaches the same value as EfA, while with the wild-type receptor the limit of the EfA value was greater than that of the EfD. Fitting the data according to the oligomerization model for the EfD produced the value of E=0.21 and n=1.70 (R²=0.8641) and for EfA-values of E=0.17 and n=2.10 (R²=0.8874) for the non-palmitoylated receptors.

It is also notable that the goodness of fit of the model to the wild-type receptor data is lower than to the mutant receptor data. The previously mentioned deficiency in the model may result in a less than ideal fit for data measured from a system with complexes composed of more than two units, as is the suggested case for the wild-type receptor.

Differences between the E values obtained for wild-type and mutated receptors suggest that non-palmitoylated receptor possesses a higher characteristic efficiency of energy transfer or a higher percentage of receptors are involved in oligomerization. Palmitoylation of the 5-HT1A receptor has been suggested to be responsible for the creation of an additional fourth intracellular loop at the receptor C-terminus [5]. The non-palmitoylated mutant will lack this loop and, therefore, may adopt a conformation which is more favorable for the FRET between the C-terminally fused fluorophores.

In the case of the acylation-deficient mutant, the oligomeric complexes were suggested to be formed by two interacting units (n=1.7 and n=2.10, for the EfD and EfA fits, respectively), while in the wild-type receptor more than two units can be involved in complex formation (n=2.95 and n=2.44, for the EfD and EfA fits, respectively). One possible explanation for such differences is the differential distribution of wild-type and mutant receptors within the plasma membrane. We have previously demonstrated that significant fraction of the 5-HT1A receptor resides in lipid rafts, while the yield of the palmitoylation-deficient receptor in these membrane microdomains is considerably reduced [24]. Furthermore, it has been shown that localization of proteins in lipid rafts may significantly increase stochastic interaction [28]. Therefore, the suggested greater number of units participating in complex obtained from the model for the wild-type receptors may results from stochastic interaction between oligomeric complexes with free receptors and/or with another complex localized in lipid microdomain. In contrast, non-palmitoylated receptors, which are excluded from lipid rafts [24], have a much lower probability for the additional interaction.

The next important question addressed in the present study was the analysis of interaction specificity. It has been reported that positive FRET signals may result not only from specific protein interactions, including receptor oligomerization, but also from randomly distributed and over-expressed FRET donors and acceptors. Based on the critical analysis of BRET data, it has been recently considered that the contribution of non-specific interactions measured by resonance energy transfer may be even larger than previously thought [32]. Interaction specificity may be analyzed by plotting energy transfer efficiency as a function of expression level at fixed donor/acceptor ratio [32]. In the case of random interaction it has been proposed that the energy transfer between two fluorophores is linearly dependent on expression level and will diminish to zero at very low concentration of fluorophores. In the case of non-random interaction, the apparent FRET efficiency should be relatively independent of concentration with an EfA intercept greater than zero. Figures 2C and 3C show the relationship between apparent FRET efficiency EfA and total surface concentration D obtained for the wild-type and acylation-deficient receptors at fixed donor to acceptor ratio. Both Figs. 2C and 3C demonstrate that FRET obtained for 5-HT1A receptors was largely independent of expression level, confirming non-random interactions between the receptors.
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