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Covalent labeling of cell-surface proteins for in-vivo FRET studies

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Abstract **Fluorescence resonance energy transfer (FRET) is a powerful technique to reveal interactions between membrane proteins in live cells. Fluorescence labeling for FRET is typically performed by fusion with fluorescent proteins (FP) with the drawbacks of a limited choice of fluorophores, an arduous control of donor–acceptor ratio and high background fluorescence arising from intracellular FPs. Here we show that these shortcomings can be overcome by using the acyl carrier protein labeling technique. FRET revealed interactions between cell-surface neurokinin-1 receptors simultaneously labeled with a controlled ratio of donors and acceptors. Moreover, using FRET the specific binding of fluorescent agonists could be monitored.**

Keywords: ACP labeling; FRET microscopy; GPCR; Donor–acceptor ratio

1. Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane cell-surface proteins involved in signal transduction and are the most important targets in the search for novel therapeutic compounds [1]. With the development of various labeling strategies, such as the site-selective introduction of fluorescent amino acids into the sequence of proteins [2], the modification of cysteines in suitable receptor mutant proteins [3] or the production of fusion constructs with fluorescent proteins (FP) [4], fluorescence techniques [5] have rapidly gained importance in the study of the function of GPCRs in vitro and in living cells. In particular, fluorescence resonance energy transfer (FRET), owing to its strong distance sensitivity in the range of 10–100 Å, was shown to be ideally suited to study both structures [2] and structural changes within [6], and interactions between [7–9] GPCR-signaling proteins tagged with suitable donor and acceptor fluorophores. In addition, FRET is of increasing importance as a readout signal for genetically encoded sensors [10] and as a means to monitor ligand binding to membrane proteins [11,12].

Restrictions often encountered in FRET studies using FPs are the choice of suitable fluorophores, the incomplete processing of fusion constructs leading to high background signals, and difficulties in achieving defined donor–acceptor (DA) ratios. In recent years several promising alternatives to FPs have been developed [13–19]. They are based on the covalent or non-covalent post-translational labeling of a fusion protein. One of these methods, the acyl carrier protein (ACP) labeling technique, makes use of the enzymatic transfer of a 4'-phosphopantetheine from coenzyme A (CoA) labeled with a fluorophore to a serine residue of ACP fused to the protein of interest [18]. Because the enzyme and the CoA substrate required for the reaction are not membrane permeable, only proteins correctly localized in the cell membrane are labeled, avoiding background signals from intracellular proteins. In addition, the ACP labeling allows the choice of optimal fluorophores for each experimental situation and thus the panel of applications is substantially broader.

Here we show the feasibility and versatility of ACP labeling for in vivo FRET studies by investigating a prototypical GPCR, the neurokinin-1 receptor (NK1R). FRET between labeled receptors and fluorescently labeled agonists allowed accurate recording of binding kinetics. Furthermore, FRET between NK1Rs simultaneously labeled with donors and acceptors at a controlled DA ratio revealed close proximity of NK1Rs in the plasma membrane.

2. Materials and methods

2.1. Material

Enhanced cyan (ECFP) and yellow (EYFP) fluorescent proteins were from Clontech; Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Dulbecco phosphate-buffered saline (D-PBS) and pCEP4 expression vector from Invitrogen; hygromycin B from Calbiochem; bovine serum albumin (BSA) from Fluka; substance P (SP) and N-terminally acetylated SP from K. Servis (University of Lausanne, Lausanne, CH); bacitracin from Serva.

2.2. Plasmid constructs and cell culture

Fusion of ACP to the extracellular N-terminus of NK1R (ACP-NK1R) is described elsewhere [18]. ECFP and EYFP were fused to the C-terminus of NK1R through a TSGGGG linker yielding NK1R-ECFP and NK1R-EYFP, respectively, and were subcloned into pCEP4.

Adherent HEK293 cells were grown in DMEM containing 2.5 v% FCS at 37 °C in a humidified atmosphere with 5% CO_2. For confocal microscopy, HEK293 cells were seeded (10^5 cells/ml) into 6-well plates

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Abbreviations: DA, donor–acceptor; FRET, fluorescence resonance energy transfer; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FP, fluorescent protein; NK1R, neurokinin 1 receptor; GPCR, G protein-coupled receptor; ACP, acyl carrier protein; SP, substance P; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; D-PBS, Dulbecco phosphate-buffered saline; BSA, bovine serum albumin

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containing a 25 mm glass coverslip and 2 ml DMEM/FCS. About 16–20 h after splitting, cells were transfected with 2.5 μg of plasmid DNA per well using the calcium phosphate technique. Imaging was performed 24–55 h after transfection in D-PBS (with 0.1% w/v BSA for ligand binding). Stable HEK293 cell lines were produced from transiently transfected cells by selection with 200 μg/ml hygromycin B.

2.3. Calcium signaling

HEK293 cells stably expressing NK1R constructs were seeded into a clear-bottom 96-well plate (Greiner) in DMEM/FCS and incubated at 37 °C and 5% CO_2. After 24 h the cells were loaded with a calcium-sensitive fluorophore (Calcium 3 assay kit, Molecular Devices) during 30 min at 37 °C. The change in fluorescence intensity at 525 nm upon addition of SP in buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $MnCl_2$, 1 mg/ml BSA, and 100 μg/ml bacitracin) was recorded on a FLEX station (Molecular Devices) upon excitation at 485 nm. The dependence of the amplitude of the calcium signal on the SP concentration was fitted with a Hill-equation.

2.4. Synthesis of SP-Cy5

N-terminally acetylated SP was reacted with 0.9 equivalent of Cy5-*N*-hydroxysuccinimide in 500 μl *N*,*N*-dimethylformamide and 50 μl 100 mM sodium carbonate, pH 8.2. The product was purified by thin layer chromatography on silica G60 (Merck) using MeOH:NH_4OH (95:5) as eluent and extraction with MeOH:H_2O:5 M HCl (85:14:1). The purity of the product evaluated by chromatography was better than 95%. Calcium-signaling assays on live cells revealed SP-Cy5 to be an agonist with pEC_{50} = 9.2 ± 0.6 for wtNK1R and 8.9 ± 0.4 for ACP-NK1R.

2.5. ACP labeling

Synthesis of CoA-Cy3 and CoA-Cy5 substrates and purification of AcpS were described previously [18]. The cells were first washed with D-PBS and then labeled in D-PBS supplemented with 10 mM $MgCl_2$, 1 μM AcpS and 5 μM CoA-substrate at 19 °C for 40 min. For double labeling CoA-Cy3 and CoA-Cy5 were mixed beforehand in the desired ratio to a total concentration of 5 μM. After labeling the cells were washed three times with D-PBS.

2.6. Confocal microscopy and FRET imaging

Laser-scanning confocal micrographs were recorded using the 458/488 nm Ar-ion or the 543/633 nm HeNe laser lines on an LSM 510 microscope (Zeiss) with a 63× (1.2 NA) water immersion objective. Detection of fluorescence signals was achieved at following settings (excitation wavelength, dichroic mirror, emission bandpass filter): ECFP (458 nm, HFT 453, BP 465-495); EYFP (488 nm, HFT 488, BP 510-560). FRET efficiencies using Cy3 as donor and Cy5 as acceptor were measured by sensitized acceptor emission. Settings for the acceptor emission *Af* (acceptor channel) were (633 nm, HFT 633, LP 650). Donor and FRET images were recorded simultaneously with excitation at 543 nm and a dichroic mirror HFT 543, the emission beam was split with a NFT 635 dichroic mirror onto two detectors with a BP 560-615 filter for donor emission *Df* (donor channel) and a LP 650 filter for acceptor emission *Ff* (FRET channel), respectively. Intensities were corrected for background.

In the following, we use the terminology of Gordon et al. [20]. The apparent FRET efficiency $E_{app,se}$ was calculated according to a method called 3-cube FRET [21]. After applying a threshold on the donor image to select the membrane comprising the labeled ACP-NK1, FRET ratios (FR), the fractional increase in acceptor emission due to FRET, were calculated on a pixel-by-pixel basis:

$$FR = \frac{Ff - S_1 Df}{S_4 Af}, \quad (1)$$

where S_1 and S_4 are cross-talk factors correcting for the donor emission detected in the FRET channel and the emission due to direct excitation of the acceptor, respectively. No additional correction factors were necessary as Cy3 is not excited at 633 nm and Cy5 does not emit in the donor channel. A histogram of FR values was then built and fitted with a Gaussian to yield the mean FR. This procedure minimized possible artefacts introduced by the threshold at low FR values. $E_{app,se}$ were calculated using:

$$E_{app,se} = (FR - 1)[\varepsilon_A(\lambda)/\varepsilon_D(\lambda)], \quad (2)$$

where ε_A (λ) and ε_D (λ) are the molar extinction coefficients of donor and acceptor, respectively, at the donor excitation wavelength (543 nm). For Cy3-Cy5, the ratio at 543 nm is 0.11. The experimentally determined $E_{app,se}$ is a function of both the true FRET efficiency E and the fraction of acceptor-labeled molecules bound to donor-labeled molecules.

Image analysis was performed using IGOR Pro 5 (Wavemetrics).

3. Results

The fusion of ACP to the N-terminal part of NK1R and its subsequent labeling did not alter the functionality of the receptor as shown in Fig. 1. First, ACP-NK1R stably expressed in HEK293 cells was still able to activate downstream signaling upon agonist binding eliciting a calcium response in a dose dependent manner (Fig. 1A). pEC_{50} values measured for SP were 10.7 ± 0.3 and 10.6 ± 0.3 for ACP-NK1R and wild-type NK1R, respectively, in close agreement with published data [22]. Second, exposure to the agonist induced internalization of the Cy5-labeled ACP-NK1R within 15 min (Fig. 1B), which is consistent with previous reports on NK1R internalization by Grady et al. [23]. No fluorescence from either CoA-Cy5 or

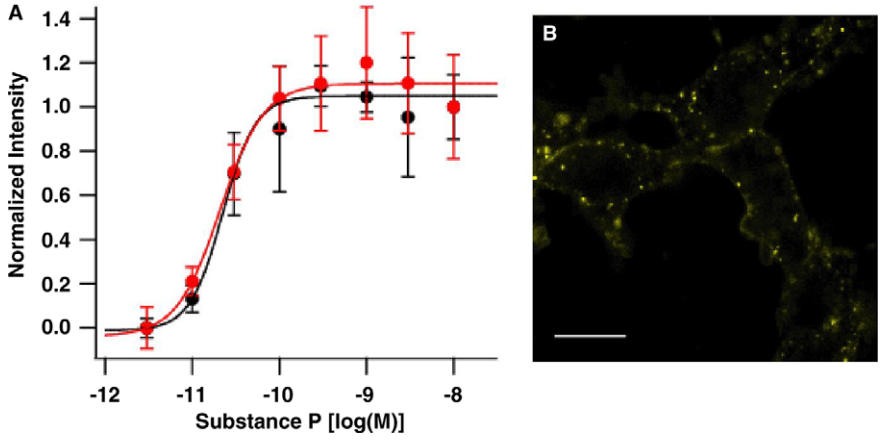

Fig. 1. SP activation of ACP-NK1R signaling. (A) Concentration–response curves of SP measured by calcium signaling on wild-type NK1R (black) and ACP-NK1R (red) expressed in HEK293 cells, yielding pEC_{50} of 10.6 ± 0.3 and 10.7 ± 0.3, respectively. (B) Fluorescence confocal micrograph showing HEK293 cells stably expressing ACP-NK1R labeled with Cy5. After 15 min of incubation with 100 nM SP at 19 °C, the receptor was internalized as revealed by the presence of bright intracellular foci. Scale bar is 10 μm.

CoA-Cy3 was observed on cells not expressing ACP-NK1R, showing that the receptors were specifically labeled by the ACP-labeling technique (see Supplementary data). Together these data demonstrate that the ACP-NK1R behaves as the wild-type receptor and that fluorescent labeling of NK1R through the ACP fusion allows observation of the authentic GPCR signal transduction processes.

GPCR-mediated signaling is initiated by ligand binding. This can be easily monitored on a living cell in real-time by FRET when receptor and ligand are labeled with donor and acceptor. The versatility of the ACP labeling method for this approach was demonstrated by labeling ACP-NK1R with Cy3 (donor) and subsequently adding SP-Cy5 (acceptor) (Fig. 2A–B). Complete co-localization of both fluorescence signals showed that all Cy3-ACP-NK1R are accessed by SP-Cy5, confirming that the ACP method exclusively labels the receptors on the cell surface. Non-specific binding of SP-Cy5 to the plasma membrane was far less than 5% of the specific binding to the NK1R as assessed from competition experiments with other non-fluorescent NK1R-specific ligands and the colocalisation with ACP-NK1R (see Supplementary data). In contrast to these results obtained using ACP labeling, similar experiments using NK1R fused to FPs suffered from strong background from intracellular receptors (see below) and from the restricted choice of the spectral region (data not shown).

The high quality of the fluorescence signals allowed monitoring of the time course of repetitive binding and dissociation of SP-Cy5 to Cy3-ACP-NK1R (Fig. 2). The time course of the increase of the fluorescence of SP-Cy5 at the cell membrane shows two distinct processes (Fig. 2C, middle): the rapid phase stems from ligand addition to the sample (Fig. 2C, top), the slower phase corresponds to the specific binding of SP-Cy5 to the NK1R as revealed by the concomitant Cy3-Cy5 FRET signal (Fig. 2C, bottom). It was not possible to determine the specific binding of SP-Cy5 to NK1R only from the fluorescence trace of SP-Cy5 (Fig. 2C, middle) because in addition to both specific and unspecific binding, it comprises a contribution from free SP-Cy5 in solution. Addition of increasing SP-Cy5 concentrations resulted in both a further increase of the fluorescence of membrane associated SP-Cy5 and a decrease of the Cy3-ACP-NK1R signal (see Supplementary data). The time course of specific binding measured by FRET revealed that after removal of SP-Cy5, about one-third of the specifically bound agonists did not dissociate from their cognate receptors (Fig. 2C, bottom). The origin of this effect might be the internalization of NK1R-bound SP-Cy5 (see also Fig. 1B) or the existence of a state of the NK1R with extremely low dissociation rate [12], which might represent receptors pre-coupled to G proteins.

Besides the monitoring of ligand binding, FRET is increasingly used to detect protein–protein interactions in living cells. For example, in GPCR research, FRET (and bioluminescence resonance energy transfer) is a method of choice to investigate receptor oligomerization in the plasma membrane [8,9]. In this type of experiments, quantitative determination of the degree of oligomerization critically relies on the possibility to vary precisely the DA ratio and to investigate its influence on the FRET signal [24]. This is usually done by cotransfection of plasmids encoding the protein of interest fused to ECFP and EYFP.

We compared the standard FP method using ECFP- and EYFP-NK1R fusions to our approach of double labeling by the ACP technique using ACP-NK1R. Representative confocal micrographs in Figs. 3A–C show the difficulties related to the

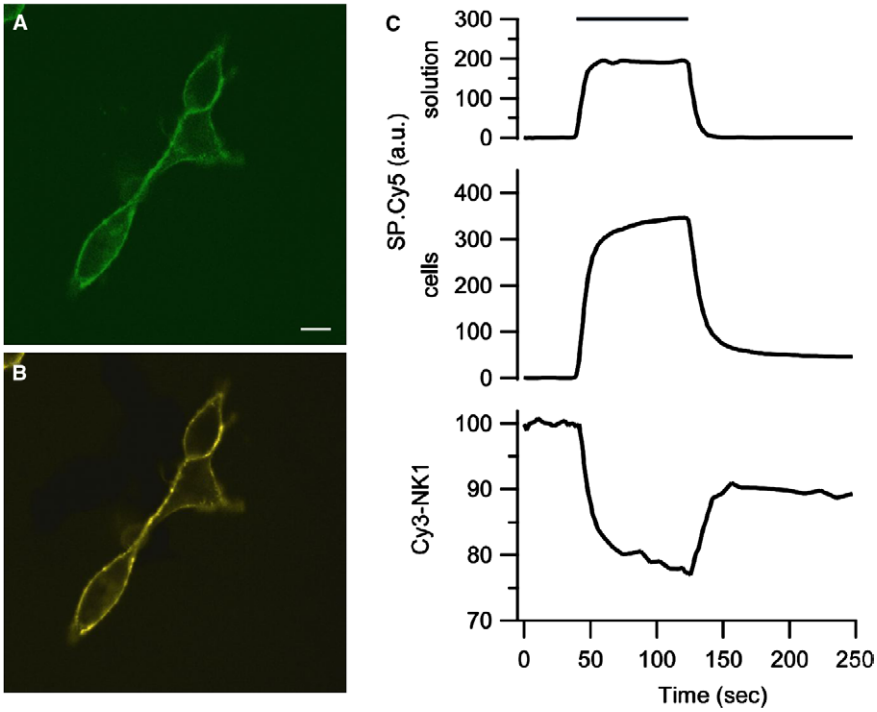

Fig. 2. SP-Cy5 binding to Cy3-labeled ACP-NK1R. Fluorescence confocal micrographs showing HEK293 cells expressing ACP-NK1R labeled with Cy3 (A) and receptor-bound SP-Cy5 upon a short incubation with 100 nM ligand (B). Scale bar is 10 μm. (C) A 116 nM solution of SP-Cy5 was applied at a few micrometers away from the cell with the pipette of a perfusion system at 19 °C. Application duration denoted by the bar length was 84 s. The time traces represent fluorescence intensities for the same region of interest using the acceptor channel of SP-Cy5 in buffer (top) and bound to the cell membrane (middle), and using the donor channel of Cy3-labeled ACP-NK1R (bottom).

use of FPs for double labeling: (i) a considerable amount of the FP-labeled NK1Rs is retained within the cell, thereby introducing a strong fluorescence background. (ii) Although a 1:1 mixture of the corresponding plasmids was used for transfection, expression levels and localizations of the receptor constructs in the cells were finally very different for both fusion proteins, making a precise determination of the DA ratio difficult. In contrast, ACP labeling using a mixture of Cy3 and Cy5 substrates resulted in the simultaneous double labeling of only surface-exposed ACP-NK1Rs, without any background fluorescence from receptors inside the cells (Fig. 3D–E). Furthermore, cellular autofluorescence did not interfere with the measurements when using dyes with longer wavelengths.

The DA ratio of Cy3- and Cy5-labeled ACP-NK1R could be precisely controlled by simultaneous double labeling using different mole fractions of the CoA-substrates. This is demonstrated in Fig. 3H, which shows that the fluorescence intensity of Cy5-ACP-NK1R depended linearly on the acceptor mole fraction. The final DA ratio after labeling can thus be varied in a controlled way by simply mixing the two dye-conjugated substrates to the desired ratio beforehand.

The high quality of the measurements on ACP-NK1R in Fig. 3 together with the absence of non-specific binding of the fluorescent CoA analogues to the plasma membrane allowed a quantitative evaluation of the FRET signal. Figs. 3D–F are typical images taken with the donor, acceptor and FRET settings, respectively. The FRET ratio (FR) was calculated on a pixel-by-pixel basis after application of an intensity threshold to select the membranes comprising the labeled NK1Rs (Fig. 3G). The average FR value of 5.75 ± 0.02 corresponds to an apparent FRET efficiency of 52.3%. This high FRET signal indicates that the NK1Rs on the cell surface are in close proximity, which could result from either GPCR oligomerization [8] or high receptor surface densities.

4. Discussion

Our results show that the ACP labeling technique is highly suitable for FRET studies of plasma membrane proteins in living cells. Compared to the very popular labeling with FPs, ACP labeling offers access to a wide range of labels and to the exclusive labeling of cell-surface proteins, thereby allowing an optimized choice of fluorophores and measurements free of the background resulting from autofluorescence or intracellular, labeled proteins. This is crucial for accurate FRET experiments.

We demonstrated the feasibility of the ACP labeling for direct measurements of specific ligand binding in a FRET-based approach as pioneered by Turcatti et al. in native membranes using suppressor tRNA technology [2] and Vollmer et al. using

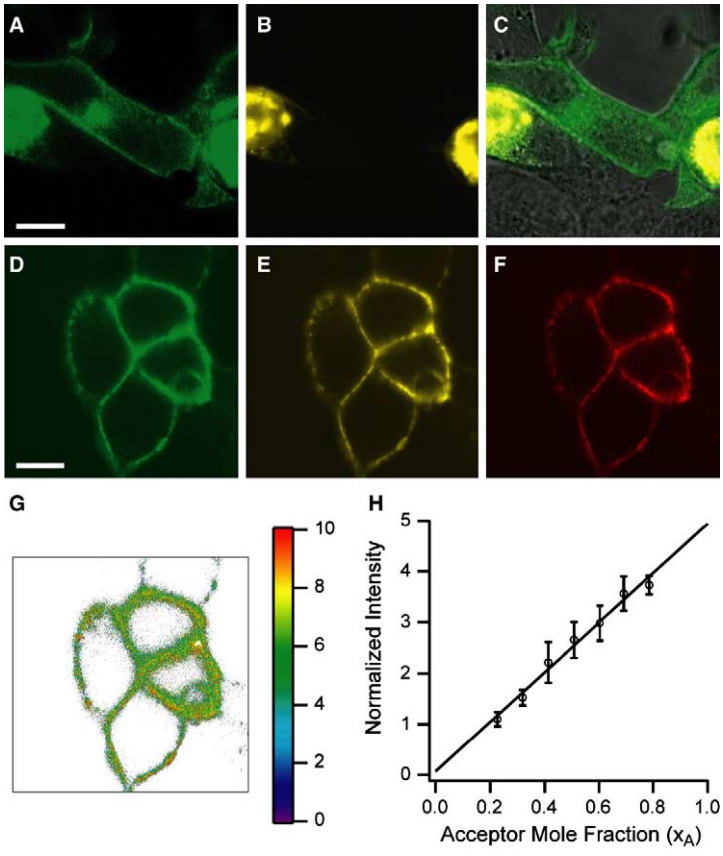

Fig. 3. Controlled double labeling of NK1R by the ACP technique for FRET. HEK293 cells were transiently transfected with a 1:1 DNA ratio of ECFP and EYFP. Fluorescence of ECFP (A) and EYFP (B) was detected using appropriate settings. (C) Transmission image with superimposed fluorescence signals from A and B. (D–F) HEK293 cells transiently expressing ACP-NK1R were labeled simultaneously with an 80%/20% mixture of Cy3 and Cy5. Cells were imaged with the appropriate settings for donor (D), acceptor (E) and FRET (F). Scale bars are 10 μm. (G) FRET ratio (FR) image calculated from the images D–F. The average FR value was 5.75 ± 0.02, corresponding to an apparent FRET efficiency of 52.3%. The color scale represents the FR value. (H) Normalized acceptor intensity (a.u.) as a function of the acceptor mole fraction used for labeling. Data points are means of 10 cells (±S.D.).

receptor-FP fusions in live cells [11]. For experiments in live cells, the ACP method presents two major advantages compared to FP fusions: (i) Improved signal-to-noise ratios can be obtained owing to the exclusive labeling of the cell-surface receptors, and (ii) an extended panel of FRET pairs can be tested with the same fusion protein, reducing tedious cloning efforts. This is of utmost interest in this context because only a limited number of FPs suitable as FRET pairs is available [25].

We further showed the benefits of ACP labeling for monitoring protein–protein interactions. The simultaneous two-color labeling of ACP-NK1R with well-defined ratios of donors and acceptors revealed a high apparent FRET efficiency, showing close proximity of the NK1Rs in the plasma membrane, whose origin will need further investigations. Similar studies using FPs suffer from poorer signal-to-noise ratios and require the co-expression of two fusion proteins at the same time in the same cell in a defined ratio, which is often difficult to achieve and is usually controlled by the DNA ratio used for transfection.

Therefore, we are confident that the many advantages offered by the ACP labeling technique, in particular the possibility of double labeling with controlled DA ratios, make it a generally applicable method for investigating cell-surface proteins by novel fluorescence techniques [5] including molecular interactions, especially homo-oligomerisation, by FRET with potential single-molecule sensitivity. We are presently extending this approach to monitor conformational changes of ionotropic receptors, which are accessible by fluorescence techniques [26,27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.02.007.

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