BRET² assay using the FDSS/µCELL: monitoring cell surface-receptor internalization and intracellular trafficking

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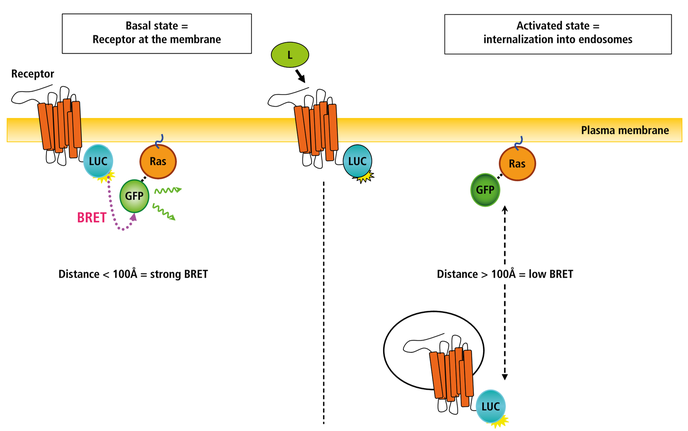
1. Introduction

A previous poster presentation was dedicated to BRET¹ technology applied to the FDSSµCell imaging plate reader and allowed us to monitor (i) the β-arrestin recruitment to a G protein-coupled receptor (GPCR) or (ii) the interaction between Shc protein and tyrosine kinase receptor TrkB (1).

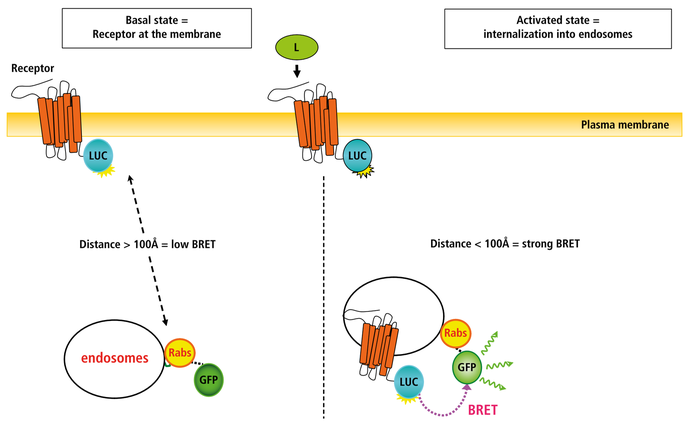
Based on a study published in 2011 (2), we recently implemented the more sensitive BRET² technology to the FDSS/µCell system for monitoring the internalization of the dopaminergic D2s-receptor (short splice form) and its intracellular trafficking. Indeed, to prevent GPCRs from acute overstimulation, their activity is regulated by a mechanism called internalization. Endocytosis of an activated GPCR from the plasma membrane into internal cellular compartments allows the receptor to be recycled to the cell surface or to be targeted for degradation in lysosomes. The D2s-receptor is considered as an indispensable target for antipsychotic medication (3) as well as for antiparkinson agents.

Endocytosis and intracellular trafficking of GPCRs is a dynamic process regulated by Rab-family GTPase activities. Indeed, more than 60 different membrane-anchored Rab proteins control a variety of important intracellular processes in membrane trafficking. For example, Rab5 is mainly localized on early endosomes and controls fusion of clathrin-coated vesicles with early endosomes. While Rab5 represents a probe for early endosome compartments, the inner cell-surface-located Kras GTPase protein can be used as a general marker for monitoring the presence of a receptor at the plasma membrane. Upon receptor activation triggering its internalization, we could monitor distance changes between the receptor and the GTPase proteins, by fusing the luciferase variant Rluc8 to the C-terminus of the D2s receptor and fusing the green fluorescent protein variant GFP² to the N-terminus of Kras and Rab5 GTPases (Cf. figure 1). We observed a BRET decrease or increase between the receptor and the Kras or the Rab5 proteins, respectively.

(a) Biosensor monitoring BRET/KRas internalization



(b) Biosensor monitoring BRET/Rab internalization:



(a) At basal state, over-expression of the two partners and their common location on plasma membrane will allow a relative proximity and a certain energy transfer. The activation of the receptor will trigger its internalization inducing an increase of the distance between the partners and a decrease of BRET.

(b) At basal state, distance is such that the BRET signal is low. Once activated, the receptor is relocated to endosomes inducing a relative proximity and an increase of BRET.

2. Experimental procedure

Rluc8 tagged-D2s receptor (C-terminal) and GFP²-tagged GTPase plasmids (N-terminal) were transiently transfected into CHO cells. We additionally coexpressed β-arrestin2 and GRK2 proteins. 48 hours after transfection, cells were washed twice with PBS plus glucose 1g/l, detached with PBS plus 3mM EDTA and resuspended in PBS plus glucose 1g/l. A Dc protein measurement assay (Bio-Rad) was performed according to the manufacturer's protocol. For BRET monitoring, cells were then distributed (70µg protein equivalent per well) in white and non treated clear-bottom 96-well micro-plates (Costar, ref. #3632) and incubated during 60 minutes at 37 °C in the presence or absence of various ligands before substrate addition. BRET between Rluc8 and GFP2 was measured immediately after the addition of the Rluc8 substrate coelenterazine 400a (5µM final) (Interchim, ref. #BB839B).

BRET² signals were collected using the FDSSµCELL (Hamamatsu Photonics), allowing to integrate successive signals detected in the 387-447 nm (Rluc8) and in the 489-532 nm (GFP2) windows by using appropriate filters. Time of integration of 0.5 second was used for measuring photons emitted by Rluc8 and GFP² (Additional parameters: sensitivity 8; samples number 4). BRET² signals were obtained by calculating the ratio of the energy emitted by GFP² over the energy emitted by Rluc8.

3. Results

3.1-Plasma membrane level (Kras) (Cf. figure 2):

following the exposure to the agonist dopamine, we observe a significant decrease of BRET signal compared to the basal (untreated cells), and this signal is totally reversed by the antagonist haloperidol. Aripiprazole promotes only a partial response (a). The stimulations by dopamine and aripiprazole are concentration-dependent and present similar potencies (b). We exposed the D2s-receptor to a series of antipsychotic and antiparkinson agents, allowing to discriminate them by their efficacy. Antipsychotics are mostly partial agonist, while antiparkinsonians show a full activity similar to dopamine. Clozapine and NDMC (N-desmethylclozapine) present a negative effect which is potentially an inverse agonist activity (c).

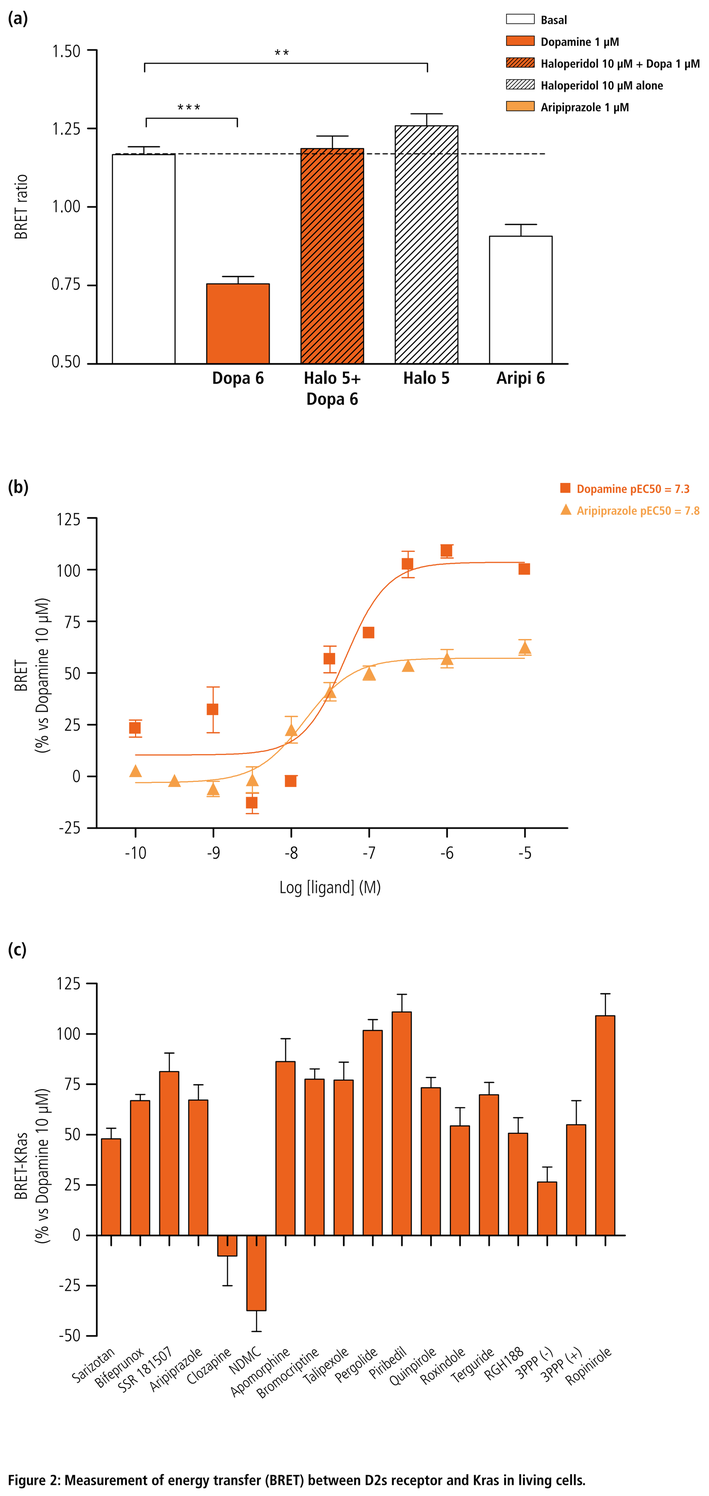


Figure 2: Measurement of energy transfer (BRET) between D2s receptor and Kras in living cells.

BRET is measured in CHO cells coexpressing D2s-Rluc8 and GFP²-Kras. Data represent the mean ± s.e.m. of at least 3 independent experiments. Statistics were performed using a "paired Student t-test".

3.2-Early endosome level (Rab5) (Cf. figure 3):

we observe a significant increase of BRET signal compared to the basal signal when we expose cells to dopamine. The antagonist haloperidol fully areverses the BRET signal induced by dopamine. Aripiprazole promotes only a partial response (a). The stimulations by dopamine and aripiprazole are concentration-dependent (b).

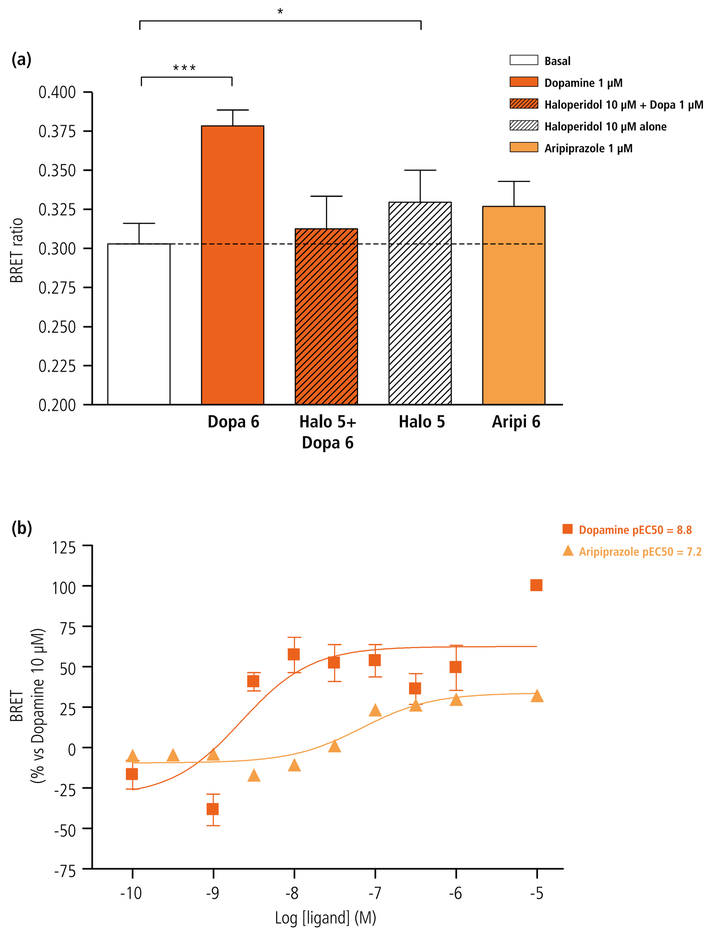


Figure 3: Measurement of BRET between D2s receptor and Rab5 in living cells.

BRET is measured in CHO cells coexpressing D2s-Rluc8 and GFP2-Rab5. Data represent the mean ± s.e.m. of at least 3 independent experiments. Statistics were performed using a "paired Student t-test".

4. Conclusion

By imagining and creating light-emitting probes based on BRET² technology and located in multisubcellular membranes, we succeeded in the development of a new tool allowing the study of GPCR pharmacology and monitoring their intracellular fate. In future times, we should be able to decipher compound-specific intracellular trafficking and discriminate different intracellular pathways, thus correlate drug behavior with specific pathways and evaluate more accurately the therapeutic impact of these specific pathways.

This work confirms the ability of the FDSSµCELL to monitor protein-protein distance variations using BRET² technology.

Bibliography

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