A modular ligand-directed approach to label endogenous aminergic GPCRs in live cells

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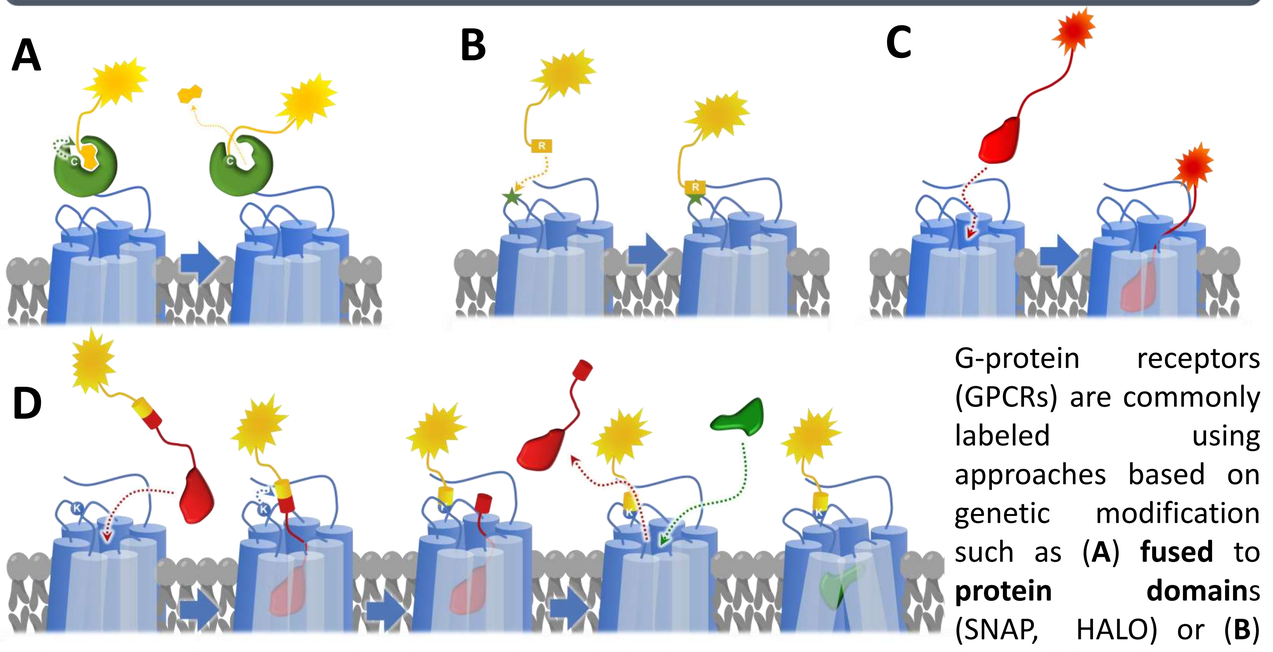
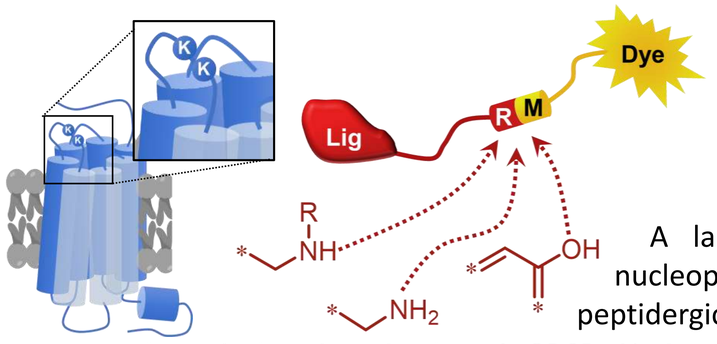
1. Labelling of G-Protein Coupled Receptors



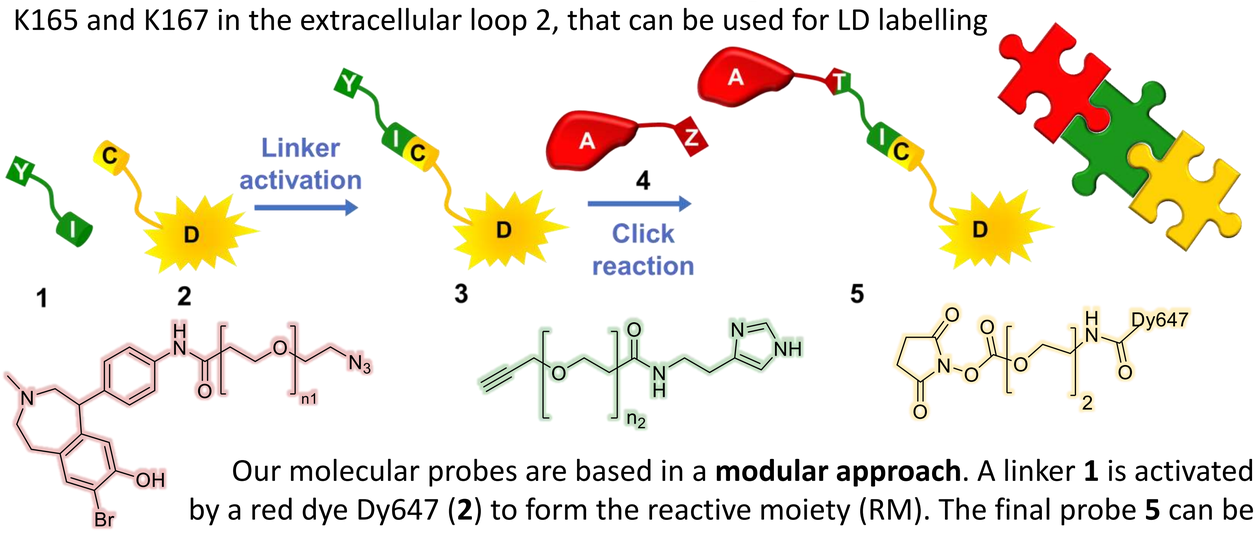
G-protein receptors (GPCRs) are commonly labeled using approaches based on genetic modification such as (**A**) **fused** to **protein domains** (SNAP, HALO) or (**B**) with **unnatural amino acids**. Thus, a dye is linked to a reactive group that will **biorthogonally** react with the modified part of the GPCR. (**C**) **Native GPCRS** can be labelled with **fluorescent ligands** or **affinity probes**, but they modify the GPCR activity. (**D**) **Ligand-directed approaches (LD)** are based on probes containing an **affinity unit** (i.e. a ligand), a **reactive moiety** and a **fluorescent dye**, which bind to the native GPCR. Typically, a natural nucleophilic residue (e.g. lysine) in the vicinity of the linker group reacts with the reactive moiety to form a covalent link with the dye and the resulting ligand can be released and washed-out, leaving a native GPCR labeled and fully functional. This receptor is able to get activated by agonists or antagonists.

2. Limitations of LD labeling and a solution for D_1 receptor



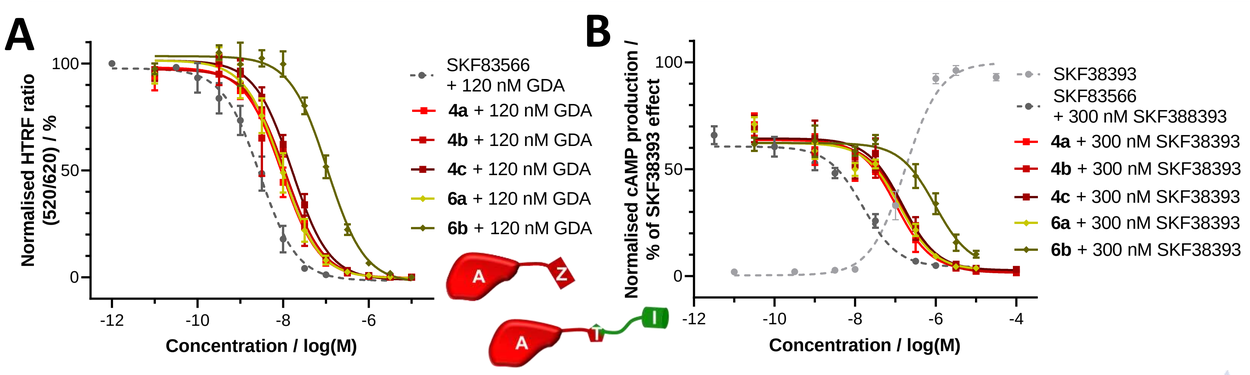
LD labelling requires the use of n**on-nucleophilic affinity units**. Amines or other nucleophilic functional groups can interact with the probe reactive moiety, inducing probe degradation.

A large number of GPCR ligands contain nucleophilic ligands, such as **aminergic**, peptidergic or other **GPCRs**. We used **Dopamine D_1** as an archetypical aminergic class A GPCR. Native hD_1 receptor contain two lysine residues K165 and K167 in the extracellular loop 2, that can be used for LD labelling



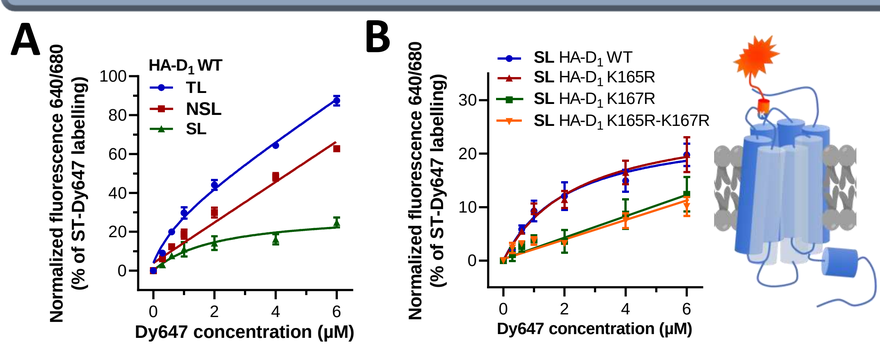
Our molecular probes are based in a **modular approach**. A linker **1** is activated by a red dye Dy647 (**2**) to form the reactive moiety (RM). The final probe **5** can be readily prepared before the labelling reaction from **3** and a long ligand **4**. Thanks to a fast and specific click reaction, the nucleophilic ligand can barely react with the activated linker before it is bound to the native target GPCR and the labelling reaction occurs.

3. Extended ligands bind D_1 receptor and are antagonists

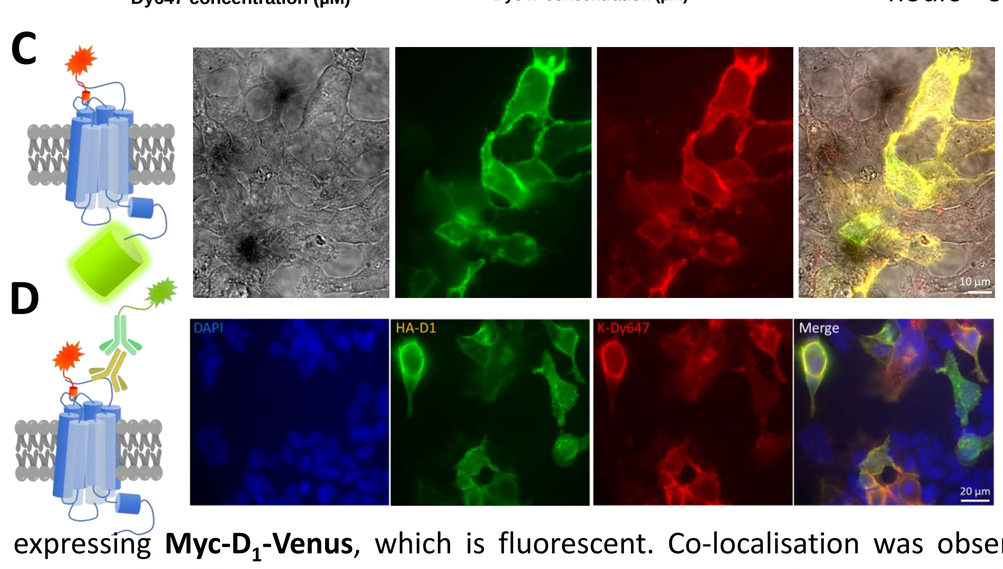


Three ligands **4** with different linker lengths and two longer ligand **6** were tested with both binding (**A**, HTRF TagLite®) and functional assays (**B**, HTRF cAMP) with hD_1. All compounds were D_1 antagonists with nanomolar affinity and the linkers were well adapted.

4. HA-D_1 was labelled with CLD probe 5 in the lys167

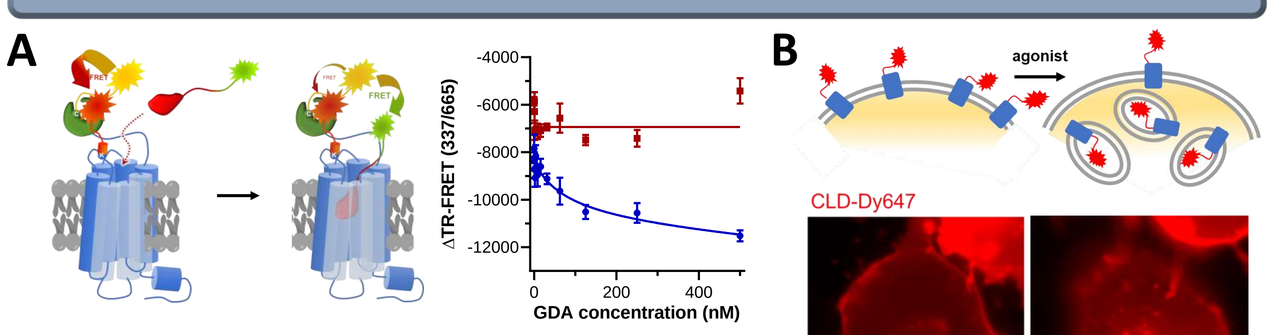


Several ligand-directed probes **5** recently prepared were tested to label HA-D1 transiently expressed in HEK293 cells. The best candidate efficiently labelled HA-D_1 with 3 hours of incubation (**A**).



(**B**) The mutant **K167R** was fully functional (not shown) and **did show labelling** by the CLD probes **5.** (**C**) D_1 labelling was observed after applying ligand-directed probes **5** in transfected HEK293 cells expressing **Myc-D_1-Venus**, which is fluorescent. Co-localisation was observed, indicating **D_1 receptor labelling**. (**D**) HA-D_1 was also labelled with **5** and D_1 was also detected by anti-HA immunofluorescence Co-localization of both dyes indicated D_1 receptor labelling.

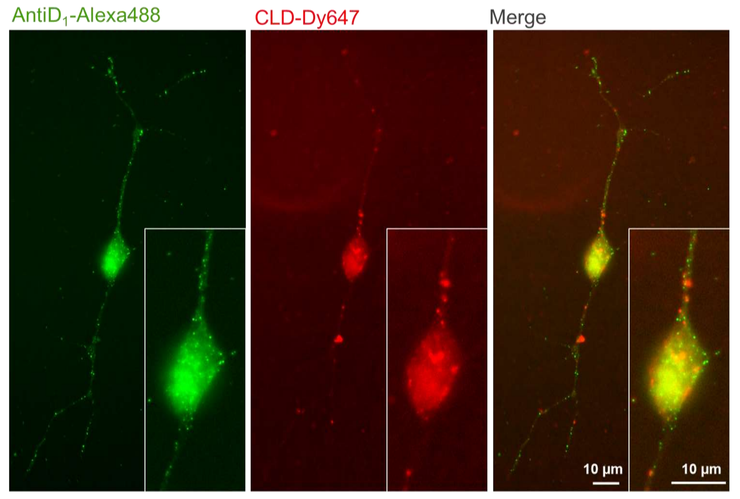
5. The D_1 orthosteric binding site is available after labelling



(**A**) F-ST-D_1 was doubly labelled with probe **5** (CLD-Dy647) and with SNAP-Lumi4Tb®. It showed TR-FRET between the two dyes. Upon application of a fluorescent green D_1 antagonist, we observed a decrease of the red fluorescence due to the formation of new TR-FRET with the green dye. This decrease of fluorescence indicated the **availability** of the **binding site**. (**B**) HEK293 cells expressing HA-D_1 labelled with **5** display fluorescence at the cell membrane, but after 30 min of SKF81297 (1 μM) application, the fluorescence is translated in the in the cytosol, indicating **labelled receptor internalization**.

6. Endogenous D_1 is labelled in neuronal cell lines

SH-SY5Y cells can be differentiated to a neuron-like cells and express **endogenous D_1 receptor**. After labelleing with **5**, cells were fixed with para formaldehyde, immuno-stained and imaged. Immunostaining fluorescence (left) and Dy647 fluorescence (center) were located mostly at the cell membrane with a decent co-localization (right).



7. Conclusions

The Click ligand-directed (CLD) labelling approach is a successful modular approach based in the preparation of the probes **5** just before the labelling protein labelling. We proved D_1 receptor labelling at K167 and we observed labelling of Myc-D_1-Venus, HA-D_1 and native D_1 from neural cells. Moreover, the binding site is available and the receptor is fully functional after CLD labelling.

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