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

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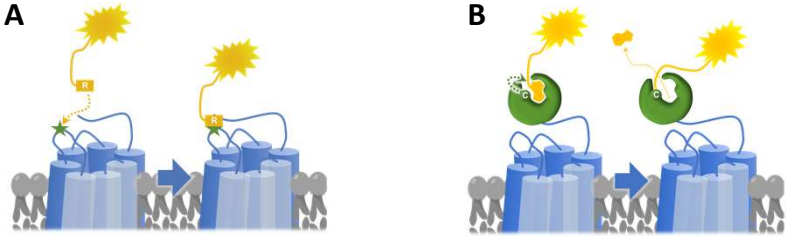
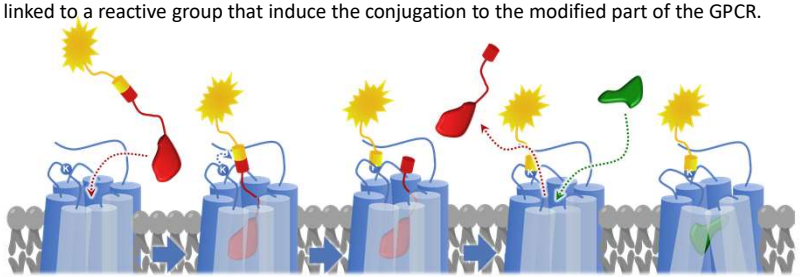
## 1. GPCR labelling approaches



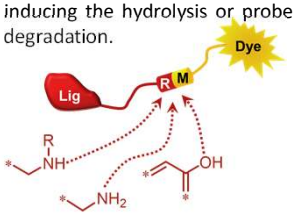

GPCR labelling through bioortogonal reactions with unnatural amino acids (**A**) or fused to protein domains (**B**, SNAP, CLIP, HALO…) is based on proteins genetically modified. Thus, a dye is linked to a reactive group that induce the conjugation to the modified part of the GPCR.

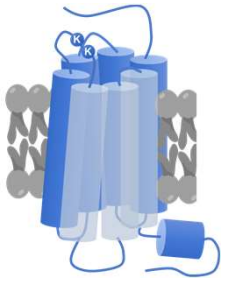


**Ligand-directed approaches (LD)** are based on probes containing a ligand, a reactive moiety and a dye, which bind to the native GPCR. Typically, a natural nucleophilic residue (e.g. lysine) in the vicinity of the linker group reacts with the reactive moiety to form a covalent link with the dye and 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. LD with nucleophilic ligands? Aminergic GPCRs

LD labelling requires the use of non-nucleophilic ligand moieties. Amines or other nucleophilic functional groups can interact with the probe reactive moiety, inducing the hydrolysis or probe degradation.

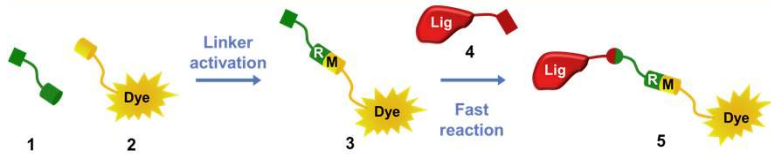


A large number of GPCR ligands contain nucleophilic ligands, such as aminergic, peptidergic or many other GPCRs.
We used dopamine D1, as an archetypical aminergic class A GPCR coupled to Gs, which is mainly expressed in central nervous system (CNS), specially in the dorsal and ventral striatum.

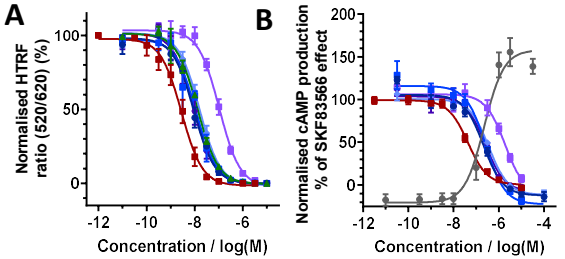
Native $hD_1$ receptor contain to Lys residues K165 and K167 in the extracellular loop 2, that can be used for LD labelling.

## 3. Lanthslider labelling approach



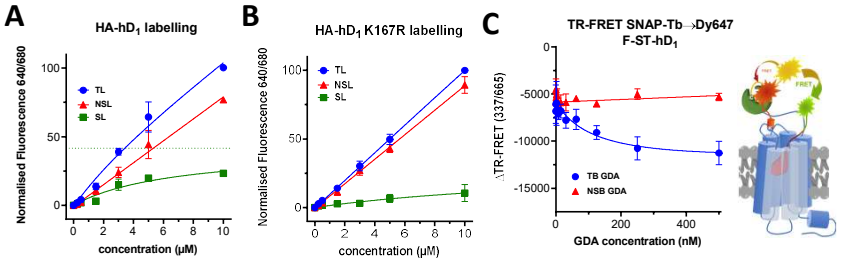

Our molecular probes are based in a modular approach. A linker (**1**) is activated by a red dye Dy647 (**2**) to form the reactive moiety (RM). The final probe **5** can be readily prepared before the labelling reaction from **3** and a long ligand (**4**). **T**hanks to a fast and specific chemical 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.

## 4. Pharmacological evaluation of ligands



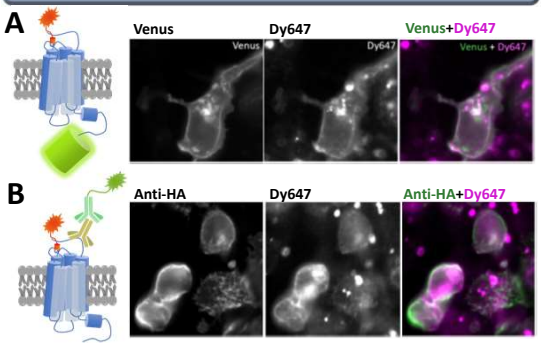

Several ligands **4** with different linker lengths were tested with both binding (**A**, HTRF TagLite®) and functional assays (**B**, HTRF cAMP) with $hD_1$. All compounds were $D_1$ antagonists with nano-molar affinity and the linkers were well adapted.

## 5. $D_1$ labelling and determination of the site of labelling




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**). The mutant K167R was fully functional (not shown) and did show labelling by the labelling probes 5 (**B**). F-ST-$D_1$ was doubly labelled with probe **5** (red dye, Dy647) and with SNAP-Lumi4Tb®. It showed continuous TR-FRET between the two dyes. Upon application of a fluorescent green D1 antagonist, we observed a decrease of the red fluorescence due to the formation of new TR-FRET with the green dye (**C**). This decrease of fluorescence indicated the availability of the binding site and was compatible with the affinity of the green $D_1$ antagonist.

## 6. Imaging labelled $D_1$ in cells




$D_1$ labelling was observed after applying ligand-directed probes **5** in transfected HEK293 cells with Myc-$D_1$-Venus, which is fluorescent (**A**). Co-localisation was observed, indicating $D_1$ receptor labelling. HA-$D_1$ was also labelled with **5** and $D_1$ was additionally detected by anti-HA immunofluorescence (**B**). Co-localization of both dyes was also observed, indicating $D_1$ receptor labelling.

## 7. Conclusions

The Lanthslider ligand-directed labelling approach is a successful modular approach based in the preparation of the probes **5** just before the labelling protein labelling.
Several $D_1$ antagonists with different linkers were tested and they show antagonism for D1 in the nanomolar range.
The probes **5** were tested and we obtained labelling with 3 hours of incubation. Myc-$D_1$-Venus and HA-$D_1$ labelling was observed by microscopy. We determined that the site of labelling is Lys167 by mutagenesis and we proved that the binding site was available after $D_1$ labelling.

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Early Career Investigators

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