An Original Approach to Measure Ligand/receptor Binding Affinity in Non-purified Samples

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Abstract

Several biochemical and biophysical methods are available to determine dissociation constants between a biological target and its ligands. Most of them require purification, labelling or surface immobilisation. However, these measurements remain challenging concerning membrane proteins because purification requires their extraction from the native lipid environment using different approaches, a process that may impact receptor conformation and functionality. We have developed a novel experimental procedure to determine binding affinities of a ligand to a membrane protein, the dopamine D2 receptor (D2R), directly from cell membrane fragments, using microscale thermophoresis (MST). Two main challenges had to be overcome: to determine the concentration of dopamine D2R in the crude sample; to find ways to minimize or account for non-specific binding of the ligand to cell fragments. Using MST, we were able to determine the D2R concentration in cell membrane fragments to be about 36.8 ± 2.6 pmol/mg. Then titration curves allowed the determination of a K_D about 5.3 ± 1.7 nM, that is very close to the reported value. Important details of the experimental procedure are detailed to allow the transposition of this novel method to various membrane proteins.

Introduction

G protein-coupled receptors (GPCRs) constitute a large family of integral membrane proteins. As they are able to induce very different intracellular signalling events upon activation by extracellular stimulus (small molecules, peptides, light, odorants, etc) they represent one of the most important drug target family. However, it is still challenging to assess their pharmacological properties in physiological environment. One of the properties that is often investigated concerns ligand affinity to GPCRs. Among the biochemical or biophysical approaches available, some strategies require labelling of one of the partners (in-solution approaches) and others require capture of one of the partners (sensor-based technology approaches). To do so, there are two general strategies: 1) to perform measurements directly on cells or cell fragments; 2) to extract the receptor from its natural environment, to isolate, purify and quantify it before measuring ligand affinity(1). While the second option allows for certain parameters to be better controlled (lipid and protein membrane composition for example), the first strategy is far more employed due to its simplicity and the fact that receptor isolation and purification can alter the native protein functionality (2). Purification frequently requires addition of tags to the native protein, changing the protein sequence. Concerning membrane protein, the solubilisation step is an additional step that can affect the protein functionality(3). Furthermore, recent findings have shown the role of membrane components on the protein functionality, in particular for GPCRs(4). It is therefore important to develop methods and protocols to characterize GPCRs in their complete native environment. One of the pioneer methods, and still of great use nowadays is the radiolabel assay. While being extremely sensitive (K_D in the picomolar range can be determined)(5) the drawbacks are numerous, including the necessity to have specific laboratory equipment, dedicated and isolated laboratory space and trained personal to safely deal with the hurdle of using radioactivity(6). To avoid such problems, within the last two decades or so, several approaches based on fluorescence measurements have been reported(7), often associated with

the development of new methods and establishment of protocols. The most well-known are based on the measurement of the fluorophore rotational speed, such as fluorescence polarization (FP), or based on energy transfer such as homogeneous time resolved fluorescence (HTRF), both of them exploiting the light properties.

Concerning FP, when a fluorescent probe is excited, light is emitted with a certain degree of polarization. When the fluorescent probe (the small ligand being the labelled partner) is bound to a large particle, such as proteins in suspension, the freedom of movement is highly decreased, which results in higher polarization of the emitted light. This change in polarization is measured as a change in FP signal(8). While this method is quite robust and allows for both equilibrium and kinetic data to be obtained, it has not been extensively used probably due to the fact that the choice of the fluorescent probe and its location along with the physical properties within the free ligand itself and when bound to the receptor have to be carefully considered (for review see Rinken(9)). More recently, microscale thermophoresis (MST) has been developed as a low sample consumption and versatile method for quantification of various protein interactions, including ligand/receptor ones. The method relies on the observation of fluorescence variation upon a sudden temperature modification, of a few degrees, induced by an infrared laser. This temperature change induces two interconnected phenomena(10). On one hand thermophoresis relates to the diffusion of the molecules induced by the temperature gradient thus generated. On the other hand, the temperature related intensity change (TRIC) describes the dimming of the dye with increasing thermal agitation. The combination of the two describes MST and the modification of intensity observed upon temperature modification is linked to the size, the charges and the hydration of the labelled molecule referred as the target. Any modification upon binding to the target will ultimately be linked to a modification of the MST signal, thus making the technique sensitive to even minute changes such as ion binding to a labelled protein. Last but not least since the technique relies on fluorescence emission it uses only a few nM of the target and utilizes a 10 µL sample cell. The sensitivity of MST allows label-free measurements, using directly tryptophan fluorescence of purified proteins(11). Interactions must be monitored between a fluorescently-labelled partner and a non-labelled one. When the protein is purified, labelling kits are commercially available to label the protein via lysine or cysteine residues(12). MST is also enough sensitive to work in complex matrices such as blood samples or cell lysates. Such approaches usually required the target to be specifically labelled. For example, a fused GFP protein has been used to investigate protein-protein interactions(13). Tagged proteins (Histidine Tag, SNAP tag) can also be specifically coupled to a fluorophore using commercially available kits(14). Finally, as for FP measurements, it is also possible to use fluorescently labelled ligands(15).

To the best of our knowledge, all MST studies involving GPCRs have been performed using purified systems(11, 16–18). Several challenges appear when working with very complex matrices, in particular determination of the concentration of the biomolecule of interest and non-specific binding to matrix components. In this paper, we propose a method to characterize ligand binding to a model GPCR, the dopamine D2R receptor (D2R), using MST with minimal manipulation of the crude protein following expression in mammalian cell plasma membrane.

Results

Determination of D2R concentration in cell membrane fragments

In the system employed in the present work, the fluorescent partner is the ligand, spiperone - Cy5 (a known D2R antagonist)(19), which is maintained at fixed concentration, while the D2R concentration is varied. As the D2R sample consists of non-purified membrane fragments (HEK cells expressing the D2R, HekD2), its concentration in samples is unknown. Therefore, the first goal was to determine the D2R concentration using MST. To do so, a titration curve has been performed with fixed spiperone – Cy5 concentration above the K_D while varying D2R concentration. As at this point, D2R concentration is unknown, it is here expressed in terms of the total protein mass obtained by bicinchonic acid assay (BCA) that ranged from 10 mg/mL to 5 µg/mL. Taking into account that one ligand binds per receptor molecule, the idea is that the signal should increase with increasing protein concentration, until reaching a maximum signal that should correspond to the point where the ratio of ligand to receptor is 1:1. After that, a plateau should be observed.

Three different spiperone - Cy5 concentrations were used for incubation with D2R membrane fragments (HekD2 diluted in the buffer): 5 (supplementary fig. S1 online), 7.5 (Figure 1A, 1B), and 12 (supplementary fig. S1 online) nM. At very low membrane fragment concentration, spiperone - Cy5 stays unbound. In presence of D2R in the membrane fragments, a linear relation between the protein concentration and the fluorescence signal is observed until it reaches a plateau. The intersect point corresponds to a condition where there is an equal concentration of D2R and spiperone - Cy5 (Figure 1C). The concentration of D2R in membrane fragments has been calculated using the intersect point of the mean curve obtained from 3 independent experiments. The calculated D2R concentration in HekD2 (antibiotics-induced) cell membrane fragments is 33.9, 37.9 and 38.8 pmol/mg of total protein with 5, 7.5 and 12 nM spiperone - Cy5, respectively (Figure 1D). The results indicate, as expected, that D2R concentration is quite similar among the three measurements (Table 1), so independent of the ligand concentration used, as expected. In parallel, the D2R concentration determined in non - induced HekD2 membrane fragments is 4 and 3 pmol/mg of total protein with 5 and 7.5 nM spiperone - Cy5, respectively. This has not been performed at 12 nM as it was already difficult to saturate D2R with 7.5 nM spiperone - Cy5 in this sample. For HekWT sample, spiperone - Cy5 binding to membrane fragments is observed for the highest total protein concentrations, but it doesn't reach a plateau (Figure 1A, 1B).

Table 1 : D2R concentration per total protein content.

	[D2R] pmol/mg	
	Hek D2	HekD2 induced
5	4	33.9
7.5	3	37.9
12	-	38.8

The D2R concentration of HekD2 induced has been fixed at 37 pmol/mg (mean of all the measurements) for the second type of experiments to be performed by MST, aiming at determining the affinity of known D2R ligands to this receptor (dose response curves).

Optimization of conditions for the measurement of dose response curves

Binding checks were performed with different ligand concentrations in absence (buffer only) or presence of membrane fragments expressing (HekD2) or not (HekWT) the D2R, to optimize the amplitude of the measured MST signal. Ideally, the difference between the signal of the samples with and without D2R should be maximal, ideally one would aim for a signal to noise ratio above 12. At a low concentration of spiperone – Cy5 of 0.125 nM, the amplitude signal between HekD2 membrane fragments and buffer is very small, as observed on figure 2A. However, when the spiperone – Cy5 is diluted in HekWT membrane fragments, rather than buffer, a larger amplitude (about 5%) of the fluorescence signal between bound and not-bound spiperone is observed. On the contrary, the amplitude of the MST signal of spiperone – Cy5 is similar in buffer or HekWT membrane fragments (unbound) for 5, 7.5 or 12 nM spiperone – Cy5, and largely lower than HekD2 (bound), as expected (supplementary fig. S2 online).

As a consequence, the dose response curve obtained by diluting directly the membrane fragments in buffer, is not optimal (Figure 2B). The MST signal magnitude difference between the highest concentration of D2R and the smallest one is very close (around 910 Fnorm), even if certain variations among them are observed. In this type of dilution, not only the concentration of D2R varies, but also the concentration of the lipids and other proteins present in the membranes. Therefore, it became clear to us that there is a need to maximize differences between the samples containing or not the D2R. For such, we decided to dilute the samples with HekWT membrane fragments (with similar total protein concentration) rather than buffer, as illustrated in figure 3A. The idea is to maintain a resembling environment, especially in terms of potential non-specific molecular interactions in all samples.

Determination of ligand binding affinity to D2R in cell membrane fragments by MST

For the dose response curve, HekD2 membrane fragments were diluted directly with HekWT membrane fragments in order to maintain a constant concentration of lipids and total proteins in each capillary, and observe an optimized MST amplitude between bound and not bound spiperone - Cy5 to D2R only.

In this measurement, spiperone - Cy5 is maintained at a fixed concentration of 0.125 nM, which is well below the expected K_D (6.6 nM, according to the supplier), while D2R concentration is varied from 10^{-12} to 10^{-8} M. Moreover, in order to discard any possibility regarding non-specific binding between the ligand and the cell membranes (as such ligands have strong affinity for lipids (20), such experiments have been carried out in absence and presence of a D2R antagonist, and known antipsychotic, haloperidol (Figure 3B). As shown in figure 4A and 4B, MST signal rises with increasing D2R concentration in absence of haloperidol (black curves). On the contrary, in presence of haloperidol, the MST signal stays unchanged (red curves). This data confirms that the MST signal increase observed in absence of haloperidol reflects the specific binding between spiperone - Cy5 and the D2R. This allows specific binding to be obtained by subtracting the non-specific binding from the total binding (Figure 4C, D). The K_D determined by fitting the

binding curve obtained from the mean MST signal without haloperidol (total binding) is 4.3 nM which is very close to the K_D determined by fitting the binding curve obtained after subtraction of the mean MST signal with haloperidol (non-specific binding) from the mean MST signal obtained without haloperidol (total binding) is 5.3 ± 1.7 nM.

Discussion

Herein, we present a protocol to measure binding constants of integral membrane proteins such as GPCRs in conditions very close to their native environment. The D2R has been used as a model GPCR, as it is a major therapeutic target, from antipsychotic drugs to anti-emetic ones. MST has been already used to determine K_D of ligand/membrane protein complexes. Generally, the protein of interest is solubilized, specifically labelled, often after purification. When non-purified, the technique requires specific labelling of the protein, for example through an histidine tag. However, in these very heterogeneous samples, it was not possible to detect any change in MST difference between bound state (with spiperone or quinpirole) and unbound state (supplementary fig. S3 online). On the other hand, when the protein is solubilized, for example using DiIsoButylene-Maleic Acid (DIBMA) copolymer, but not purified, this polymer results in strong non-specific binding of the fluorescent ligand spiperone -Cy5 (supplementary fig. S4 online). In this paper, the D2R has been expressed in mammalian cell culture and studied, in suspension, in membrane fragments, without any need for solubilisation, purification, or labelling procedures. Instead, a fluorescent ligand, available for the receptor of interest, has been used as the fluorescent tracer. In this type of samples, K_D values are generally determined using binding kinetics available by homogeneous time-resolved fluorescence (HTRF)(21) or fluorescence polarization(8). The MST instrument is actually not ready to perform binding kinetics, so the dissociation constant can only be determined directly by dose response curves or indirectly by competition assays. The strategy developed in this paper is to determine first the concentration of the integral membrane protein in the samples (in this case the D2R) and then the dissociation constant without any further purification or characterization step, using MST. Determining the membrane protein concentration in the sample using a fluorescent ligand requires the use of a ligand concentration higher than the K_D, but less than the concentration in the protein sample. These conditions lead to a saturation regime, where all the receptor is depleted from the solution due to binding to the fluorescent ligand until there is no more free fluorescent ligand(22). The intersect between the linear progression of Fnorm with increasing D2R concentration and the plateau corresponds to the concentration of D2R, according to the stoichiometry of the reaction (Figure 1C). As we were not sure that the titration regime with 5 nM final concentration of spiperone - Cy5 was obtained, because it is close to the expected ligand K_D, we repeated this experiment with higher ligand concentrations: 7.5, and 12 nM. The fact that the use of such ligand concentrations did not affect the quality of the binding curves obtained, this let no doubt on the binding regime that we observed. Moreover, the D2R concentration calculated in the three conditions was very much comparable (Table1), with a mean of D2R concentration of 36.8 ± 2.6 pmol/mg, further reinforcing the data obtained. D2R levels in non-induced HekD2 were too low to be used in our setup since unspecific event toward the membrane occurs at concentration estimated for the binding to D2R, as highlighted by the similarity between HekWT (green) and HekD2

(blue) (Figure 1A, 1B, SI1). Thus induced cells were used in our study. Plus, the concentration of D2R in the highest concentration of membrane fragments is close to the K_D, so it's not evident if the saturation regime is obtained. To determine the K_D, the MST response is also plotted against various concentrations of D2R, but the concentration of the fluorescent ligand is ten times lower than the concentrations used for the titration of D2R. At this concentration, a same MST response is observed for spiperone-Cy5 in presence of HekD2 or buffer (figure 2A). An important point of the experimental procedure has been implemented: HekD2 fragments need to be diluted in HekWT cell fragments to limit any additional potential variation, except D2R concentration, in samples since spiperone-Cy5 exhibits strong non-specific binding to hydrophobic environments as lipids. In this configuration, only the concentration of D2R varies across the dilution, while the concentration in total protein (other than the D2R) and lipid membrane remain stable. Using this experimental procedure, only the specific binding is observed. To investigate if non-specific binding occurs, a high concentration of haloperidol, a non-labelled D2R antagonist, was added to the different dilution wells. After subtraction of non-specific to total binding, the K_D has been determined to be 5.3 ± 1.7 nM, which is very close to the reported values, provided by the commercial supplier (6.6 nM)(23), or by another group (3.9 nM)(24). This paper describes for the first time a protocol to determine the K_D between a ligand and a GPCR directly in membrane fragments, without any solubilisation, purification, or labelling step, using MST only. This novel methodology may be implemented to determine K_D of various membrane proteins, allowing an alternative strategy to the panel already available.

Methods

Materials

Fetal bovine serum (FBS), trypsin/EDTA (0.05%/0.02%), ethylene diamine tri acetic acid (EDTA), Dulbecco's Phosphate Buffered Saline (DPBS), Protease inhibitors cocktail (PI), Tween 20, Tetracyclin were purchased from Sigma-Aldrich (Munich, Germany). Bicinchonic acid protein assay kit has been purchased from Thermoscientific. Blasticidin and Hygromycin B were obtained from Invivogen. Tag-Lite® dopamine D2 receptor red antagonist (spiperone - Cy5) was purchased from Cisbio Bioassays. The total protein concentration has been determined using the Pierce™ BCA protein assay kit provided by ThermoFischer Scientific.

Mammalian cell culture

Hek293T cells (HekWT) and the stably transfected D2R-expressing Hek-293T (HekD2) cells, were kindly provided by Prof. Jonathan A. Javitch (Department of Pharmacology, Columbia University, New York, USA). Both cell lines were maintained in DMEM supplemented with 10% FBS in a water-saturated atmosphere (37°C, 95% air, 5% CO2). To enhance D2R expression level from HekD2 cell line, cells were incubated 24 h with the following antibiotic cocktail: hygromycin 0.1 mg/mL, tetracyclin 0.01 mg/mL, blasticidin 0.015 mg/mL.

Preparation of membrane fragments

When cells are at 80% confluence in T175 cell culture flasks, they were dissociated with 1.5 mL DPBS containing 1 mM EDTA. Cells were collected by centrifugation and pellets were incubated in PBS 20 mM, EDTA 2 mM, PI, for 30 min on ice. Samples were homogenized by 100 up and down using Potter homogenizer on ice and centrifuged 10 min 400 x g at 4°C. Pellets were collected and a second time homogenized with Potter on ice and centrifuged 10 min 400 x g at 4°C. Supernatants were centrifuged 45 min at 20 000 x g at 4°C to pellet membrane fragments. The pellets were then collected in a smaller volume with PBS, tween 0,05%, PI in order to obtain a total protein concentration of ~10 mg/mL, as controlled by BCA for each samples. Samples are stored at -80°C before use.

Microscale thermophoresis

MST experiments were performed on a Monolith NT. 115 (NanoTemper Technologies GmbH) using a red filter set. All dilutions were prepared ensuring that no other gradient (salt, glycerol, DMSO, etc) was created during buffer mixing. To minimize adsorption of the sample to material, low retention tips and tubes were used, and Tween 20 at 0.05% was added to the PBS, buffer used to dilute all components. After mixing the different components, the samples were incubated at room temperature for 30 to 60 min before loading into standard capillaries (Nanotemper Technologies). For binding check, HekD2 membrane fragments, buffer, or HekWT, adjusted to HekD2 regarding total protein concentration, was mixed with an equal volume of spiperone - Cy5 to obtain a final concentration of 0.125, 5, 7.5, 12 nM. After an incubation time of 1 hour, capillaries are loaded and the LED is set to 20% for 0.125 nM, and 1% for 5, 7.5 and 12 nM, using medium MST power. For receptor titration assay, the protein sample at various concentrations ranging from ~10 mg/mL total protein to ~ 5 µg/mL is mixed to a fixed concentration of fluorescent ligand. The fluorescent ligand, spiperone - Cy5, is added at a final concentration of 5, 7.5, or 12 nM to each dilution points. An incubation time of 1h is taken before capillary loading. The LED power is set to 1% and MST power to “medium”. The intersect point has been determined using Excel, from the curves obtained from the mean of three replicates, as shown in figure 2B. In binding affinity assays, samples obtained from HekD2 are serially diluted 1:1 (v:v) in HekWT adjusted at the same total protein concentration, before adding spiperone - Cy5 at a final concentration of 0.125 nM, with or without haloperidol 10 µM (for competition assays). After 2h incubation time, capillaries are loaded. The LED power is set to 20% and MST power to “medium”. The K_D has been determined using the one site - specific binding equation from GraphPad software, from the curves obtained from the mean of three replicates. In all MST protocols, MST-on time is measured at 1.5 s after infrared laser heating.

Declarations

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Author contributions statement

R. B., E. R., and I. A. conceived the experiment(s), A.D. and E. R. conducted the experiment(s), E. R. and P. S. analysed the results and prepared the figures. All authors reviewed the manuscript.

Competing interests

The author(s) declare no competing interests.

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Figures

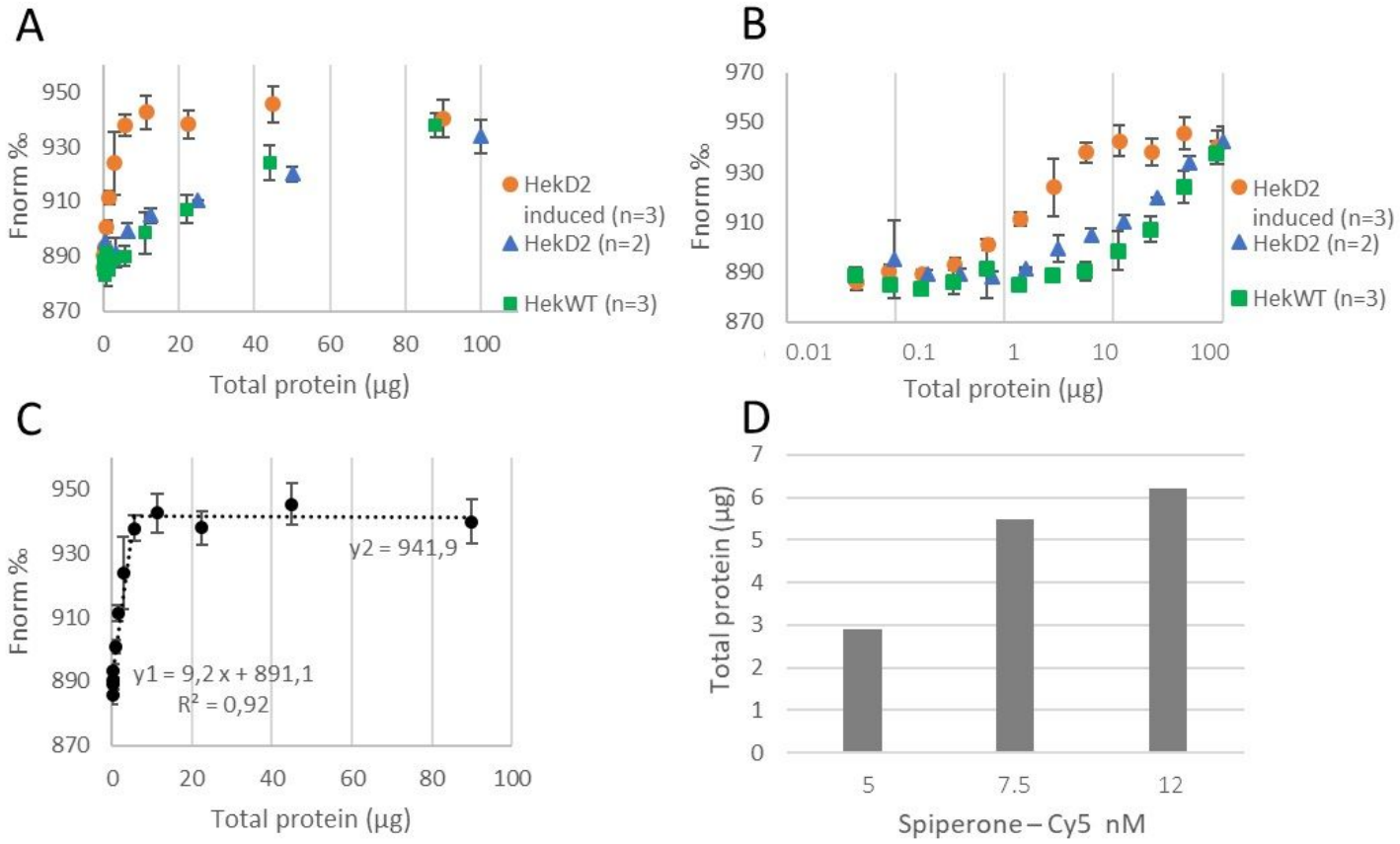

Figure 1

Determination of D2R concentration in cell membrane lysates using MST. Membrane fragments were serially diluted 1 : 1 and incubated 1h at room temperature with a fixed concentration of spiperone - Cy5 at 7.5 nM (A, B). Titration was performed with membrane fragments obtained from induced HekD2 (orange), incubated 24h with antibiotics (for details see materials and methods), non-induced HekD2 (blue) and HekWT (green) cells. Total protein mass was measured by BCA assay, and reported in a logarithmic scale (A) or linear scale (B). The intersect point has been determined using the equations of the two curves obtained from 3 independent replicates, considering y1 = y2 (C). The value of x constitutes the total protein concentration corresponding to the spiperone - Cy5 concentration (7.5 nM in this example). The value of x expressed in total protein mass increases when the concentration of spiperone - Cy5 is increased (D).

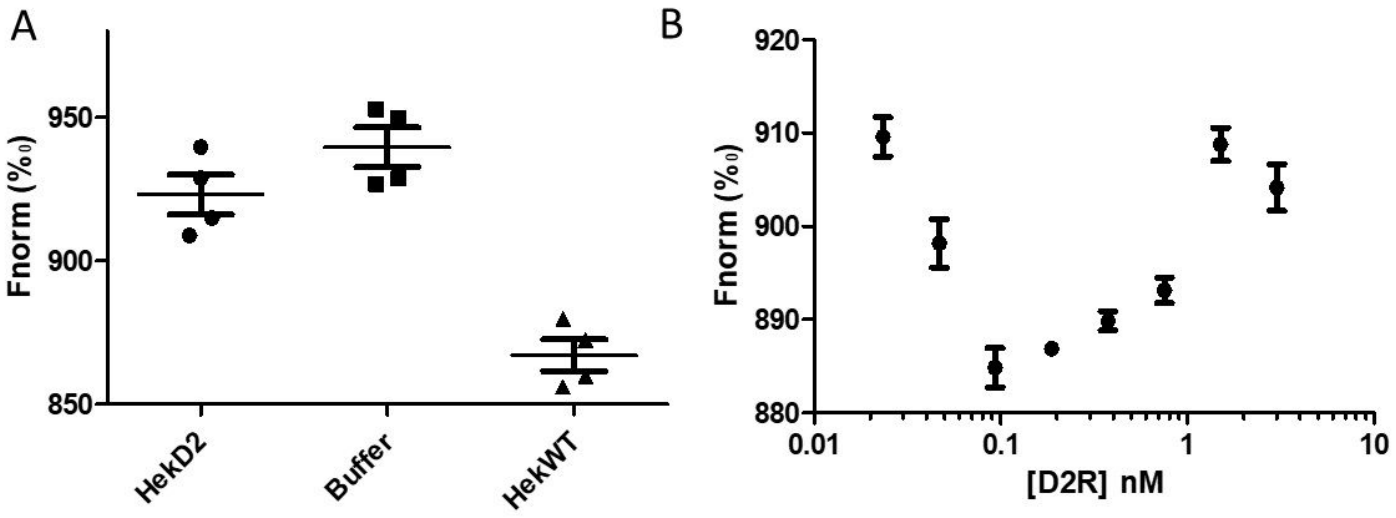

Figure 2

Binding check before saturation curve. The binding check allows the comparison of MST signal for 0.125 nM spiperone - Cy5 in presence of induced HekD2, buffer or HekWT (A). The total protein concentration was set similar in HekD2 and HekWT. Saturation curve obtained when HekD2 membrane fragments were diluted in buffer with 0.125 nM spiperone - Cy5 (B).

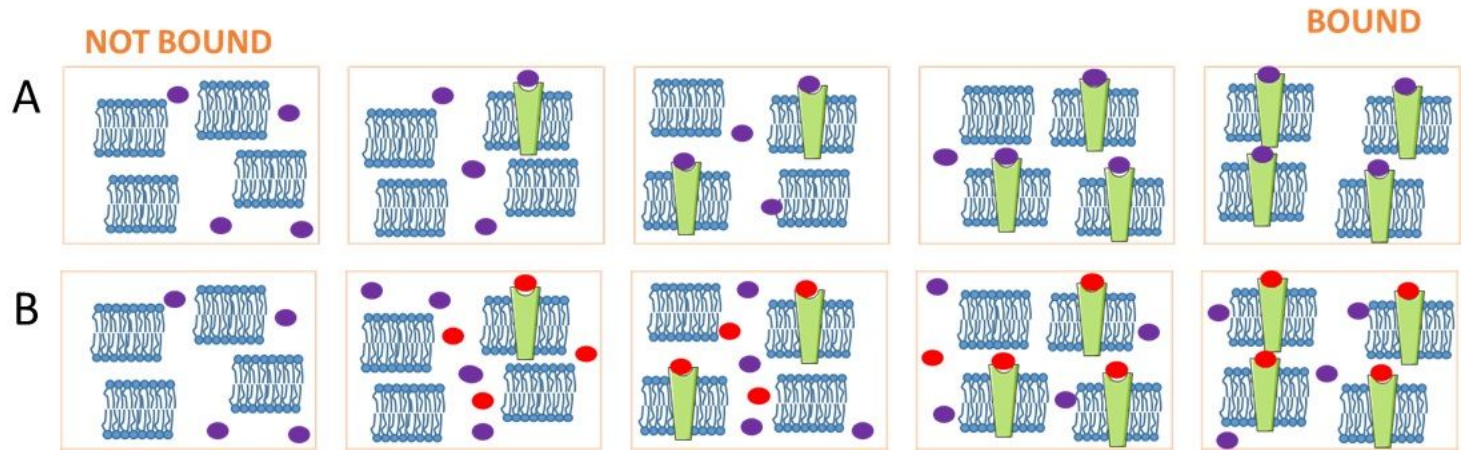

Figure 3

HekD2 membrane fragments (D2R in green) are diluted in HekWT membrane fragments (A). To characterize the presence or not of non-specific binding, a high concentration of non-labelled D2R antagonist, haloperidol (red), is added in each dilution point to compete with labelled ligand (violet) (B).

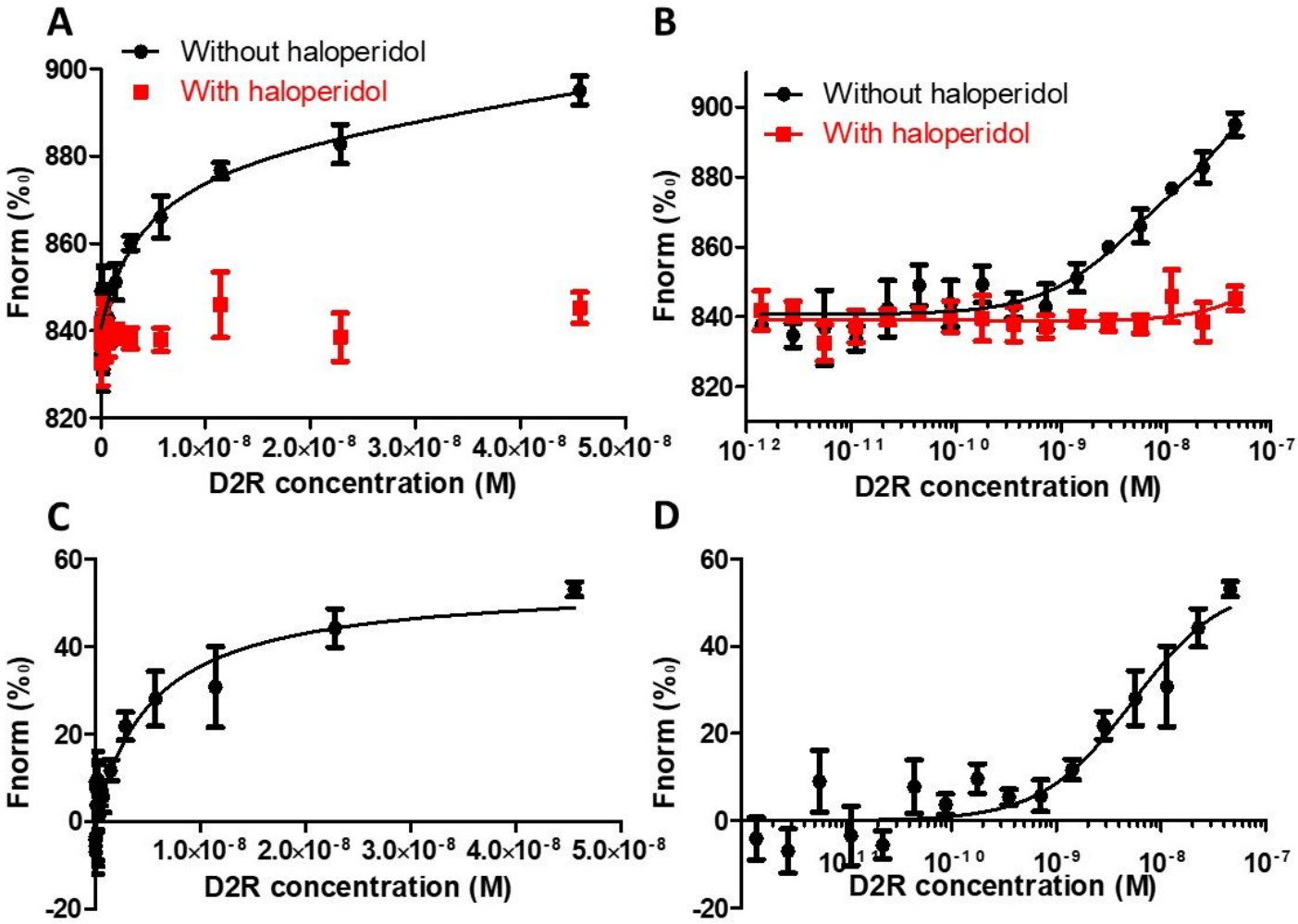

Figure 4

Dose response curves obtained using MST. Saturation curves were obtained with various D2R concentrations and a fixed concentration of 0.125 nM spiperone - Cy5, in presence (red) or absence (black) of 10 µM haloperidol (A, B, n=4). The normalized fluorescence (Fnorm) obtained in presence of haloperidol is subtracted from the signal obtained in absence of haloperidol for each individual experiment in order to obtain specific binding (C, D, n=4).

Supplementary Files

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