[^{3}H]-Spiperone Saturation Binding to Dopamine D_2, D_3 and D_4 Receptors

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[Abstract] This protocol is intended for use in 96 well plates (1,200 µl wells) but it can similarly be applied to standard test tubes (Levant, 2007). D_2, D_3, and D_4 dopamine receptors are members of the D_2-like class of dopamine receptors. They can be studied using the radioligand [^{3}H]-spiperone, which is an antagonist binding to D_2, D_3, and D_4 receptors with comparable affinity. A saturation assay can be used to determine the affinity of a radioligand to a receptor (K_d) and to determine the total number of receptors present in the assay (B_{max}). If saturation binding experiments are performed in the absence and presence of a fixed concentration of another, not radiolabeled ligand, it can also be determined whether the other ligand acts in a competitive manner. If the specific radioactivity is low (tritiated) relative to the affinity of the radioligand (< 1 nM), a high assay volume (≥ 1 ml) is required to avoid ligand depletion; this is of particular importance if a receptor source with high expression density is used (*e.g.* expressed recombinant receptors). To obtain reliable estimates of these parameters at least 6 different concentrations of radioligand must be tested, but particularly when a receptor is first detected in a given tissue or cell type a greater number of concentrations are helpful. The incubation time and temperature are chosen to allow formation of equilibrium between association and dissociation with the receptor for both radioligand and competitor. Each experiment can be divided into different steps such as assay preparation, membrane preparation, incubation, filtration, counting of the samples and data analysis. To minimize experimental error all assays are performed at least in duplicate. Radioligand dilutions should be prepared to cover the desired concentration range. Optimally these concentrations should cover both the high range (corresponding to 5-10x K_d and hence saturation of the receptor) and the low range (around K_d), so that both K_d and B_{max} can reliably be estimated without undue extrapolation. At each radioligand concentration total and non-specific binding should be determined; the agent used for the definition of non-specific binding (NSB) should be chemically (different family) and physically (avoid combination of two lipophilic compounds) distinct from the radioligand to avoid artifacts. For discussion of specific benefits of chosen assay conditions see van Wieringen *et al*. (2013) (copy can be obtained from the author).

Materials and Reagents

1. Radioligand [^{3}H]-spiperone (*e.g.* PerkinElmer, catalog number: NET565250UC)
2. Butaclamol (*e.g.* Sigma-Aldrich, catalog number: 55528-07-9)
3. Receptor-containing membrane suspension
4. Whatman GF/C filters (*e.g.* PerkinElmer, catalog number: 6005174)
5. Poly(ethyleneimine) solution (PEI) (*e.g.* Sigma-Aldrich, catalog number: 9002-98-6)
6. Scintillation cocktail (*e.g.* PerkinElmer, catalog number: 6013641)
7. Tris-HCl
8. KCl
9. $CaCl_2$
10. $MgCl_2$
11. Destilled water
12. Assay buffer (see Recipes)
13. Wash buffer (see Recipes)
14. NSB solution (see Recipes)
15. Radioligand solutions (see Recipes)
16. Radioligand Dilutions (see Recipes)

Equipment

1. 96 well plates (polysterene)
2. Cell harvester (*e.g.* PerkinElmer)
3. Ultra-Turrax® (IKA, model: 0001602800) or similar disperser
4. Water bath
5. Scintillation counter

Software

1. Prism (Graphpad Software, San Diego, CA, USA) or similar

Procedure

A. Before the assay

1. Prepare assay and wash buffer.

2. With some receptor sources PEI-pretreated filterplates may improve the TB/SB ratio: Prepare 0.1% PEI solution and pipet 100 µl/filter on the filterplate, subsequently place in refrigerator (4 °C) for at least 2 h.
3. The experiment is performed in 96 well plates (polysterene) with a total assay volume of 1,000 µl (450 µl assay buffer + 200 µl radioligand + 200 µl of assay buffer or 5 µM (+)-butaclamol + 150 µl membrane preparation).
4. Prepare NSB solution.
5. Prepare radioligand solutions.
6. Membrane preparation.

   Prepare receptor-containing membrane suspension according to preparation protocol. Re-homogenize suspension in small volume (< 2 ml) using short burst of Ultra-Turrax. Dilute to desired protein concentration and to yield a total volume of about 4 ml per 24 data point experiment. The protein concentration of the membrane suspension should be chosen so that a robust specific binding signal is obtained but at the same time total binding should be < 10% (even better < 5%) of free radioligand concentration. Protein content can be assayed by a variety of essays, *e.g.* Bradford (1976). Prepare the membrane suspension initially in ice.
7. Pre warm all solutions for 15 min in 25 °C water bath.
8. Final preparation (Table 1), add components to wells in following order:
   a. 450 µl assay buffer.
   b. 200 µl assay buffer (TB) or 200 µl 5 µM butaclamol (NSB).
   c. 150 µl membrane suspension.
   d. Start reaction by adding 200 µl radioligand.

Table 1. Pipetting scheme for sample on microtiter plate

T1	T1	T2	T2	T3	T3	T4	T4	T5	T5	T6	T6
TB1	TB1	TB2	TB2	TB3	TB3	TB4	TB4	TB5	TB5	TB6	TB6
NSB 1	NSB 1	NSB 2	NSB 2	NSB 3	NSB 3	NSB 4	NSB 4	NSB 5	NSB 5	NSB 6	NSB 6

B. During the assay

1. Incubate for 120 min at 25 °C in water bath.
2. Terminate reaction by rapid vacuum filtration over Whatman GF/C filters using a cell harvester. Wash filter 10 times with ice-cold wash buffer.

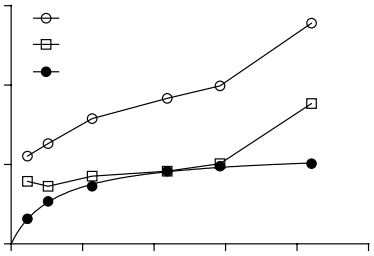
C. After the experiment

1. Add aliquots of 50 µl radioligand to wells of counting plate to determine total radioactivity.
2. Dry, *e.g.* in oven for 2 h.
3. Place sticker on bottom plate. Add scintillation cocktail (20 µl) to filters, place sticker on top of plate and count in a scintillation counter, allow adequate time (15 min) before counting samples.

D. Data analysis

The following calculations are required to derive K_d and B_{max} from the data.

1. Carefully inspect raw data for consistency of replicates. Do not light-heartedly eliminate apparent 'outliers' from the data set.
2. Subtract mean non-specific binding from each replicate of total binding to obtain specific binding.
3. Transform totals to molar concentration of radioligand in the assay, and measured bound values (total binding, non-specific binding and specific binding) to molar amount of ligand based upon the specific radioactivity of the radioligand and the efficiency of the scintillation counter. A representative experiment with D_2 receptors may look like this.



4. Plot specific binding (y-axis) vs. concentration of radioligand (x-axis).
5. Analyze data by non-linear iterative curve fitting using a rectangular hyperbolic function using one of many available iterative curve fitting programs, *e.g.* Prism.
6. Molar amount of B_{max} can be corrected for tissue content, mostly the amount of protein per well (mostly yielding fmol/mg protein); alternative normalization *e.g.* for tissue wet weight, number of cells or DNA content are possible.
7. As internal quality check, determine the fraction of total binding from total radioactivity in the assay. This needs to be < 10% (better < 5%) as otherwise non-equilibrium conditions may exist. In the latter case, membrane concentration in the assay can be reduced if the measured signal remains robust. Alternatively, assay volume can be increased as this will

increase total amount of radioligand (concentration constant) but not amount bound (membrane protein amount constant).

8. In the past when no computer assisted data analysis was available K_d and B_{max} were estimated with the Rosenthal plots, better known as Scatchard plots (Rosenthal invented them but Scatchard made them famous). In these plots the specifically bound radioligand is on the x-axis and is plotted against the ratio of bound/unbound radioligand on the y-axis. The x-intercept corresponds to B_{max} and the negative reciprocal of the slope corresponds to K_d. Rosenthal/Scatchard plots can still be useful to visualize that data points indeed fall on a linear line but the estimates derived from this are less reliable than those from iterative curve fitting (the assumptions of linear regression used in these plots don't meet because x and y are not independent of each other), making this an outdated analysis.
9. If saturation binding experiments are performed in the absence and presence of an inhibitor, the data can be used to test for a competitive nature of the inhibitor. This is the case if the presence of inhibitor changes the K_d but not the B_{max} of the radioligand.

Recipes

1. Assay buffer
   50 mM TRIS: TRIS-HCl: 6.6 g and TRIS base 970 mg (or only 6.04 g TRIS base/L)
   5 mM KCl: 373 mg/L
   2 mM $CaCl_2$: 220 mg/L
   2 mM $MgCl_2$ (6 H_2O): 410 mg/L
   pH 7.4
2. Wash buffer
   50 mM TRIS: TRIS-HCl (33 g/5 L) and TRIS base (4.85 g/5 L) (or only 30.22 g/5 L TRIS base)
   pH 7.4
3. 0.1% PEI (only for D_3 receptor assay)
   Prepare 0.1% solution, PEI is delivered as a 50% solution, pipet 1 ml from this with a syringe and add to 9 ml aqua dest. to get 5%, pipet 400 µl PEI 5% + 19.6 ml destilled water to get PEI 0.1%
4. NSB solution
   3.98 mg butaclamol HCl/10 ml assay buffer yields $1 \cdot 10^{-3}$ M (prepare aliquots of this)
   Dilute this 1:200 to obtain 5 µM in solution, *i.e.* 1 µM final concentration in assay
5. Radioligand solutions

The intended radioligand concentration range in the assay should be chosen to cover both the expected K_d and about 5-10x K_d (K_d spiperone in transfected cells according to literature ≈ 0.05 nM, 0.35 nM and 0.07 nM for D_2, D_3 and D_4, respectively). The stock solution to be prepared needs to be 5x the assay concentration. Thus, the stock solution should be 25-50x K_d. The following is an example calculation based on a specific activity of 16.2 Ci/mmol radioactive of the radioactive stock solution for a D_2 receptor assay. This needs to be adapted for the other subtypes based on their K_d values and for each batch of radioligand and its specific activity.

[^{3}H]-spiperone concentration = 1 mCi/ml/(16.2 Ci/mmol x 1,000 mCi/Ci) = 61.7 µM

Highest concentration needed (10 x K_d x 5) = 10 x 0.05 x 5 = 2.5 nM

2.5 ml x 2.5 nM = 0.00625 nmol [^{3}H]-spiperone needed

(0.00625 nmol)/61.7 x 10^3 nM = 0.1013 µl of [^{3}H]-spiperone solution needed for 2.5 ml

Thus 1 µl [^{3}H]-spiperone solution in 25.0 ml assay buffer to yield a concentration of 2.5 nM.

A free tool for such calculations can be found at www.graphpad.com/quickcalcs/chemMenu/.

6. Radioligand Dilutions (Over a 100-fold Range) (Table 2).

Table 2. Dilution scheme to for radioligand working solutions

Number	Relative concentration	Preparation
1	100	2.5 ml of radioligand at the highest concentration
2	66.67	Mix 800 µl from tube 1 + 400 µl assay buffer
3	50	Mix 1,000 µl from tube 1 + 1,000 µl assay buffer
4	25	Mix 1,000 µl from tube 3 + 1,000 µl assay buffer
5	12.5	Mix 1,000 µl from tube 4 + 1,000 µl assay buffer
6	6.25	Mix 1,000 µl from tube 5 + 1,000 µl assay buffer

Acknowledgments

This protocol is the adaptation of a protocol originally published by Levant (2007).

References

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