Exploring the effect of radioligand depletion on affinity determinations in the dopamine D2 binding assay

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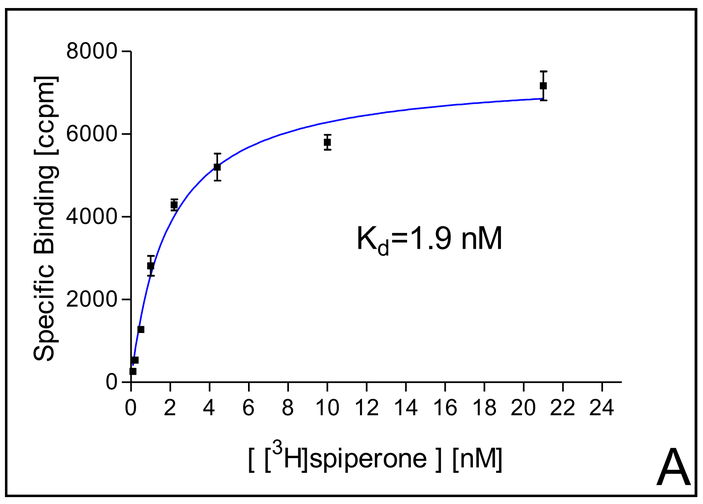
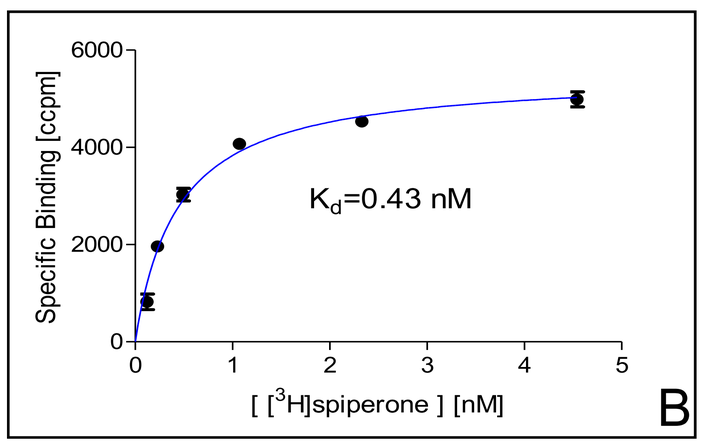
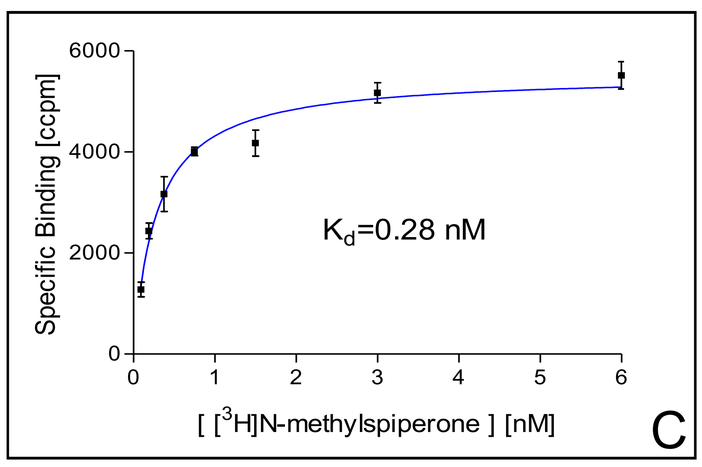
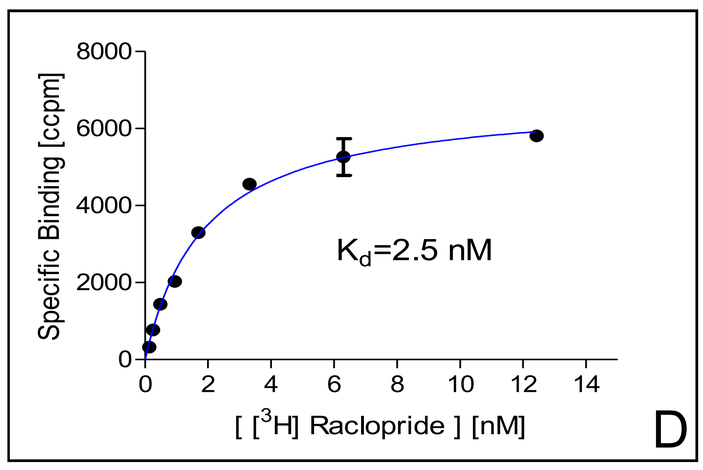
INTRODUCTION In order to extend capability of our academia-based platform to dopamine D2 receptors as a main target for antipsychotics, we adopted D2 radioligand binding assay for the format of 96 well microplate. This required miniaturiaztion of the experiment, but at the same time increased the risk of radioligand depletion. Radioligand depletion is a phenomenon in which the free ligand concentration is significantly reduced which complicates the interpretation of binding data. Recommended "golden rule" of avoiding ligand depletion is that the concentration of receptors should be less than 10% of the ligand's K_d, such that less than 10% of the ligand will be bound to receptor. This problem is particularly acute for binding assays with the use of high-affinity radioligands, and is often ignored by investigators leading to substantial errors in the obtained values of affinity measurements. In the case of D2 receptors, the commonly used radioligand [^{3}H] Spiperone is an ultra high affinity binder of low specific activity and the golden rule of 10% necessitate the use of extremely low receptor concentration. This results in low levels of bound radioactivity and low sensitivity of the assay, so a conflict between practicality and correct practice arises because the assay conditions are not compatible with miniaturized format. In this report, we present data obtained in radioligand binding assays for the human D2 receptor expressed in HEK293 cells, exploring the effect of radioligand depletion on affinity determinations for three different radioligands. We compared our experimental data with those presented in literature, and finally, we developed experimental protocol in 96-well format devoid of ligand depletion, obtaining accurate affinity constants in competition experiments.









Representative saturation binding curves:

A. [^{3}H]Spiperone binding in 0.25 ml volume assay

B. [^{3}H]Spiperone binding in 0.5 ml volume assay

C. [^{3}H]NMSP binding in 0.25 ml volume assay

D. [^{3}H]Raclopride binding in 0.25 ml volume assay

MATERIALS and METHODS

Chemicals [^{3}H]Spiprerone (15 Ci/mmol), [^{3}H]N-methyl-spiperone (67.6 Ci/mol), [^{3}H]Raclopride (74,4 Ci/mol) were purchased from PerkinElmer. Butaclamol, Ritanserin and Olanzapine were purchased from Sigma-Aldrich; Clozapine, Remoxipride and Haloperidol from Tocris. Other chemicals were obtained from commercial sources and were of analytical grade.

Biological material Membranes were prepared from our human embryonic kidney (HEK) 293 cells stably transfected with human D2 (Long) receptor cDNA.

Ligand binding assays For [^{3}H]Spiprerone binding crude membrane preparations were incubated in two volumes of assay buffer (0.5 and 0.25 ml) in 37°C for 1 h. For [^{3}H]NMSP and [^{3}H]Raclopride bindings 0.25 ml assay volumes were used. Composition of assay buffer was: 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl. Non-specific binding was defined with the use of 5 μM butaclamole. The incubations were terminated by the rapid filtration through Whatman GF/B fibre filters (in the case of 0.5 ml assay volumes) or unifilter plates (PerkinElmer) and subsequent washing with ice-cold buffer using Brandel or Unifilter harvester. Scintillation cocktail was added and the radioactivity determined in scintillation counters: Beckman 6500 and MicroBeta.

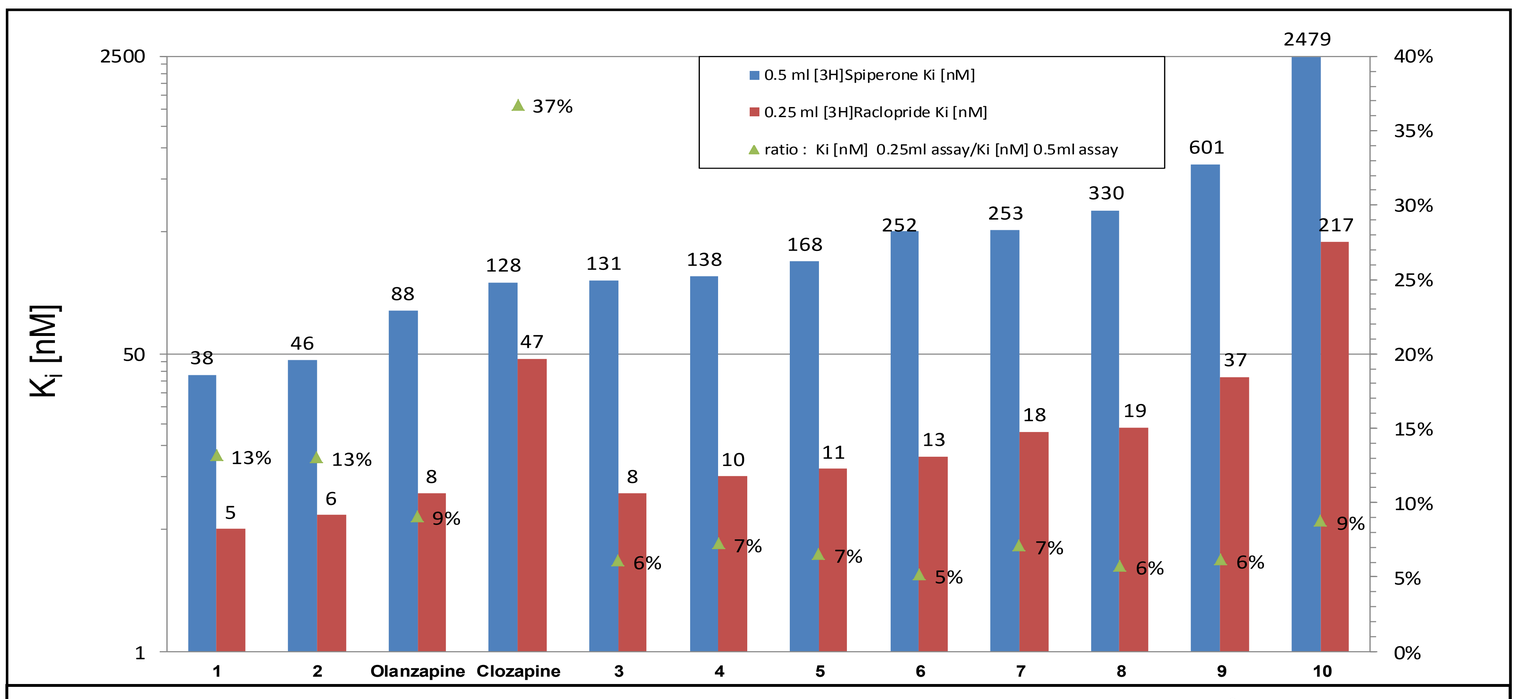
RESULTS For the [^{3}H]Spiperone binding in the assay of 0.25 ml total volume, the depletion over the 30% was observed and determined K_d values of radioligand were exceeded several times. Similarly, in the competition binding experiments the affinities of standard drugs (Olanzapine and Clozapine) were underestimated. Increasing the volume of assay buffer to 0.5 ml lowered depletion to the 22%, but both the K_d value of [^{3}H]Spiperone and K_i values for investigated standard drugs, have still been distorted. Using [^{3}H] NMSP (which has higher specific activity than [^{3}H] Spiperone) in the 0.25 ml assay volume, the level of depletion was again to high to obtain correct values of K_d in saturation experiments and accurate affinity measurements in competition assays (Table 1). Only the use of [^{3}H]Raclopride, which characterized higher K_d value and has high specific activity, eliminated the depletion (the level of bound radioligand did not exceed 10%) and determined values of K_d for radioligand as well as K_i for five standard drugs were in agreement with verified literature data (Table 1 and 2).

Table 1.

Radioligand	K_d value [nM] (Assay volume [ml])		Reference K_d value [nM]
	(0.25)	(0.5)	
[^{3}H]Spiperone	1.9	0.43	0.07 [9]
[^{3}H] NMSP	0.28	---	0.25 [10]
[^{3}H]Raclopride	2.5	---	3 [11]

Table 2.

Standard drug	Measured K_i value [nM] (Assay volume [ml])				Reference K_i value [nM]		
	[^{3}H] Spiperone		[^{3}H] NMSP	[^{3}H] Raclopride	[^{3}H] Spiperone	[^{3}H] NMSP	[^{3}H] Raclopride
	(0.25)	(0.5)	(0.25)	(0.25)			
Olanzapine	266	88	67	13	20 [3]	34 [2]	10 [1]
Clozapine	1472	128	385	31	44 [4]	256 [2]	63 [5]
Ritanserin	156	---	49	7	12 [6]	N/A	N/A
Remoxipride	---	---	---	75	---	---	80 [8]
Haloperidol	---	---	---	1	---	---	0.7 [7]



Direct comparison of results obtained in competition experiments carried out in the volume of 0.5 ml and [^{3}H]Spiperone with those performed in the 96-well microplates with the use of [^{3}H]Raclopride. Averaged ratio of determined K_i values in both experiments was 10%.

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