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PROBING THE EXISTENCE OF G PROTEIN-COUPLED RECEPTOR DIMERS BY POSITIVE AND NEGATIVE LIGAND-DEPENDENT COOPERATIVE BINDING

Laura ALBIZU, Marie-Noëlle BALESTRE, Christophe BRETON, Jean-Philippe PIN, Maurice MANNING, Bernard MOUILLAC, Claude BARBERIS, Thierry DURROUX

Institut de Génomique Fonctionnelle LA M-NB, J-PP, BM, CB, TD
CNRS UMR 5203, Montpellier F-34094, France, INSERM U 661, Montpellier F-34094 France, Université de Montpellier 1, Université de Montpellier 2, Montpellier F-34094, France.

Laboratoire de Neuroendocrinologie du Développement CB
Département de Physiologie, UPRES-EA 2701, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq CEDEX.

Department for Biochemistry and Cancer Biology MM
Medical University of Ohio, Toledo, OH 43614-5804, USA.

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Running Title : Cooperative binding probes GPCR dimer existence

Correspondence address: Thierry DURROUX, Institut de Génomique Fonctionnelle, CNRS UMR 5203, 141 rue de la Cardonille, 34094 Montpellier CEDEX 5, France. Tel : 33-467-14-29-16 ; Fax : 33-467-54-24-32; E-mail : tdurroux@igf.cnrs.fr

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Abbreviations

GPCR : G protein coupled receptor
AVP : arginine vasopressin
OT : oxytocin
BSA: bovine serum albumin
HA tag: hemaglutinin tag
6HIS tag: hexa-histidine tag
GTP: guanosine triphosphate
FRET: fluorescence resonance energy transfer
HTRF: homogeneous time resolved fluorescence
HO-LVA: 4-OH-Phenylacetyl-D-Tyr(Me)^2, Phe^3, Gln^4, Asn^5, Arg^6, Pro^7, Arg^8-$NH_2{}^9$
OTA: d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Orn^8, Tyr-$NH_2{}^9$]vasotocin

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Abstract

An increasing amount of ligand binding data on G protein-coupled receptors (GPCRs) is not compatible with the prediction of the simple mass action law. This may be related to the propensity of most GPCRs, if not all, to oligomerize. Indeed, one of the consequences of receptor oligomerisation could be a possible crosstalk between the protomers which in term could lead to negative or positive cooperative ligand binding. We prove here that this can be demonstrated experimentally. Saturation, dissociation and competition binding experiments were performed onto vasopressin and oxytocin receptors expressed in CHO or Cos-7 cells. Linear, concave and convex scatchard plots were then observed depending on the ligand used. Moreover, some competition curves exhibited an increase of the radiotracer binding for low concentrations of competitors suggesting a cooperative binding process. These data demonstrate that various vasopressin analogs display either positive or negative cooperative binding. Because positive cooperative binding cannot be explained without considering receptor as multivalent, these binding data support the concept of GPCR dimerization process. The results which are in good accordance with the predictions of previous mathematical models, suggest that binding experiments can be used to probe the existence of receptor dimers.

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G protein-coupled receptors (GPCRs) have classically been considered as functional monomeric proteins, their activation resulting from the stoichiometric binding of one ligand per receptor. However for the last two decades, pharmacological data obtained on various GPCR models are not compatible with such a model. For instance, negative cooperative binding has been reported for various class A GPCRs such as muscarinic (Christopoulos and Kenakin, 2002; van Koppen and Kaiser, 2003), or dopamine (Armstrong and Strange, 2001) receptors, and more recently for glycoprotein hormone receptor (Urizar et al., 2005) and chemokin receptors (El-Asmar et al., 2005). In addition, very few studies have reported positive cooperative binding, for instance on muscarinic receptors (Mattera et al., 1985; Wreggett and Wells, 1995), and on opioid κ–δ heterodimers (Jordan and Devi, 1999). Finally, apparent variations in the maximal binding capacity on a given preparation (Armstrong and Strange, 2001; Serradeil-Le Gal et al., 1996; Wreggett and Wells, 1995) and discrepancies in the ligand affinity estimations, both depending on the radioligand used, have been described.

A large amount of data have shown that many GPCRs, if not all, have the propensity to oligomerize (Terrillon and Bouvier, 2004). One expected consequence of receptor oligomerisation is a possible crosstalk between the protomers which can lead to negative and positive cooperative ligand binding. Various theoretical models have considered receptors as multivalent complexes (Armstrong and Strange, 2001; Durroux, 2005; Franco et al., 2005; Mattera et al., 1985; Wreggett and Wells, 1995). Few of them, including ours, have analyzed the impact of receptor crosstalk within a dimer on ligand binding (Durroux, 2005; Franco et al., 2005). Predictions show that ligand-induced negative or positive cooperativities result in modifications of both shapes and slopes of saturation and competition binding curves (Durroux, 2005). As a consequence, crosstalk between protomers can also explain apparent variations in the maximal binding capacity in saturation experiments performed with various

ligands on a given preparation, and discrepancies in the ligand affinity estimations according to the radioligand used (Durroux, 2005).

Whereas theoretical models have considered different possibilities of cooperative binding, it has not been experimentally demonstrated so far that for a given receptor, different selective ligands can bind with various cooperative processes. In order to investigate such an hypothesis, we performed saturation, dissociation and competition binding experiments on vasopressin and oxytocin receptors expressed in Cos-7 and CHO cells with various selective ligands. We focused more specifically on four peptidic ligands: the natural agonists, vasopressin (AVP) and oxytocin (OT), and two antagonists: the linear vasopressin antagonist HO-LVA (Barberis et al., 1995), and the cyclic antagonist OTA (Breton et al., 2001; Elands et al., 1988b). We carefully examined the data which were obviously not compatible with the predictions of the ligand/receptor interaction model which follows the simple mass action law.

We therefore demonstrate that negative and positive cooperative binding can be observed with different ligands on vasopressin and oxytocin receptors, further validating mathematical models and demonstrating that receptors dimerize.

Materials and Methods

Drugs

[^{3}H]AVP (60-80 Ci/mmol), [^{3}H]OT (33 Ci/mmol) were from Perkin Elmer Life Science (Courtaboeuf, France) Products. 4-OH-Phenylacetyl-D-Tyr(Me)^2, Phe^3, Gln^4, Asn^5, Arg^6, Pro^7, Arg^8-NH_2^{9}, (HO-LVA) (Barberis et al., 1995), d(CH_2)$_5$[Tyr(Me)^2, Thr^4, Orn^8, Tyr-NH_2^{9}]vasotocin (OTA) (Elands et al., 1988b), d(CH_2)$_5$[D-Thi^2, Thr^4, Orn^8, Eda^9 ←Tyr^{10}]vasotocin (Manning et al., 2001), [Phe^2, Orn^8]vasotocin were synthesized in the

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laboratory of Dr Manning. We performed radio-iodinations of HO-LVA and OTA to give [^{125}I]-HO-LVA and [^{125}I]-OTA respectively, as previously described (Barberis et al., 1995; Elands et al., 1988a). GTP-y-S was from Sigma-Aldrich (Saint Quentin, France).

Cell culture

The CHO cell lines which stably express the human vasopressin V1a or V1b receptors or the human oxytocin receptor, were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum and 100 units/ml penicillin and streptomycin in an atmosphere of 95 % air and 5% CO2 at 37°C.

Cos-7 cells were transiently transfected by electroporation as previously described (Cotte et al., 2000). Briefly, electroporation was performed with 1µg of a pRK5 containing HA-tagged or 6His-tagged human V1a receptor and 9µg of empty vector. Membrane preparations were made as follows.

Membrane Preparation

Culture dishes of CHO or Cos-7 cells expressing the human vasopressin V1a, V1b or oxytocin receptors were washed twice in PBS without calcium and magnesium and cold lysis buffer (15mM Tris:HCl, 2mM $MgCl_2$, 0.3mM EDTA, pH 7.4) was added. Cells were scraped with a rubber, homogenized with a polytron and centrifuged at 100g for 5 minutes at 4°C. Supernatants were recovered and centrifuged at 44000g for 30 minutes at 4°C. Pellets were resuspended in a suspension medium (50mM Tris:HCl, 5mM $MgCl_2$, pH 7.4) and centrifuged at 44000g for 30 minutes at 4°C. Pellets were resuspended in an appropriate volume of the same buffer. For each membrane preparation, protein content was evaluated, membranes were then aliquoted and frozen in liquid nitrogen.

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Homogeneous Time-Resolved Fluorescence-Fluorescence Resonance Energy Transfer (HTRF-FRET) Assays

HTRF-FRET experiments were performed as described (Maurel et al., 2004). These experiments involve the transfer of energy from an europium cryptate pyridine-bipyridine as the donor fluorophore to an Alexa Fluor 647 as the acceptor fluorophore. We used the monoclonal anti-6His antibody and the monoclonal anti-HA antibody (12CA5) labeled with the different fluorophores kindly provided by Eric Trinquet and Hervé Ansanay (Cis Bio Int. Research). After transfection, 100, 000 Cos-7 cells/well were dispatched into a black 96-well assay plate (Costar) in 100µl of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Twenty four hours later, Cos-7 cells or membrane preparations (25µg/well) expressing the indicated 6His- or HA- tagged human V1a receptor were incubated in 100µl of Tris/Krebs buffer (20mM Tris-HCl pH7.4, 118mM NaCl, 5.6mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7mM KCl, 1.8 mM $CaCl_2$) supplemented with 0.1% bovine serum albumine and containing 1nM europium cryptate-PBP- and 1nM Alexa Fluor 647-labeled antibodies. As a negative control, Cos-7 cells or membranes were incubated with only the donor fluorophore-labeled antibody. After a 8h incubation at 4°C, fluorescence emissions were measured both at 620nm and at 665nm on a RubyStar fluorometer (BMG Labtechnologies) equipped with a nitrogen laser as excitation source (337nm). A 400-µs reading was measured after a 50-µs delay to eliminate the short-lived fluorescence background from the specific signal. The fluorescence intensities measured at 620nm and at 665nm correspond to the total europium cryptate emission and to the FRET signal respectively. The specific signal was calculated using the following equation : $\Delta F = (R - Rneg)/(Rneg)$. R is the ratio (fluorescence 665nm / fluorescence 620nm) x 10^4 calculated in wells incubated with both donor- and acceptor-labeled antibodies whereas Rneg is the same ratio for the negative control.

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Binding Assay

For saturation experiments, affinities of [^{125}I]-HO-LVA and [^{125}I]-OTA were determined from hot saturation experiments. Membranes (1-3µg/assay) were incubated with increasing concentrations of radioactive tracer (5 pM to 500 pM for [^{125}I]-HO-LVA and 20 pM to 2 nM for [^{125}I]-OTA) for 1h at 30°C. For each concentration of tracer, non specific binding was determined by the addition of an excess of HO-LVA or OTA, respectively. Bound ligand fractions were separated from the free ligand either by filtration or centrifugation. When performed by filtration, we used Whatman GF-C filters preincubated in polyethylenimide (5µl/ml for [^{125}I]-HO-LVA) or bovine serum albumin (10mg/ml for [^{125}I]-OTA). Filtration was performed on a Brandel apparatus. Radioactivity on the filter was counted on a Gamma counter Cobra (Hewlett Packard). Each assay was performed in triplicate. When centrifugation was used, 1ml of ice-cold medium containing 100 mM Tris:HCl, 10 mM $MgCl_2$, BSA 1mg/ml, pH 7.4 was added to the assay at the end of the incubation. Samples were centrifuged at 22,000g for 5 min and supernatants were discarded. Pellets were washed with 1ml of the same medium, centrifuged at 22000g for another 5 min. Supernatants were removed and radioactivity in the pellet was counted on a Gamma counter Cobra (Hewlett Packard) as explained above.

Affinities of [^{3}H]AVP and [^{3}H]OT were determined from hot and cold saturation experiments. CHO membranes (8-15 µg) were incubated with [^{3}H]AVP or [^{3}H]OT (1-2nM) plus increasing concentrations of AVP or OT (1 pM to 1µM). Non specific binding was determined by the addition of a large excess of AVP or OT (1µM). Bound and free ligand fractions were separated by filtration or centrifugation as mentioned above. Each assay was performed in triplicate. Radioactivity was counted on a Beta-counter (Hewlett Packard).

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For competition experiments, membranes were incubated with [^{125}I]-HO-LVA (50-80 pM) or [^{3}H]AVP (1-2nM) and increasing concentrations of unlabeled ligand ranging from 1 pM to 1µM. Non specific binding was determined with an excess of HO-LVA (1µM) or AVP (1µM). Bound radioactivity was determined as described above.

All binding data were analyzed with the program Kell (Biosoft). Fitting parameters for saturation experiments were determined using a non-linear curve-fitting routine to the Hill equation $B = Bmax\ [1 + (Kd/\ [L])^{n}]^{-1}$ where Bmax is the maximal binding, L is the concentration of labeled ligand, Kd is the equilibrium dissociation constant for the labeled ligand and n is the Hill coefficient (Segel, 1975). Because of a potential crosstalk between protomers within a dimer, the Hill coefficient was allowed to vary.

For dissociation experiments, membranes were preincubated in a volume of 100 µl for 40 min at 30°C in the presence of [^{3}H]AVP or [^{3}H]OT (0.5-1.5 nM). 3ml of incubation medium (Tris 50 mM, $MgCl_2$ 5 mM, BSA 1mg/ml) with or without unlabeled AVP or OT (1µM or 10 µM) were then added at different times. The addition of 3ml of incubation medium induces a tracer dilution with a factor of 31. We checked that in such new equilibrium conditions, less than 10% of the binding sites were still able to be labeled by the tracer. The data are presented as the percentage of specific binding at time zero of dissociation (B_0). At each dissociation time, bound radioactivity was determined as described above. The dissociation rate, k_{-1}, is determined by the slope of the curve when fitting the data with the equation $Ln\ [B/B_0] = k_{-1}\ t$ in which t is time, B is the specific binding at time t, B_0 is the specific binding at time zero of dissociation.

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Results

Predictions from mathematical models

A few mathematical models have been proposed to describe ligand binding on dimeric receptors (Christopoulos and Kenakin, 2002; Durroux, 2005; Franco et al., 2005; Mattera et al., 1985; Wreggett and Wells, 1995). Consequences of a positive or a negative cooperative ligand binding between the binding sites of protomers within a dimer have been analyzed (Durroux, 2005). As illustrated in Figure 1, the profiles of the saturation and competition curves depend on the existence and on the nature of the cooperativity between the two binding sites of a dimer.

The predicted saturation curve obtained with a radioligand L_1* (middle panels, L_1=L_2) exhibit Hill coefficient of 1 and a linear Scatchard curve in the absence of any cooperativity (Figure 1a). By contrast, negative (Figure 1b) and positive (Figure 1c) cooperative bindings result in saturation curves with slope factors less or greater than 1 respectively and in concave and convex Scatchard plot respectively.

In addtition, predicted competition curves obtained with a radioligand L_1* and a competitor L_2 different of L_1 display slopes of 1 in the absence of any cooperative binding (figure 1a, lower panel). By contrast, negative and positive cooperativities result in curves with slope factors less or greater than 1, respectively. Moreover, the patterns of the competition curves can be complex : curves which exhibit two plateaus (Figure 1b) or an increase in the binding of the tracer for low concentrations of competitors (Figure 1c) can be observed for negative or positive cooperative binding, respectively. An unexpected consequence of the models is that the ligand affinity estimation can be different depending on the radio tracer used to perform competition experiments (Durroux, 2005).

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In order to validate the model, we performed binding experiments on membrane preparations from cells expressing vasopressin and the oxytocin receptors.

FRET experiments prove the existence of vasopressin receptor dimers.

In order to demonstrate the presence of dimers at the cell surface and in membrane preparations, we performed homogeneous time-resolved FRET experiments as previously described by Maurel and collaborators (Maurel et al., 2004). In this assay, vasopressin receptors were tagged with hemagglutinin (HA) or 6-histidine (6His) sequences and FRET signals were measured on intact cells or on membrane preparations with anti-HA and/or anti-6His monoclonal antibodies labeled with the fluorescent probes europium cryptate-pyridine bipyridine or Alexa Fluor® 647.

First, cells expressing HA-tagged vasopressin V1a receptors were incubated with europium cryptate-pyridine bipyridine and Alexa Fluor® 647 labeled anti-HA monoclonal antibodies. A FRET signal was measured at 665 nm (the emission wavelentgh of Alexa Fluor® 647) after excitation of europium cryptate-pyridine bipyridine at 337 nm (Figure 2, white bars). This signal was significantly higher than that measured in mock-transfected cells, indicating that HA-tagged vasopressin V1a receptors homodimerized.

Second, we performed FRET experiments on membrane preparations. A FRET signal was recorded when membranes which coexpressed HA-tagged and 6His-tagged V1a receptors (V1a (HA) / V1a (6His)) were incubated with Alexa Fluor® 647 labeled anti-HA and europium cryptate-pyridine bipyridine labeled anti-6His monoclonal antibodies (Figure 2, black bars). By contrast, no significant signal was recorded from mock membrane preparations or from a membrane preparation mix (mix V1a (HA) + V1a (6His)) which expressed either HA-tagged V1a receptors or 6His-tagged V1a receptors. The lack of FRET signal on the membrane mix was not due to a too low expression level of receptors. Indeed a

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specific FRET signal was detected when each membrane preparations which either expressed HA-tagged (V1a (HA)) or 6His-tagged (V1a (6His)) V1a receptors were incubated with europium cryptate-pyridine bipyridine and Alexa Fluor® 647 labeled anti-tag monoclonal antibodies. Then these data indicated that the FRET signal recorded on membranes which coexpressed HA-tagged and 6His-tagged V1a receptors (V1a (HA) / V1a (6His)) was specific and reflected receptor dimerization. Similar results have been obtained with oxytocin receptors (data not shown).

Saturation assays display ligand cooperative binding on vasopressin receptor.

We carried out hot and cold saturation experiments with tritiated vasopressin or tritiated oxytocin from CHO cells expressing either V_{1a} or V_{1b} or oxytocin receptors. As illustrated in Figure 3, all saturation experiments exhibited curves with a slope factor less than 1 and a concave Scatchard plot (Figure 3, insets). The fits of the experimental curves with a Hill coefficient which was allowed to vary (i.e. when considering a potential cooperativity between the binding sites), are significantly better than those obtained when only one binding site type was considered (absence of cooperative binding). The Hill coefficient and the Kd values with [^{3}H]AVP are 0.45 and 85 nM for the V_{1a} receptor (Figure 3a) and 0.27 and 85 nM for the V_{1b} receptor (Figure 3b). The fitting procedures gave a Hill coefficient of 0.38 and an affinity constant of 129 nM for the oxytocin receptor (Figure 3c).

Because these results can be an argument in favour of the existence of a negative cooperativity between binding sites or in favour of the co-existence of two receptors conformations with different affinities, we also investigated negative cooperative using dissociation kinetics experiments (Christopoulos et al., 1997; De Meyts, 1976; Springael et al., 2006; Urizar et al., 2005). The results illustrated in Figure 4 show that the dissociation rates of [^{3}H]AVP on the vasopressin V_{1b} (Figure 4a) and [^{3}H]OT on the oxytocin receptor

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(Figure 4b) are promoted by an excess of unlabeled AVP (1µM) or OT (1µM). The fits of the data on a semilogarithmic plot (Figure 4, insets) provide the dissociation rates of [^{3}H]AVP on the vasopressin V_{1b} of 0.0024 min^{-1} and 0.0063 min^{-1} without or with an excess of unlabeled vasopressin, respectively (Figure 4a, inset). Similarly, the dissociation rates of [^{3}H]OT on the oxytocin receptor are 0.0168 min^{-1} and 0,0067 min^{-1} in the absence or the presence of an excess of unlabeled oxytocin (Figure 4b, inset). Moreover, because AVP has been described as a partial agonist for oxytocin receptor (Chini et al., 1996), we also performed dissociation experiments with [^{3}H]AVP (Figure 4c) and showed that as for OT, the dissociation rate is mulitplied by 2.6 in the presence of an excess of unlabeled vasopressin (Figure 4c, inset).

In the next step, we respectively carried out hot saturation experiments with iodinated antagonists and the vasopressin V1a receptor. Typical results obtained with two antagonists are illustrated in Figure 5. Figure 5a illustrates a saturation curve obtained with [^{125}I]-HO-LVA, a vasopressin antagonist (Barberis et al., 1995) on membranes from CHO cells expressing V_{1a} receptors, and the resulting Scatchard plot is almost linear (Figure 5a, inset, continuous line) with a Hill coefficient of 0.97 and a dissociation constant, Kd, of 22 pM. Fitting the data when considering a potential cooperative binding or not, did not significantly change the Kd and Hill coefficient values (Table 1).

On the contrary, a saturation experiment performed with and [^{125}I]-OTA, a mixed vasopressin V1a/oxytocin receptor antagonist (Breton et al., 2001; Elands et al., 1988b) exhibited a curve with slope factors greater than 1 (Figure 5b) and a convex Scatchard plot (Figure 5b, inset). The best fit was obtained when considering cooperativity between the binding site and gave Hill coefficient and a Kd values of 1.29 and 302 pM, respectively. Mean values for the Hill coefficient and the dissociation constant obtained from 7 independent experiments are 1.2 $\pm$ 0.035 and 0.35 $\pm$ 0.13 nM, respectively (Table 1).

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The Kd estimations for the binding of [^{125}I]-HO-LVA, [^{3}H]AVP and [^{125}I]-OTA obtained when considering the absence of cooperative binding (Table 1, "independent binding site" analysis) are in the same range than those previously reported (Breton et al., 2001; Mouillac et al., 1995a). It is noteworthy that the differences between the Kd estimations given by the two analyses are obviously negligible when the Hill coefficient is around 1 (Table 1), but can be large when the Hill coefficient differs from 1. Moreover the nature of the cooperative binding is ligand-dependent.

Competition assays exhibit ligand cooperative binding on vasopressin receptor.

We carried out competition experiments with various ligands on CHO cells which expressed V_{1a} receptors and we observed ligand binding which are not compatible with the classical model of ligand binding on a receptor (i.e. a single ligand binds to a monomeric receptor).

Firstly, we performed competition experiments between [^{125}I]-HO-LVA and vasopressin on CHO cell membranes expressing human V_{1a} receptors. Slope factor values are below 1 and are frequently between 0.7 and 0.8 suggesting a negative cooperativity between the binding sites. Typical experimental data are presented in Figure 6a and are fitted with the following equation:

$$(\text{specific binding}) = Bg + ((Bmax - Bg) / (1+([L]/IC_{50})^{n}))$$

in which Bg corresponds to the background binding, Bmax to the maximal binding, [L] to the ligand concentration, IC_{50} to the concentration of a competitor which induces a decrease of 50% of the specific binding of the labeled ligand, and n to the Hill coefficient. The grey curve corrresponds to the best fit obtained with the equation in which n is set to 1, assuming that no cooperativity exits between the binding sites. The black curve has been obtained with the

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same equation but the Hill coefficient is allowed to vary. The best fit is obtained with a n equal to 0.75.

Secondly, competition experiments were performed with [^{125}I]-HO-LVA as radioligand and an analog of OTA, d(CH_2)$_5$[D-Thi^2, Thr^4, Orn^8, Eda^9 ←Tyr^{10}]vasotocin, which exhibits potent oxytocic antagonist effects (Manning et al., 2005; Manning et al., 2001). We observed an increase in the binding of [^{125}I]-HO-LVA for low concentrations of the competitor. Indeed low concentrations of d(CH_2)$_5$[D-Thi^2, Thr^4, Orn^8, Eda^9 ←Tyr^{10}]vasotocin induced a 25 % increase of [^{125}I]-HO-LVA binding (Figure 6b). The mean amplitude of the increase calculated from 3 independent experiments was 15% $\pm$ 5. Although this increase was generally of small amplitude (it did not exceed 20 % of the binding of the tracer in the absence of competitor), it was nevertheless frequently observed with [Phe^2, Orn^8]vasotocin, a vasopressin V_{1a} agonist (Berde et al., 1964) and numerous other analogs on membranes of CHO cells expressing human V_{1a} receptors.

Thirdly, we observed that the estimation of the affinity of a ligand for a receptor can vary depending on the method of its evaluation. As reported in Table 2, when considering the classical model of ligand binding, vasopressin had a K_d value of 0.7 $\pm$ 0.2 nM when estimated by saturation and a K_i value of 3.4 $\pm$ 1.1 nM for the human vasopressin V_{1a} receptor (Cotte et al., 2000) expressed in Cos-7 cells, when measured by competition experiments with [^{125}I]-HO-LVA as radiolabeled ligand (Table 2). This discrepancy can be greater in somes cases. For instance, when experiments are performed on the Q185A V_{1a} mutant which displays a loss of affinity for agonists and antagonists (Cotte et al., 2000), the K_d and K_i values are 46 $\pm$ 7 nM and 810 $\pm$ 148 nM, respectively (Table 2). Such discrepancies have also been observed for two other mutations on rat vasopressin V_{1a} receptor, K128A and Q131A, which have been shown to be involved in the binding site of ligands. For the mutated K128A and Q131A

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vasopressin V_{1a} receptors, vasopressin has K_d values of 1.2 nM and 11.4 nM respectively and K_i values of 266 $\pm$ 108 nM and 168 $\pm$ 87 nM when estimated by displacement of [^{125}I]-HO-LVA (Barberis et al., 1993; Mouillac et al., 1995a) (Table 2). Such discrepancies in K_i estimations have also been reported with the vasopressin agonist [Phe^{2}Orn^8]VT depending on whether the competition experiments were performed with [^{3}H]AVP or [^{125}I]-HO-LVA (Barberis et al., 1993; Mouillac et al., 1995a).

Lack of effects of GTP-γS

We then verified whether cooperative binding could be due to the coupling of V1a receptor to G proteins. Two studies have reported that the cooperative binding on myocardial muscarinic receptor are GTP dependent (Mattera et al., 1985; Wreggett and Wells, 1995). On membrane preparations of CHO cells expressing the V1a receptors, addition of GTP-γS (200µM), a non hydrolysable analog of GTP, did not significantly modified the profiles of saturation and Scatchard curves (Figure 7).

Discussion

In this study, we provide evidences that different ligands bind to vasopressin and oxytocin receptors by various cooperative processes. We showed that [^{125}I]-HO-LVA binding results in a saturation plot with a Hill coefficient of 1, suggesting that it binds to one class of binding sites. By contrast, [^{3}H]AVP binding exhibits a saturation curve with a Hill coefficient significantly lower than 1, suggesting the existence of various binding sites. The heterogeneity in the binding sites might correspond to conformations coupled or not to G

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protein although we cannot observe a significant effect of GTP-γ-S, a non-hydrolysable GTP analog, on vasopressin binding.

Dissociation kinetic experiments which are a classical procedure to demonstrate negative cooperativity (Christopoulos et al., 1997; De Meyts, 1976; Springael et al., 2006; Urizar et al., 2005) indicated that an excess of vasopressin or oxytocin promotes the dissociation of [^{3}H]AVP or [^{3}H]OT bound to the vasopressin and oxytocin receptors. The increases in the dissociation rates are not large when compared to those reported for the chemokine receptors (Springael et al., 2006). Two different hypotheses can be formulated to explain these results: (i) there is a very strong negative cooperativity between the binding sites within a homodimer. The second ligand molecule has then a very low affinity for the second binding site (greater than 1µM) and an excess of ligand at 1µM or 10µM is not enough to induce an important increase in the dissociation rate; (ii) the receptor exists under different conformations, and dimers in which binding sites negatively crosstalk constitute one of these conformations.

However, the hypothesis of the existence of a binding site heterogeneity is not compatible with the positive cooperative bindings revealed (i) by convex saturation curve with the antagonist [^{125}I]-OTA and (ii) by bell-shaped competition curve with [^{125}I]-HO-LVA and d(CH$_2$)$_5$[D-Thi^{2}, Thr^{4}, Orn^{8}, Eda^{9} ←Tyr^{10}]vasotocin or [Phe^{2}, Orn^{8}]vasotocin. Similar positive cooperative bindings have only been reported for the muscarinic receptor (Mattera et al., 1985; Wreggett and Wells, 1995).

Cooperative ligand binding on GPCR has been extensively reviewed (Christopoulos and Kenakin, 2002; Schwartz and Holst, 2006). By contrast to the negative cooperativity, positive cooperativities imply that the receptor should behave as a multivalent complex (Christopoulos and Kenakin, 2002; Mattera et al., 1985; Wreggett and Wells, 1995). However

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the binding of two [^{125}I]-OTA ligands in the same pocket or in neighbouring pockets on a single vasopressin V1a monomer seems to be unlikely since computational modelization of the vasopressin V_{1a} receptor, based on receptor mutagenesis approaches (Mouillac et al., 1995a; Mouillac et al., 1995b), and on photolabeling strategies (Breton et al., 2001; Phalipou et al., 1997; Phalipou et al., 1999), has predicted that only one vasopressin or its analogs are buried in a binding pocket defined by the seven transmembrane domains of the receptor (Mouillac et al., 1995a).

On the contrary, the binding of two ligands on the two protomers within a dimer is highly probable. Using the FRET strategy previously described (Liu et al., 2004; Maurel et al., 2004), we have shown that vasopressin receptors in our model are able to dimerize and that dimers are present at the cell surface. It confirms the results previously obtained (Terrillon et al., 2003). Moreover the binding data reported in the present paper are in accordance with the predictions from the mathematical models (Durroux, 2005) and more specifically for what concerns positive cooperativity. [^{125}I]-OTA binds to the V1a receptor in a positive coooperative manner. As mentioned above, this can only be explained by considering the binding complex as multivalent. Binding of [^{125}I]-OTA on one protomer should promote the binding of a second ligand on the second protomer. Although [^{125}I]-HO-LVA is a peptidic vasopressin V1a antagonist as [^{125}I]-OTA, no significant positive or negative cooperativity has been observed for its binding, suggesting that the cooperative binding does not exclusively depend on the agonistic or antagonistic nature of the ligand.

By contrast, the curvilinear saturation curve with [^{3}H]AVP and [^{3}H]OT can result from a negative cooperativity between the protomers within vasopressin and oytocin receptor homodimers. It is noteworthy that this negative cooperativity can be at the origin of the variation of the maximal binding depending on the ligand used to perform the saturation experiments (Serradeil-Le Gal et al., 1996). Because the linear part of the saturation curve

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obtained with [^{3}H]AVP is often considered to fit the Scatchard curve, the resulting maximal binding which in fact corresponds to the saturation of the higher affinity binding site, is underestimated. However the determination of the maximal binding when considering the whole curve remains difficult since the precision of the last points of the curve is low.

In addition, we observed negative cooperative bindings with vasopressin and oxytocin, the two natural agonists, on V1b and OT receptors when expressed in Cos-7 or CHO cell line. Recently, strong negative cooperative agonist bindings on glycoprotein hormone receptor (Urizar et al., 2005) and on chemokin receptors (El-Asmar et al., 2005) have been associated to the propensity of the receptors to dimerize. By contrast a positive cooperative binding of U69593 (a κ agonist) and DPDPE (a selective δ agonist) on the opioid κ–δ heterodimer has been reported (Jordan and Devi, 1999).

It should also be pointed out that an unobvious consequence of ligand binding on a dimeric receptor can be the loss of a competitive binding characteristics and this might explain discrepancies in the affinities of ligands depending on the ligand used as tracer (Durroux, 2005). We observed such discrepancies in the measurement of the affinities of ligands on wild type V1a receptor and this is worsened on the mutated K128A and Q131A V1a receptors. How can such mutations affect the competitive binding properties of receptors remains unclear. Because no computational dimeric receptor structure model has been proposed, it is then difficult to propose new hypotheses to describe crosstalk between protomers within a dimer and the role of particular residues. Similarly a discrepancy between K_d and K_i has been reported for the binding of raclopride on the D_2 receptor when experiments were done in the absence of sodium ions (Armstrong and Strange, 2001). Moreover, recently, Griffante and collaborators reported that the discrepancies in the affinity of SSR149415 for the OT receptor observed in two studies (Griffante et al., 2005; Serradeil-

Le Gal et al., 2002) could be attributed to the radioligand chosen in the OT receptor binding assays. This might be of great importance for drug screening. It strongly suggests that the determination of the value of the ligand dissociation constant from IC_{50} (the concentration of competitor for which the binding of the tracer is 50 % of its binding in the absence of competitor) is inaccurate when ligand bindings are not strickly competitive.

In conclusion, we showed that ligand binding exhibits cooperative process and that the nature of the cooperativities observed is ligand-dependent. We provided evidence which definitely demonstrates cooperative binding of vasopressin, oxytocin and their analogs on the vasopressin and oxytocin receptors. These cooperativities can be only explained if receptors are considered as multivalent receptor oligomers and are in very good accordance with predictions from the mathematical models which consider binding of ligands on dimer. The binding properties of GPCR can provide strong evidence of receptor dimerization and their analysis can be a valuable tool to study dimers in native tissues.

Oligomerization of GPCR has been shown to play a role in receptor ontogeny and targetting, in pharmacological properties, in receptor internalization and more precisely in the fate of the internalized receptor (for a review see (Milligan, 2004; Terrillon and Bouvier, 2004)). Here, we showed that the binding of a first ligand can modulate the binding of a second one. Whether this step can constitute another level of regulation in signal transduction and then in physiological function remains to be established.

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Footnotes:

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Figure Legends

Figure 1: **Outlines of the predictions of the mathematical model**: Binding of ligands on dimeric receptor is considered in three different contexts: the ligands L1 and L2 bind to the binding sites without any cooperativity (a), with a negative (b) or a positive (c) cooperativity. In the middle panels, L1 and L2 are identical and the conditions correspond to saturation experiments. The saturation and Scatchard curves (inset) exhibit Hill coefficient equal to 1 (a), lower than 1 (b) or greater than 1 (c). In the lower panels, L1 and L2 are assumed to be different and the resulting competition curves are illustrated in the three ccooperativity contexts. The absence of cooperativity result in curve with a slope factor equal to 1, while negative and positive cooperativities result in curves with slope factor lower or greater than 1, respectively. It is noteworthy that the intermediate plateau in (b) can be more or less marked according to the parameter used for the mathematical simulation. In the same way, the amplitude of the hump in (c) which corresponds to an increase of the binding of the tracer is variable according to the parameters used in the simulation.

Figure 2: **Time resolved FRET experiments performed on Cos-7 cells expressing tagged V1a receptors**. FRET experiments on cell surface targeted receptors (white bars): cells expressing HA-tagged V1a receptors or not (mock) were incubated with europium cryptate-pyridine bipyridine and Alexa Fluor® 647 labeled anti-HA monoclonal antibodies as described in the Experimental Procedures section. FRET experiments on total receptors expressed in Cos-7 cell membranes (black bars): membranes expressing tagged V1a receptors were incubated with europium cryptate-pyridine bipyridine and Alexa Fluor® 647 labeled anti-tag monoclonal antibodies as indicated in the figure. The condition V1a (HA)/V1a (6His) corresponds to membranes in which HA-tagged V1a and 6His-tagged V1a receptors are

coexpressed; mix V1a (HA) + V1a (6His) corresponds to a mix of membranes which expressed either HA-tagged V1a or 6His-tagged V1a receptors. The conditions V1a (HA) and V1a (6His) correspond to membranes which expressed only HA-tagged or 6His-tagged V1a receptor, respectively. The FRET signal was measured at 665 nm after excitation at 337 nm. The illustrated experiments are representative of at least three experiments. Values are means ± SD of triplicates. The FRET signal (ΔF %) was calculated as described in the Experimental Procedures section

Figure 3: **Saturation experiments performed with [^{3}H]AVP and [^{3}H]OT on CHO cells expressing vasopressin and oxytocin receptors.**

Cold and hot saturation experiments with [^{3}H]AVP (a and b respectively), and cold saturation experiments were performed with [^{3}H]OT (c) on membranes of CHO expressing V1a (a), V1b (b) or OT (c) (10-15µg protein/assay). Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means ± S.E. of triplicates. Data were fitted using the non-linear curve-fitting routine of the computer software Kell (Biosoft) to the Hill equation: $B = Bmax\ [1 + (Kd/\ [L])^n]^{-1}$ where Bmax is the maximal binding, L is the concentration of labeled ligand, Kd is the equilibrium dissociation constant of the labeled ligand, and n is the Hill coefficient. Insets, Scatchard plots obtained from the corresponding saturation curves. The fitting parameters obtained for Kd and n are 85 nM and 0.45 (a) 85 nM and 0.27 (b) and 129 nM and 0.38 (c) for the V_{1a},V_{1b} and oxytocin receptors respectively.

Figure 4: Dissociation of [^{3}H]AVP and [^{3}H]OT from vasopressin and oxytocin receptors expressed in CHO cells.

Membrane were incubated for 40 min at 30°C with [3H]AVP (a, c) or [3H]OT (b) (0.5-1.5 nM). At a given time, 3ml of incubation medium containing or not an excess of vasopressin (1µM) (a, c) or oxytocin (1µM) (b) were added. The bound fractions were determined by filtration. Data are presented as the percentage of specific binding at time zero of dissociation. Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means $\pm$ S.E. of triplicates. Insets : in order to determine dissociation rates, data are presented in a semilogarithmic plots. The dissociation rate, k_{-1}, is given by the slope of the curve when fitting the data with the equation $\text{Ln}\,[B/B_0] = k_{-1}\,t$ in which t is time, B is the specific binding at time t, B_0 is the specific binding at time zero of dissociation. The values of the dissociation rates in the absence or the presence of an excess of unlabeled ligand are respectively 0.0024 min^{-1} and 0.0063 min^{-1} **(a), 0.0168** min^{-1} and 0.067 min^{-1} (b) and 0.0076 min^{-1} and 0.0201 min^{-1} (c).

Figure 5: Saturation experiments performed with iodinated antagonists on CHO cells expressing vasopressin V1a receptors.

Hot saturation experiments with [^{125}I]-HO-LVA (a) and [^{125}I]-OTA (b) were performed on membranes of CHO cells expressing the human vasopressin V1a receptor. Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means $\pm$ S.E. of triplicates. Data were fitted using the non-linear curve-fitting routine of the computer software Kell (Biosoft) to the Hill equation: $B = Bmax\,[1 + (Kd/[L])^n]^{-1}$ where Bmax is the maximal binding, L is the concentration of labeled ligand, Kd is the equilibrium dissociation constant of the labeled ligand, and n is the Hill coefficient. Insets, Scatchard plots obtained from the corresponding saturation curves. The fitting parameters

obtained for Bmax, Kd and n are 0.0126 nM, 0.022 nM and 0.97 (a) and 0.044 nM, 0.302 nM and 1.29 (b), respectively.

Figure 6: **Competition experiments performed on CHO cells expressing V1a receptor.**

a, Competition experiments between [^{125}I]HO-LVA and AVP on membranes of CHO cells expressing human vasopressin V1a receptor. Membranes were incubated in the presence of 60 pM [^{125}I] HO-LVA and increasing concentration of AVP (1 pM to 1µM) for 1 hour at 30°C. Bound radioactivity was separated from the free ligand fraction by filtration. Experimental data (closed squares) are fitted using the equation: Bound = Bg + ((B_{max} – Bg) / (1+([L]/IC50)^n)) with n set to 1 (grey curve) or when n is allowed to vary (black curve).

b, Competition experiments between the antagonist [^{125}I]-HO-LVA and the agonist d(CH_2)$_5$[D-Thi^2, Thr^4, Orn^8, Eda^9 ←Tyr^{10}]vasotocin, a structural analog of vasotocin on membranes of CHO cells expressing human V1a receptors. Membranes were incubated in the presence of 60 pM [^{125}I] HO-LVA and increasing concentrations of d(CH_2)$_5$[D-Thi^2, Thr^4, Orn^8, Eda^9 ←Tyr^{10}]vasotocin (1 pM to 1µM) for 1hr at 30°C. Bound radioactivity was separated from the free ligand fraction by filtration. Each curve is representative of at least three independent experiments performed in triplicate samples. Values are means ± S.E of triplicates.

Figure 7: **Absence of any effect of GTP-γ-S on binding of [^{3}H]AVP**.

Cold saturation experiments with [^{3}H]AVP were peformed on membranes of CHO cells expressing V1a receptors which exhibit cooperative binding. Membranes (8-15µg per assay) were incubated with (black squares) or without (white squares) GTP-γ-S (100 µM). Each

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curve is representative of at least three independent experiments. Values are means ± S.E. of triplicates.

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	"Cooperative binding site" analysis		"Independent binding site" analysis (n=1)	
Ligand	Kd (nM)	Hill Coefficient (n)	Kd(nM)	Nb. exp.
[^{125}I]-HO-LVA	**0,031 ± 0.005**	0.94 ± 0.06	**0,028 ± 0,004**	5
[^{3}H] AVP	**70 ± 35**	0.52 ± 0.03	**3.8 ± 2**	3
[^{125}I]-OTA	**0.35 ± 0.13**	**1.20 ± 0.035**	**0.58 ± 0.18**	**7**

Table 1: Affinities and Hill coefficients determined after fitting the saturation and Scatchard curves when performing saturation experiments with [^{125}I]-HO-LVA, [^{3}H] AVP and [^{125}I]-OTA on membranes of CHO cells expressing human V_{1a} receptors. Two data analyses were performed. The first one ("cooperative binding site" analysis) has taken into account a possible cooperativity between ligand binding sites, the second one ("independent binding site" analysis) considers binding sites as independent binding sites and does not consider any potential cooperativity (the Hill coefficient n is equal to 1). The right column indicates the number of experiments.

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	Kd (nM)	Ki_{AVP} (nM)
Labeled ligand / Receptor	[^{3}H] AVP	[^{125}I] HO-LVA
Human W.T. V_{1a}	0.7 ± 0.2	3.4 ± 1.1
Human Q185A V_{1a}	46 ± 7	810 ± 148 *
Rat K128A V_{1a}	1.2 **	266 ± 108 ***
Rat Q131A V_{1a}	11.4 **	168 ± 87 ***

Table 2: Affinities of AVP determined by saturation experiments (Kd) or by competition experiments (Ki) with [^{125}I] HO-LVA as radiotracer on membranes of Cos-7 cells expressing vasopressin V1a receptor.

* : values from (Cotte et al., 2000)

* : values from (Barberis et al., 1993)

** : values from (Mouillac et al., 1995a)

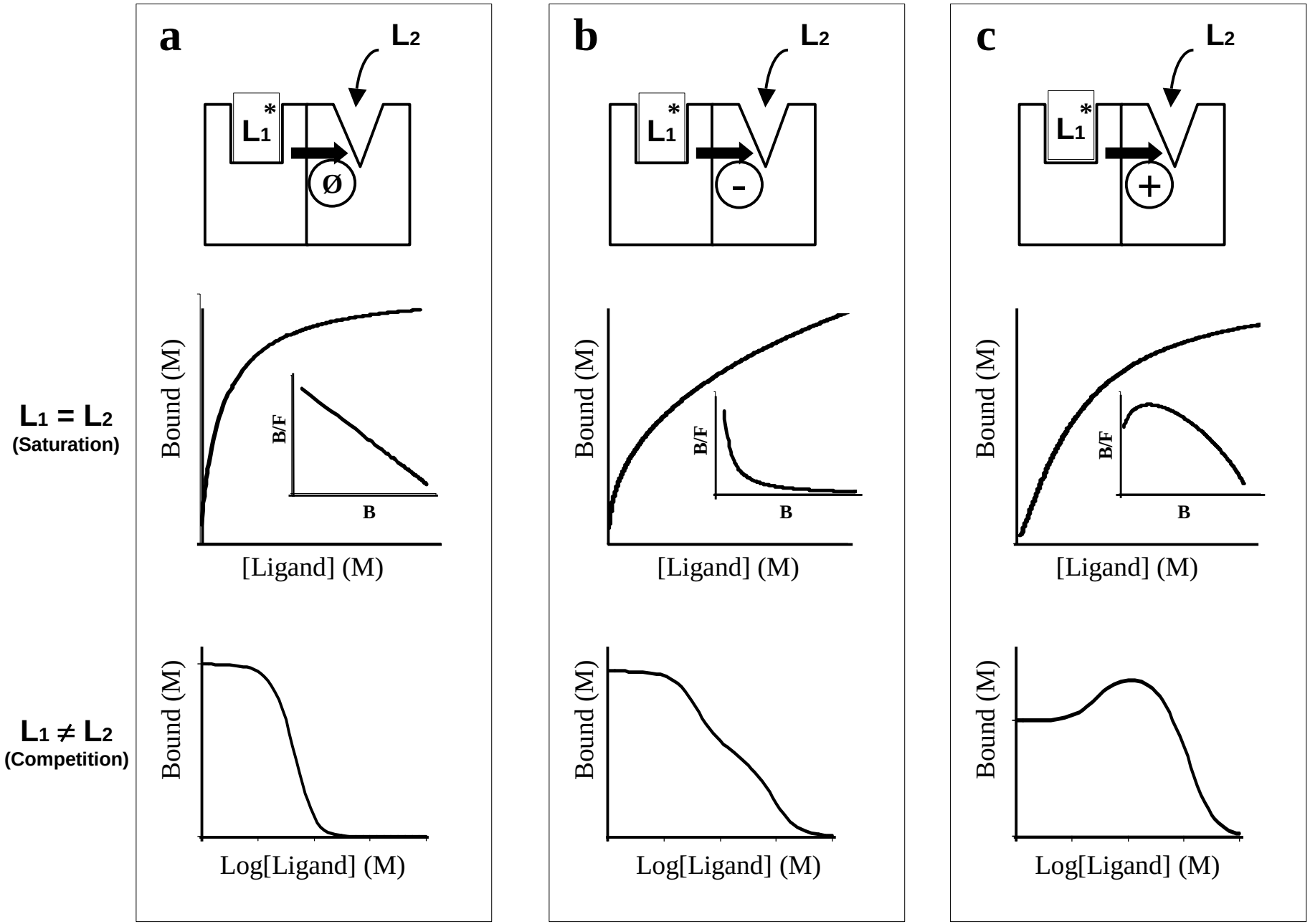

Figure 1

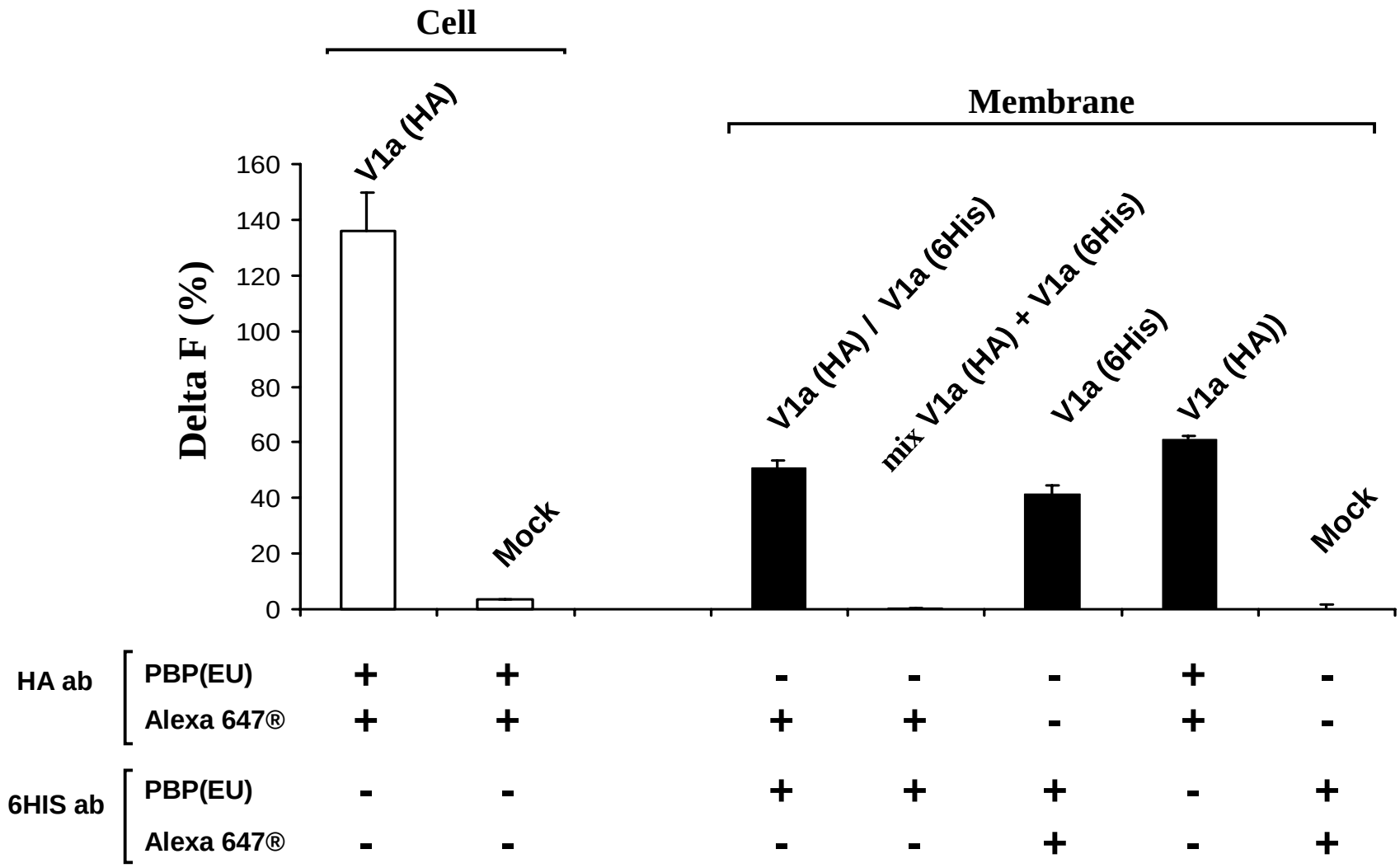

Figure 2

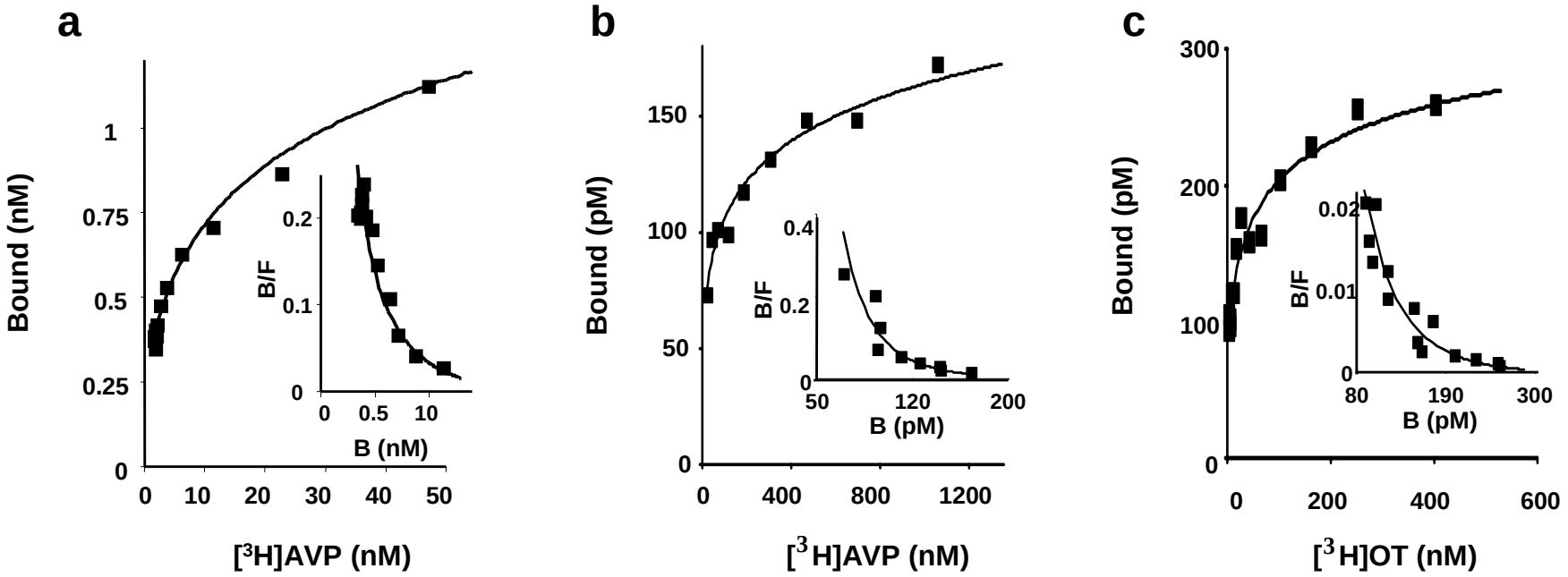

Figure 3

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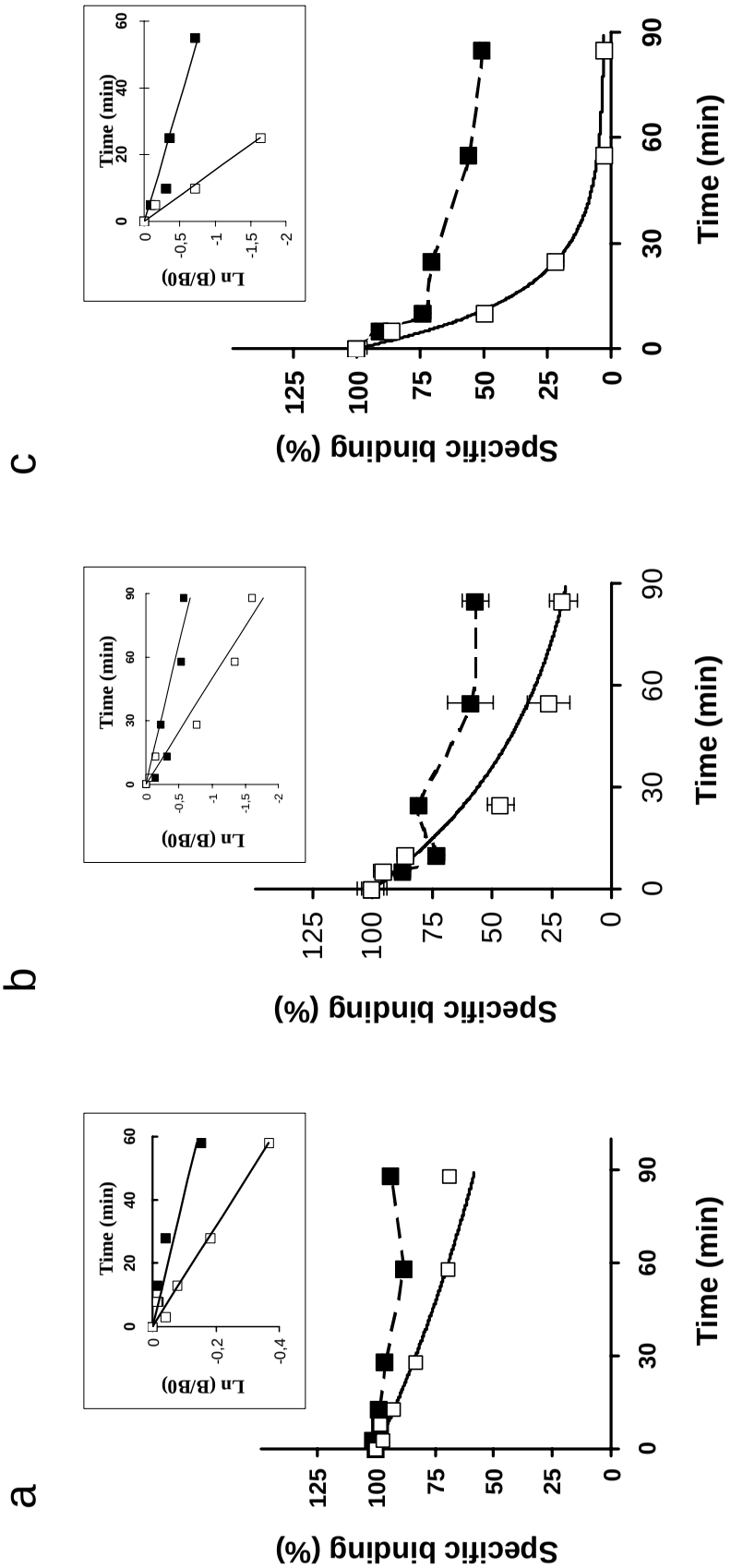

Figure 4

Figure 5

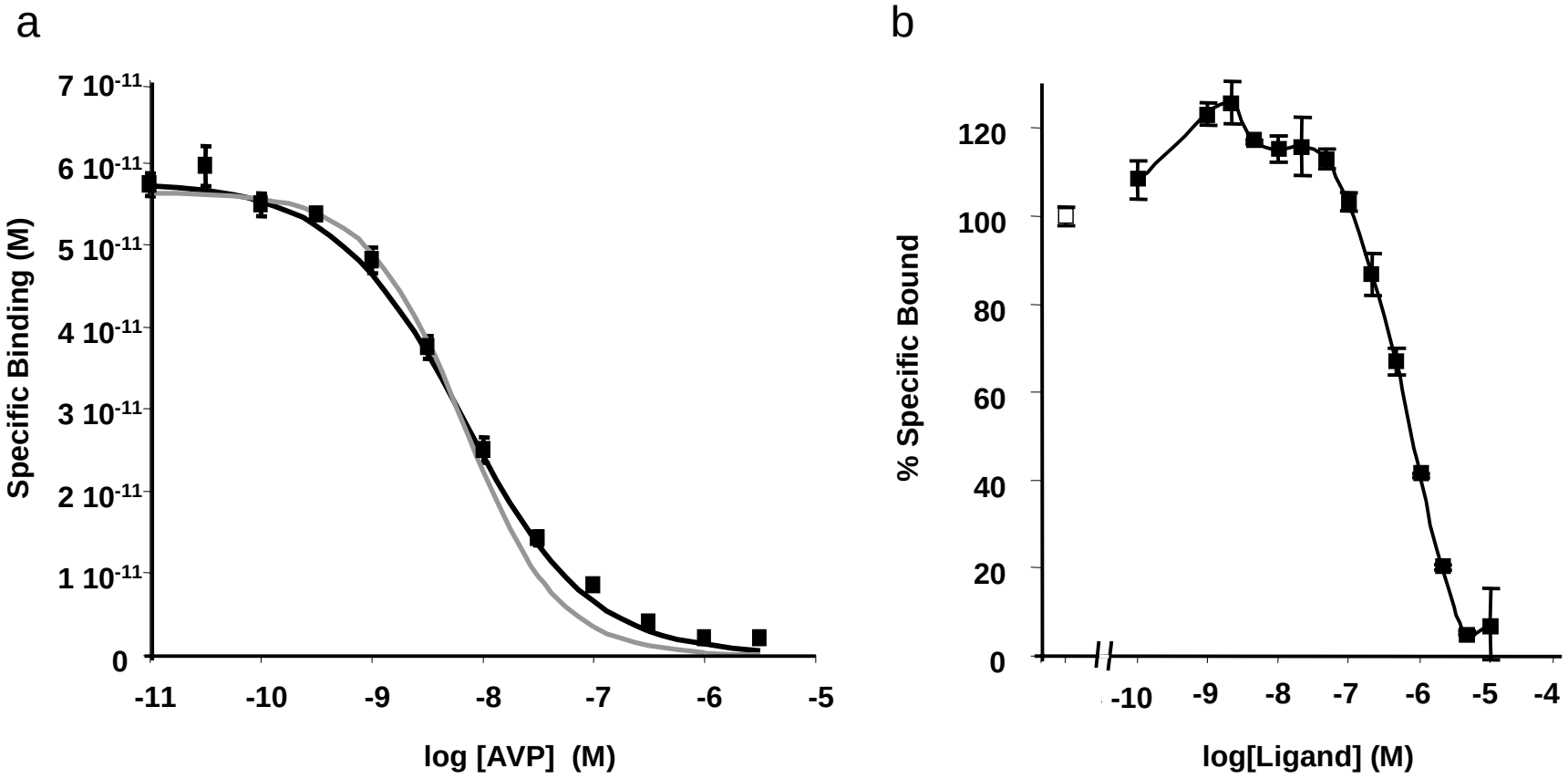

Figure 6

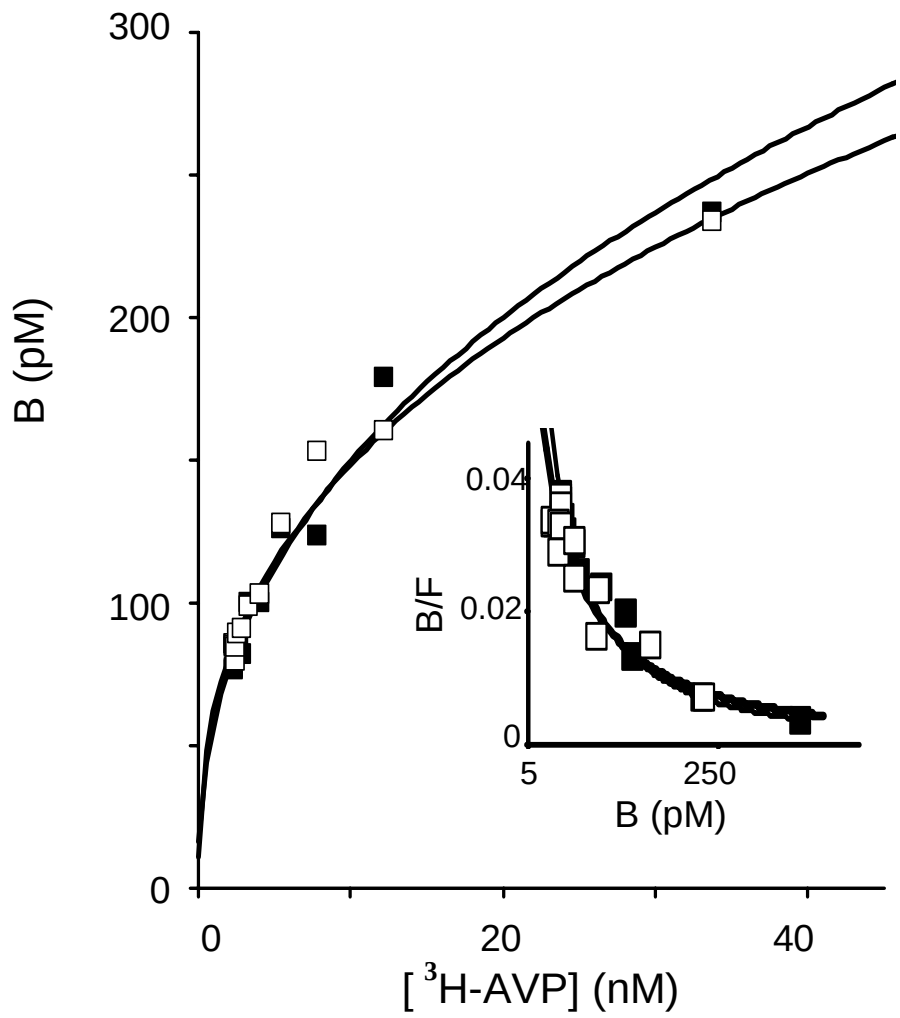

Figure 7