Author's Accepted Manuscript

Distinctive binding properties of the negative allosteric modulator, [^{3}H]SB269,652, at recombinant dopamine D_3 receptors

Irene Fasciani, Ilaria Pietrantoni, Mario Rossi, Clotilde Mannoury la Cour, Gabriella Aloisi, Francesco Marampon, Marco Scarselli, Mark J Millan, Roberto Maggio

PII: S0014-2999(17)30786-0
DOI: https://doi.org/10.1016/j.ejphar.2017.12.002
Reference: EJP71549

To appear in: *European Journal of Pharmacology*

Received date: 24 October 2017
Revised date: 27 November 2017
Accepted date: 4 December 2017

Cite this article as: Irene Fasciani, Ilaria Pietrantoni, Mario Rossi, Clotilde Mannoury la Cour, Gabriella Aloisi, Francesco Marampon, Marco Scarselli, Mark J Millan and Roberto Maggio, Distinctive binding properties of the negative allosteric modulator, [^{3}H]SB269,652, at recombinant dopamine D_3 receptors, *European Journal of Pharmacology,* https://doi.org/10.1016/j.ejphar.2017.12.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Distinctive binding properties of the negative allosteric modulator, [^{3}H]SB269,652, at recombinant dopamine D_3 receptors

Irene Fasciani[1a], Ilaria Pietrantoni[1a], Mario Rossi[2], Clotilde Mannoury la Cour[3], Gabriella Aloisi[1], Francesco Marampon[1], Marco Scarselli[4], Mark J Millan[3], Roberto Maggio[1]

[1] Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy

[2] Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, United States

[3] Centre for Innovation in Neuropsychiatry, Institut de Recherches Servier, Croissy sur Seine, France

[4] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy

[a] These authors contributed equally to the work.

Author for correspondence:

Dr. Roberto Maggio

Department of Biotechnological and Applied Clinical Sciences

University of L'Aquila

Via Vetoio Coppito 2, 67100 L'Aquila (Italy)

Tel +39-0862-433587

Fax +39-0862-433523

Email: roberto.maggio@univaq.it

Accepted manuscript

ACCEPTED MANUSCRIPT

Abstract

Recently, employing radioligand displacement and functional coupling studies, we demonstrated that SB269,652 (N-[(1r,4r)-4-[2-(7-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-1H-indole-2-carboxamide) interacts in an atypical manner with dopamine D_3 receptor displaying a unique profile reminiscent of a negative allosteric ligand. Here, we characterized the binding of radiolabelled [^{3}H]SB269,652 to human dopamine D_3 receptor stably expressed in Chinese Hamster Ovary cells. Under saturating conditions, SB269,652 showed a KD value of ≈ 1 nM. Consistent with high selectivity for human dopamine D_3 receptor, [^{3}H]SB269,652 binding was undetectable in cells expressing human dopamine D_1, D_{2L} or D_4 receptors and absent in synaptosomes from dopamine D_3 receptor knockout *vs*. wild-type mice. In contrast to saturation binding experiments, the dissociation kinetics of [^{3}H]SB269,652 from human dopamine D_3 receptors initiated with an excess of unlabelled ligand were best fitted by a bi-exponential binding model. Supporting the kinetic data, competition experiments with haloperidol, S33084 (a dopamine D_3 receptor antagonist) or dopamine, were best described by a two-site model. In co-transfection experiments binding of SB269,652 to dopamine D_3 receptor was able to influence the functional coupling of dopamine D_2 receptor, supporting the notion that SB269,652 is a negative allosteric modulator across receptor dimers. Hovever, because SB269,652 decreases the rate of [^{3}H]nemonapride dissociation, the present data suggest that SB269,652 behaves as a bitopic antagonist at unoccupied dopamine D_3 receptor, binding simultaneously to both orthosteric and allosteric sites, and as a pure negative allosteric modulator when receptors are occupied and it can solely bind to the allosteric site.

Keywords

Dopamine, receptor binding study, receptor dimerization

Accepted manuscript

ACCEPTED MANUSCRIPT

1. Introduction

Dopamine D_3 receptors are members of the dopamine D_2-like family of G protein-coupled receptors (GPCRs) that signal *via* the $G\alpha_i$ class of GTP-binding proteins to inhibit adenylyl cyclase and activate diverse kinase signaling cascades (Sokoloff and Le Foll, 2017). They are critically involved in a number of physiological processes such as the control of cognition, mood and motor behaviour (Beaulieu and Gainetdinov, 2011). Accordingly, dopamine D_3 receptors may be a target for the treatment of neuropsychiatric disorders and, due to their enrichment in mesolimbic dopaminergic projection areas, they are of particular interest for the potential control of schizophrenia (Gross and Drescher, 2012; Millan et al., 2016) and drug addiction (Heidbreder and Newman 2010; Joyce and Millan, 2005).

Conventionally, and by analogy to dopamine D_2 receptors, the design of dopamine D_3 receptor ligands has focused on the orthosteric binding site (Pich and Collo, 2015). However, in recent years, allosteric modulation of GPCR activity has attracted interest as an alternative route towards the development of selective and well-tolerated drugs for use either alone or together with other classes of agent. An attractive feature of positive and negative allosteric modulators is their concentration-response relationship, which plateaus, and permits the development of treatments with a broad therapeutic dose-range avoiding the risk of excessive activation or blockade of receptor-signalling: this underpins the interest in their clinical application either alone or as adjunctive treatments (Smith and Milligan, 2010; Hudson et al., 2013; Christopoulos, 2014).

Recently, by employing a broad and complementary range of cellular approaches, we revealed that the novel tetrahydroisoquinoline derivative, SB269,652, behaves as an atypical antagonist at dopamine D_3 and dopamine D_2 receptors (Silvano et al., 2010). In particular, SB269,652 potently abolished the specific binding of [^{3}H]nemanopride and [^{3}H]spiperone to dopamine D_3 receptors while only weakly and partially (by approximately 20-30%) inhibited the radioligand binding to dopamine D_2 receptors. However, when [^{3}H]nemanopride and [^{3}H]spiperone were employed at 10-fold higher concentrations, SB269,652, even at very high concentrations, could only sub-maximally

Accepted manuscript

inhibit their specific binding at dopamine D_3 receptor. Similarly, SB269,652 potently blocked dopamine D_3 receptor-mediated activation of $G\alpha_{i3}$, however, when concentrations of dopamine were increased 10-folds, from 1 µM to 10 µM, SB269,652 inhibited dopamine-induced stimulation of $G\alpha_{i3}$ only submaximally, indicating, once more, that SB269,652 behaved also as a negative allosteric modulator. Furthermore, the binding kinetics of [^{3}H]nemonapride and [^{3}H]spiperone at dopamine D_2 and dopamine D_3 receptors were clearly modified in the presence of SB269,652 compared with the two orthosteric antagonists, haloperidol and sulpiride. Taken together, these results strongly suggest that SB269,652 behaves as a negative allosteric modulator at dopamine D_2 and dopamine D_3 receptors - the first to be identified. The allosteric nature of this compound was subsequently confirmed by Lane et al. (2014); Shonberg et al., (2015); Mistry et al. (2015) for dopamine D_2 receptor and by Kumar et al. (2017), for dopamine D_3 receptor, and now SB269,652 has become a leading compound in the synthesis of new allosteric drugs (Rossi et al., 2017).

In view of this interest in allosteric modulators of dopaminergic receptors and more specifically, SB269,652, in the present work, this compound was radiolabelled in order to characterize its putatively allosteric actions at recombinant human dopamine D_3 receptor.

2. Materials and methods

2.1. Radiolabelled compounds.

SB269,652 is a N-[(1r,4r)-4-[2-(7-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-1H-indole-2-carboxamide. It has two diastereoisomers due to the presence of a cyclohexane moiety in its structure; the trans diastereoisomer was used in this work and was radiolabelled with tritium to a specific activity of 26 Ci/mmol. SB269,652 was synthesized by *G. Lavielle* (Servier, Paris, France). [^{3}H]Nemonapride (85.5 Ci/mmol) and N-[^{3}H]methylscopolamine (83 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Accepted manuscript

ACCEPTED MANUSCRIPT

2.2. Construction of dopamine D_3D110A mutant, and dopamine D_3-trunk and dopamine D_2-trunk receptor fragments.

The human dopamine D_3 receptor was used to make the dopamine D_3D110A and the human dopamine D_3-trunk receptor mutants. In the D_3D110A construct, aspartate 110 in transmembrane region III was replaced by alanine. The dopamine D_3-trunk receptor fragment was made by inserting a STOP codon into the dopamine D_3 receptor after amino acid isoleucine 290 and by removing the C-terminal part of the intracellular loop 3 and transmembrane regions VI and VII. The dopamine D_2-trunk receptor fragment was generated as described in (Scarselli et al., 2000). The plasmids were transiently transfected in COS-7 cells by the DEAE-dextran chloroquine method.

2.3. Stably-transfected CHO-D_3, CHO-D_{2L}, CHO-D_1 and CHO-M_4 cell lines and transiently transfected COS-7 cells.

CHO-D_3-DHFR$^-$ cells (from now on named CHO-D_3) were provided by Pierre Sokoloff (Sokoloff et al., 1992). CHO-D_{2L} and CHO-D_1 cells were generated by transfecting the human dopamine D_{2L} or human dopamine D_1 receptors, respectively, in wild type CHO cells (Aloisi et al., 2011). CHO-hM_4 (human muscarinic M_4 receptor) cells were prepared as described previously in Maggio et al., 1995. COS-7 cells were used for transient transfections. Briefly, cells were seeded at a density of 5 x 10^5 per 100-mm dish and 24 h later incubated with the DEAE-dextran chloroquine transfection reagent and with the respective plasmids as indicated for each experiment (Picchietti et al., 2009). The total amount of DNA used for each transfection was 4 μg (2 μg for each plasmid in co-transfection, unless otherwise specified.

2.4. Evaluation of the affinities of SB269,652 at human dopamine D_3 receptor, human dopamine D_{2L} receptor, and human dopamine D_{2S} receptor and at other classes of binding site.

Accepted manuscript

ACCEPTED MANUSCRIPT

SB269,652 affinities at various classes of dopaminergic receptors and other sites were determined using conventional procedures. All protocols used for determination of affinities of SB269,652 at the multiple classes of cloned receptors have been documented previously (Millan et al., 2008). The cell lines and radioligands used for determination of affinities are summarized in Table 4.

2.5. Membrane preparation and binding assays.

Confluent plates were washed twice with cold 0.9 % NaCl solution and lysed in ice-cold hypotonic buffer (1 mM Na-HEPES and 2 mM EDTA). After 20 min in ice, the cells were scraped off the plate and centrifuged at 35,000 g for 20 min. The lysed cell pellet was homogenized with Polytron homogenizer in the binding assay buffer (50 mM TRIS-HCl pH 7.4, 155 mM NaCl and 0.01 mg/ml bovine serum albumin). Equilibrium binding experiments of [^{3}H]SB269,652 and [^{3}H]nemonapride to cells membranes were carried out at 30°C for 3 h in a final volume of 1 ml. The bound ligand was separated from the unbound ligand using glass-fibre filters (Whatmann, GF/B) with a Brandel Cell Harvester, and the filters were counted with a scintillation β-counter. Nonspecific binding was calculated in the presence of 2 mM dopamine. To calculate the dissociation rate constants (K_{off}) 20 µl of membranes were loaded with 5 nM [^{3}H]SB269,652 for 2 h at 30°C and then diluted 100 times in presence of excess haloperidol (100 nM) or dopamine (1 mM). To calculate the apparent association rate constant (K_{obb}), [^{3}H]SB269,652 was used at a concentration of 1.6 nM. Binding to native dopaminergic receptors was performed using conventional procedures in male Wistar rats, 45 days old (Harlan, Italy) and in 90 days old, male, wild-type and dopamine D_3 receptor (D_3-/-) knockout C57Black mice. Experiments were performed following CEE procedures (directive/n.86/609) for the Care and Use of Laboratory Animals. Briefly, brain regions were dissected at 4°C and synaptosomal pellets (P2) fractions prepared as previously reported (Di Cara et al., 2011). The synaptosomal pellet was homogenized with a

Accepted manuscript

ACCEPTED MANUSCRIPT

Polytron homogenizer in the binding assay buffer, centrifuged and washed twice at 35,000 *g* for 20 min. Membrane suspensions were used for binding studies.

2.6. Adenylyl cyclase (AC) assays

Twenty-four h after transfection, cells were transferred into 24-well plates and cultured for additional 24 h before running the adenylyl cyclase assays. In brief, the cells were incubated in fresh media for 2 h (0.25 ml/well) in the presence of 5 µCi/ml [^{3}H]adenine. The media was then replaced with 0.5 ml/well of Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4, 0.1 mg bovine serum albumin, and the phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (0.5 mM) and RO-20-1724 (0.5 mM).

AC activity was stimulated by addition of 10 µM forskolin in the presence or absence of quinpirole. The antagonist SB269,652 or SB277,011A were added 20 min before the agonists.

After 10 min incubation at 30°C, the medium was removed and the reaction terminated with perchloric acid containing 0.1 mM unlabelled cAMP. The acid was then neutralized with KOH. The amount of [^{3}H]cAMP produced was determined by a two-step column separation procedure, as described previously in Maggio et al., 2003.

2.7. Fitting and analysis of data.

K_D and B_{max} values of [^{3}H]SB269,652 and [^{3}H]nemonapride, and B_{max} values of [^{3}H]methylscopolamine, were calculated in direct saturation experiments, whereas inhibitory concentration $(IC)_{50}$ values were calculated in competition binding experiments, that were fitted by a one or two sites binding models. Kinetics experiments were fitted to mono or bi-exponential binding models. The K_{on} was calculated by the formula [$K_{on} = (K_{obb} - K_{off})/L$], where L is the concentration of the radioligand. Statistics were run with iterative, non-linear least-squares regression analysis using OriginPro 7.5 software (OriginLab Corporation, Northampton, MA,

Accepted manuscript

USA). The goodness-of-fit of one or two sites models in equilibrium and kinetics binding experiments was compared using F-tests with significance set at $P < 0.05$.

3. Results

3.1. Saturation binding experiments of [^{3}H]SB269,652 and [^{3}H]nemonapride in CHO cell membranes expressing dopamine D_3 receptor.

Binding of [^{3}H]SB269,652 to CHO-D_3 membranes was saturable with a K_D of 0.983 ± 0.067 nM (Fig. 1A) and a Hill coefficient not significantly different from 1 (1.106 ± 0.051). The mean B_{max} calculated in three independent experiments was 5,204 ± 138 fmol/mg of protein (about 1.2 x 10^6 receptor/cell). In parallel experiments, [^{3}H]nemonapride gave a similar B_{max} value of 5,456 ± 120 fmol/mg of protein (Table 1).

3.2. Competition binding experiments with [^{3}H]SB269,652 in stable transfected CHO-D_3 cells.

As shown in Fig. 1B, [^{3}H]SB269,652 competition binding curves with the high selective dopamine D_3 receptor antagonist S33084 were best fitted with a two site binding model ($F_{2,42}$ = 38.19 → $P < 0.0001$). On average, 43.6% of the sites had an IC_{50} of 0.271 ± 0.057 nM while the remaining sites had an IC_{50} of 44.48 ± 26.36 nM (Table 2). Data from competition binding experiments with haloperidol (Fig. 1C), were also best fitted with a two sites binding model ($F_{2,39}$ = 10.09 → $P = 0.0003$), with approximately, 43.7% of the sites showing an IC_{50} of 3.401 ± 1.675 nM and the remaining sites an IC_{50} of 150.9 ± 57.43 nM (Table 2). Competition binding experiments with the agonist dopamine, were again best fitted with a two sites binding model ($F_{2,30}$ = 8.13 → $P = 0.0015$) (Fig. 1D). On average, 51.4% of the binding sites had an IC_{50} of 0.544 ± 0.195 µM while the remaining sites had an IC_{50} of 10.69 ± 9.387 µM (Table 2).

3.3. Kinetic binding experiments of [^{3}H]SB269,652 in stable transfected CHO-D_3 cells.

Accepted manuscript

ACCEPTED MANUSCRIPT

Dissociation kinetics of [^{3}H]SB269,652 from CHO-D_3 cell membranes, performed by dilution were best fitted with a one-phase exponential decay model that resulted in a K_{off} value (expressed in min^{-1}) of 0.03 ± 0.002 (Table 3 and Fig. 2A). Conversely, dissociation kinetics performed by dilution and addition of an excess of haloperidol or dopamine were best fitted by a two-phases exponential decay model ($F_{2,27} = 14.06 \rightarrow P < 0.0001$ and $F_{2,27} = 33.43 \rightarrow P < 0.0001$, respectively) (Fig. 2B and C) with K_{off} values of fast and low kinetics of 0.241 ± 0.094 and 0.024 ± 0.001 for haloperidol and 0.416 ± 0.089 and 0.256 ± 0.003 for dopamine, respectively (Table 3). In the presence of dopamine, fast dissociation of [^{3}H]SB269,652 accounts for 41% of the bound receptors, while with haloperidol this fraction dropped to 20% (Table 3). Interestingly, dissociation kinetics performed in the presence of an excess of cold ligands resulted in association kinetics that were best fitted by a two-phase exponential growth function (Fig. 2D). The two apparent association rate constants in the presence of 1.6 nM [^{3}H]SB269,652 were: $K_{obb}1 = 0.553 \pm 0.407$ and $K_{obb}2 = 0.037 \pm 0.007$ min^{-1} (Table 3). Notably, the fast association rate constant accounted for 24.16% of the saturated receptors.

3.4. [^{3}H]SB269,652 binding to dopamine D_3D110A receptor mutant, to co-transfected dopamine D_3D110A/D_3-trunk receptor and to co-transfected dopamine D_3D110A/D_2-trunk receptor.

In this set of experiments, we tested whether aspartate 110 in the third transmembrane region of dopamine D_3 receptor was important for [^{3}H]SB269,652 binding, inasmuch this type of mutation in the analogous residue (aspartate 114) of dopamine D_2 receptor leads to a total loss of both agonist and antagonist binding (Sukalovic et al., 2015). As shown in Table 2, [^{3}H]SB269,652 (up to 10 nM) did not bind to dopamine D_3D110A receptor mutant expressed in COS-7 cells. Analogous results were obtained with [^{3}H]nemonapride. In order to exclude that the lack of binding to radioligands was due to incorrect folding and expression of dopamine D_3D110A receptor mutant on the plasma membrane, we coexpressed the dopamine D_3D110A receptor mutant with a receptor

ACCEPTED MANUSCRIPT

fragment containing the first five transmembrane regions of dopamine D_3 receptor (dopamine D_3-trunk receptor), and bearing the correct aspartate residue 110 in the third transmembrane region. As observed previously (Scarselli et al., 2003), if the dopamine D_3D110A receptor mutant is correctly expressed on the membrane, the truncated fragment should rescue the binding to the radioligands. Even though only of modest efficiency, the binding of [^{3}H]SB269,652 and [^{3}H]nemonapride was indeed rescued when the dopamine D_3D110A receptor mutant was co-transfected with the dopamine D_3-trunk receptor fragment (Table 1). Conversely, only binding to [^{3}H]nemonapride was rescued when dopamine D_3D110A receptor mutant was co-transfected with the analogous truncated fragment derived from the dopamine D_2 receptor (dopamine D_2-trunk receptor). This shows that the chimeric, reconstituted dopamine D_2/D_3 receptor has low affinity for [^{3}H]SB269,652 (Table 1).

3.5. Binding of [^{3}H]SB269,652 at other classes of dopamine receptor.

[^{3}H]SB269,652 up to 10 nM did not bind to membranes generated from CHO-hD_{2L} and CHO-hD_1 cells nor it did bind to COS-7 cells transiently transfected with the human dopamine D_4 receptor (Table 1). Hence, it interacted specifically and only with recombinant dopamine D_3 receptor.

3.6. Binding of [^{3}H]SB269,652 to membranes from rat and mouse striatal synaptosomes.

The affinity of [^{3}H]SB269,652 was tested in membranes from synaptosomes prepared from rat and mouse striatum. The maximal binding capacity of [^{3}H]SB269,652 in rat synaptosome membranes was 61.2 ± 11.3 fmol/mg protein. The K_D, 421.7 ± 66.5 pM, was highly correlated with that obtained with cloned human dopamine D_3 receptor. In order to verify whether this binding was specific to dopamine D_3 receptor, parallel binding experiments were performed in synaptosomes of wild type and knockout dopamine D_3 receptor (D_3–/–) mice. These experiments showed that [^{3}H]SB269,652 was able to bind to the synaptosomes from wild type mice with a B_{max} of 71.1 ±

Accepted manuscript

ACCEPTED MANUSCRIPT

8.37 fmol/mg protein and a K_D of 471.7 ± 112.9 pM (Fig. 3), whereas no binding was detected in synaptosomes from dopamine D_3 receptor (D_3−/−) KO mice.

3.7. Binding affinity of SB269,652 to multiple GPCRs.

Interestingly, [^{3}H]SB269,652 displayed some specific binding also for the hM_4 muscarinic receptor. In particular, at a radioligand concentration of 10 nM of [^{3}H]SB269,652, a specific binding of 89.5 ± 13.7 fmol/mg was detected using CHO-hM_4 cell membranes, accounting for nearly 16% of the B_{max} calculated with the specific muscarinic antagonist N-[^{3}H]methylscopolamine (Table 1). No specific binding was detected on the other four classes of muscarinic receptor (data not shown). In view of this observation, we tested the affinity of cold SB269,652 for diverse classes of GPCR in comparison to hM_4 and other subtypes of muscarinic receptor. As shown in Table 4, SB269,652 binds with high affinity to dopamine D_3 receptor and displayed lower affinity at dopamine D_2 receptors, yet it also revealed significant affinity for muscarinic hM_2 and hM_4 receptors, displaying IC_{50} values of 213 nM and 60.2 nM, respectively. These data are in line with those outlined above attained with the radiolabelled [^{3}H]SB269,652 at hM_4 receptors.

Accepted manuscript

3.8. Influence of SB269,652 and SB277,011A upon quinpirole-induced inhibition of forskolin-stimulated ACVI activity at co-transfected dopamine D_2/D_3 receptor.

In order to address the effect of SB269,652 on dimeric dopamine D_3 receptor, we co-transfected dopamine D_3 and D_2 receptors in COS-7 cells. Significantly, these two receptors have been shown to interact with each other and to form heterodimers (Scarselli et al., 2001; Pou et al., 2012). The activity of these two receptors can be easily distinguished in functional assays, as quinpirole inhibits forskolin mediated stimulation of ACVI only in COS-7 cells that co-express the dopamine D_2 receptor and the ACVI, whereas it has no effect in COS-7 cells co-transfected with the dopamine D_3 receptor and the ACVI (Fig. 4A). In COS-7 cells co-expressing both dopamine D_2 and

ACCEPTED MANUSCRIPT

dopamine D_3 receptors and ACVI, the inhibitory effect of quinpirole on forskolin mediated activation of ACVI increased by four-fold in potency (Fig. 4A).

The effect of SB269,652 on the ability of quinpirole to inhibit ACVI activity was tested on COS-7 cells transfected with either dopamine D_2 receptor alone, or co-transfected with dopamine D_2 and dopamine D_3 receptors. We observed that SB269,652 inhibited with very low potency and efficacy the action of quinpirole in dopamine D_2 receptor transfected cells (Fig. 4B), whereas it was markedly more efficacious and potent at COS-7 cells co-expressing both dopamine D_2 and dopamine D_3 receptors (Fig. 4B). Importantly, even though there was an increase in the ability of SB269,652 to counter the actions of quinpirole in co-transfected dopamine D_2/D_3 receptor COS-7 cells, the inhibition was only partial and reached a plateau, the competitive antagonist SB277,011A, completely reversed the influence of quinpirole on forskolin-stimulated ACVI activity, both in dopamine D_2 receptor and in cotransfected dopamine D_2/D_3 receptor cells (Fig. 4C). SB277,011A was likewise more potent at cotransfected dopamine D_2/D_3 receptor cells.

Accepted manuscript

ACCEPTED MANUSCRIPT

4. Discussion

In previous work we showed that SB269,652 displayed an atypical and allosteric-like pattern of interaction at dopamine D_3 receptor and, less potently, at dopamine D_2 receptor. The present study extends these observations in demonstrating that radiolabelled [^{3}H]SB269,652 manifests high affinity, saturable, reversible and specific binding to recombinant human dopamine D_3 receptor, and it shows likewise an unusual and distinctive binding profile consistent with a predominantly allosteric type of interaction.

Whereas [^{3}H]SB269,652 showed a strong interaction with the human dopamine D_3 receptor, by displaying a B_{max} very similar to that of the prototypical radiolabel, [^{3}H]nemonapride (about 5 pmol/mg), the compound was not able to bind to the human dopamine D_1, dopamine D_2 and dopamine D_4 receptors underlying the specificity of its interaction for dopamine D_3 receptor. The specific recognition of dopamine D_3 *vs.* dopamine D_2 receptors extended to native populations. Thus, [^{3}H]SB269,652 bound to dopamine D_3 receptor sites in synaptosomal preparations of the striatum from wild type mice, with an affinity comparable to that observed for cloned human dopamine D_3 receptor, whereas binding was no detected in synaptosomes from dopamine D_3 receptor knockout mice.

In saturation binding experiments, [^{3}H]SB269,652 recognized an apparently homogeneous population of binding sites whereas, in kinetic experiments, the pattern of results was more complex. Thus, when [^{3}H]SB269,652 dissociation was initiated by dilution, it revealed a binding behaviour consistent with a one binding site model. However, when [^{3}H]SB269,652 dissociation was triggered by dilution and the addition of excess of the ligands, dopamine and haloperidol, a two-ligand binding site model best described its behaviour. These observations are reminiscent of those acquired with other radioligands - including those that do not necessarily act *via* an allosteric mechanism. For example, the opioid antagonist, [^{3}H]naloxone benzoylhydrazone, binds to a single population of μ-opioid receptors in saturation binding experiments whereas kinetic studies reveal a biphasic dissociation (Brown and Pasternak, 1998). Similar results have been published for the

Accepted manuscript

ACCEPTED MANUSCRIPT

muscarinic receptor antagonists, N-[^{3}H]methylscopolamine and [^{3}H]quinuclidinyl benzilate. In equilibrium binding experiments, these compounds bound to homogenous population of receptors yet, in kinetic experiments, the unlabelled ligands generated biphasic dissociation curves (Jakubík et al., 2000; Novi et al., 2003). This notion is also underlined by May et al. (2011) in work with adenosine A_1 and A_3 receptors, which demonstrated that the dissociation rate constants of the fluorescent agonist ABA-X-BY630 markedly increased in the presence of orthosteric agonists or antagonists. Moreover, these authors proposed an innovative method for detecting cooperative interactions amongst two topographically distinct binding sites based on the dissociation kinetics of a tracer ligand in the absence *vs* presence of an additional, cold ligand. Accordingly, for the present studies with [^{3}H]SB269,652, an acceleration of its dissociation from dopamine D_3 receptor in the presence of the orthosteric antagonist, haloperidol, or the agonist, dopamine would be consistent with a negative type of cooperativity between the radioligand on one hand and the cold ligand on the other. Taken together, these observations underpin the importance of performing kinetic studies with competing cold ligands to unveil otherwise-hidden, multiple sites or affinity states for a radioligand.

In line with the kinetic experiments discussed above, competition binding isotherms with haloperidol, the highly selective dopamine D_3 receptor antagonist S33084 (Cussac et al., 2000; Millan et al., 2000a, 2000b) and dopamine were in each case consistent with two populations of binding sites. Taken together these competition binding and kinetic data suggest that [^{3}H]SB269,652 recognizes either multiple sites or multiple affinity states of the dopamine D_3 receptor.

The conserved aspartate residue in transmembrane region 3 of amine receptors plays a pivotal role in the docking of ligands to the orthosteric binding site (Vaidehi et al., 2014). Dopamine D_2 receptors mutated at this residue lose the ability to bind orthosteric agonists and antagonists. An analogous mutation in the dopamine D_3 receptor, dopamine D_3D110A receptor mutant, prevented the binding of both [^{3}H]SB269,652 and [^{3}H]nemonapride. These results might in theory be

Accepted manuscript

explained either by a lack of ability of the mutant receptor to bind these radioligands or by the lack of its expression at the plasma membrane. This issue was addressed by complementing the dopamine D_3D110A receptor mutant with truncated mutants of dopamine D_3 or dopamine D_2 receptors this approach mirrors a previous work, in which, the ligand-binding and functional properties of truncated receptors were rescued by co-expressing complementary non-functional GPCR fragments (Scarselli et al., 2000, 2003). In particular we found that the binding of both [^{3}H]SB269,652 and [^{3}H]nemonapride was restored when dopamine D_3D110A receptor mutant was co-transfected with the dopamine D_3-trunk receptor fragment. The fragment consists of the first 5 transmembrane regions of the dopamine D_3 receptor and by interacting with the dopamine D_3D110A receptor mutant recreates a wild-type orthosteric binding site. [^{3}H]nemonapride binding was likely restored in cells co-transfected with dopamine D_3D110A receptor mutant and dopamine D_2-trunk receptor mutant, whereas [^{3}H]SB269,652 showed no binding, which is consistent with the high selectivity of SB269,652 for dopamine D_3 receptor. These results underpin the concept that high affinity binding of [^{3}H]SB269,652 to dopamine D_3 receptor depends on the presence of an intact orthosteric binding site.

Binding of [^{3}H]SB269,652, at least in part, to the orthosteric site of the dopamine D_3 receptor raises the issue of how this compound exerts its allosteric properties. One intriguing explanation may be found in the now broadly-accepted concept of GPCR homo- hetero- and higher order oligomerisation (Maggio et al., 2007; Scarselli et al., 2013, 2016; Shivanandan et al., 2014), which has been well-documented for dopamine D_3 receptor (Marsango et al., 2015, 2017; Pou et al., 2012). Dimerization can lead to crosstalk between protomers resulting in a cooperative pattern of ligand binding between them (Smith and Milligan, 2010).

As mentioned above, dopamine D_3 receptor form homo- (Karpa et al., 2000; Marsango et al., 2015; Nimchinsky et al., 1997; Pou et al., 2012) and heteromers (Fiorentini et al., 2010; Maggio and Millan, 2010; Maggio et al., 2015; Scarselli et al., 2001; Pou et al., 2012) in transfected cells as well as in native tissues. Clearly then, the formation of dopamine D_3 receptor homomers could account

Accepted manuscript

ACCEPTED MANUSCRIPT

for the kind of binding behaviour observed with [^{3}H]SB269,652 in kinetic and competition experiments. That is, [^{3}H]SB269,652 likely binds to the orthosteric site of one protomer and allosterically modify the binding of ligand to the other protomer.

To test this idea, we co-transfected dopamine D_3 and dopamine D_2 receptors inasmuch they have been shown to form heteromers (Scarselli et al., 2001; Pou et al., 2012), and can easily be distinguished in functional assays. In particular, when SB269,652 was tested at dopamine D_2 receptor alone, a modest reduction of quinpirole-induced inhibition of forskolin-stimulated ACVI activity was achieved at the higher concentrations of the drug. Conversely, when the dopamine D_3 receptor was co-transfected with dopamine D_2 receptor a much stronger inhibition and an increase in potency was obtained with SB269,652, even though the inhibition reached a plateau. As quinpirole did not have any effect on dopamine D_3 receptor alone, the data on co-transfected dopamine D_3 and dopamine D_2 receptors imply that SB269,652 binds to the dopamine D_3 receptor to inhibits the dopamine D_2 receptor via the dimer. Remarkably, a shift in potency was also observed with SB277,011A suggesting that mechanisms of cross inhibition can be relevant for other antagonists, potentially pure orthosteric ligands.

This potential mechanism of action is supported by the work of Lane et al. (2014) on dopamine D_2 receptor. They proposed that SB269,652 acts as a bitopic ligand composed of two bridged pharmacophores that simultaneously bind to the orthosteric and allosteric sites of the same receptor protomer. Importantly, they fragmented SB269,652 into two pharmacophores: 1), the 7-cyano-tetrahydroisoquinoline (7CN-THIQ) moiety that contains the tertiary amine, the part of the molecule that is important for the interaction with the conserved aspartic acid of the amine receptors in the orthosteric site and 2), the indole-2-carboxamide moiety that binds to an allosteric site of the dopamine D_2 receptor in a non-competitive manner (Fig. 4). Furthermore, Shonberg et al. (2015) focusing on the three main chemical constituents of SB269,652 (Fig. 4), found that: a) the 7CN-THIQ binding to the orthosteric site of dopamine D_2 receptor is important for the orientation and binding of the indolcarboxamide to the allosteric site, and even subtle modifications of 7CN-THIQ

Accepted manuscript

ACCEPTED MANUSCRIPT

affect the functional affinity and negative allosteric cooperativity of the molecule; b) the indole-2-carboxamide moiety of SB269,652 is important for its allosteric effect: SB269,652 analogues where indolic NH groups are substituted with others that are unable to generate hydrogen bonds lose the ability to allosterically interact with the receptor; c) linker length is critical for the allosteric effect of SB269,652 analogues. Lane et al. (2014) proposed that the binding of SB269,652 to one protomer of a dopamine D_2 receptor dimer changes its binding properties at the orthosteric site of the other promoter of the dimer. According to this concept, SB269,652 behaves as a competitive antagonist at receptor monomers yet as a negative allosteric modulator across receptor dimers. This hypothesis – that seems to apply to dopamine D_3 receptor - is supported by the above-discussed data with the dopamine D_3D110A receptor mutant, which lacks the aspartic acid in the orthosteric site crucial for binding of the tertiary amine in the 7CN-THIQ moiety of [^{3}H]SB269,652. Moreover, in a previous study using chimeric dopamine D_2/D_3 receptor (Silvano et al., 2010), we showed that the extracellular loop II of the dopamine D_3 receptor plays a pivotal role in binding SB269,652, which further supports the concept that this loop could bind the indole-2-carboxamide fragment of this compound.

While this hypothesis is compelling, it does not necessarily account for *all* the results generated with SB269,652 at dopamine D_3 receptor. Thus, we previously demonstrated that SB269,652 does not behave as a *classic* negative allosteric modulator: at high concentrations, in fact, it reduces radioligand dissociation rate constants at dopamine D_2 and dopamine D_3 receptors (Silvano et al., 2010). Conversely, as shown herein, dopamine and haloperidol accelerated the dissociation of a fraction of [^{3}H]SB269,652 from CHO-D_3 cell membranes. The most parsimonious explanation to reconcile these apparently contrasting results would be that SB269,652 binds in two ways: when the orthosteric site is occupied by a ligand, SB269,652 would still be able to physically interact with the monomer through the receptor allosteric site and it would physically occlude the orthosteric site so that competitive antagonists would be unable to readily leave (or enter) the orthosteric site (Fig. 5A). The lack of binding of [^{3}H]SB269,652 to the dopamine D_3D110A receptor mutant, in which

Accepted manuscript

ACCEPTED MANUSCRIPT

the bitopic interaction of the radioligand is prevented, could be explained by the much lower affinity of the radioligand bound to the sole allosteric site. On the other hand, when the receptor is unoccupied, SB269,652 would bind as a bitopic ligand to both orthosteric and allosteric binding sites of a single dopamine D_3 receptor protomer, and orthosteric ligands would increase its dissociation by binding to the sibling protomer in the corresponding dimer (Fig. 5B). The allosteric properties of SB269,652 would then strictly depend on the ligand bound to the sibling protomer. In this context, the recognition of a single population of binding sites in saturation binding experiments and in studies of dissociation kinetic started by dilution, suggest that when SB269,652 binds to one protomer in a dimer, it does *not* alter the binding of molecules of SB269,652 to the other protomer. A similar mechanism of binding and allostery has been described for the antagonist methoctramine at muscarinic M_2 receptors (Jakubík et al., 2014).

Intriguingly, despite high selectivity for human dopamine D_3 receptor over other classes of dopamine receptors (see above), [^{3}H]SB269,652 was found to bind membranes prepared from CHO cells expressing human muscarinic M_4 receptor. In addition, in a broader screen (competition experiments) of GPCRs, despite a lack of interaction with multiple subtypes of monoaminergic receptor, cold SB269,652 revealed significant affinity for muscarinic M_4 receptor and, albeit less potently, muscarinic M_2 receptor. It would be interesting to determine the functional properties of SB269,652 at muscarinic receptors and to determine whether it behaves as an allosteric modulator and/or as an orthosteric ligand. While the interaction of SB269,652 suggests caution in its use as a cold ligand to probe the function of dopamine D_3 receptor *in vivo*, the loss of [^{3}H]SB269,652 binding in dopamine D_3 receptor knockout mice nonetheless underscores its interest as a radioligand for their characterisation at appropriately low concentrations and it could even become a candidate for probing dopamine D_3 receptor occupancy by techniques such as Positron Emission Tomography.

Accepted manuscript

ACCEPTED MANUSCRIPT

Author contributions

Participated in research design:

Fasciani, Maggio

Conducted the expreiments:

Fasciani, Pietrantoni, Rossi, Mannoury la Cour, Aloisi, Marampon

Wrote or contributed to the writing of the manuscript:

Scarselli, Rossi, Millan, Maggio

This research was founded by Institut de Recherches Servier.

.

Accepted manuscript

References

Aloisi, G., Silvano, E., Rossi, M., Millan, M.J., Maggio, R., 2011. Differential induction of adenylyl cyclase supersensitivity by antiparkinson drugs acting as agonists at dopamine D(1)/D(2)/D(3) receptors vs D(2)/D(3) receptors only: parallel observations from co-transfected human and native cerebral receptors. Neuropharmacol. 60 (2-3), 439-445.

Beaulieu, J.M., Gainetdinov, R.R., 2011. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol. Rev. 63 (1), 182-217.

Brown, G.P., Pasternak, G.W., 1998. ^{3}H-Naloxone benzoylhydrazone binding in MOR-1-transfected Chinese Hamster Ovary cells: evidence for G-protein-dependent antagonist binding. J. Pharmacol. Exp. Ther. 286 (1), 376-381.

Christopoulos, A., 2014. Advances in G protein-coupled receptor allostery: from function to structure. Mol. Pharmacol. 86 (5), 463-478.

Cussac, D., Newman-Tancredi, A., Sezgin, L., Millan, M.J., 2000. [^{3}H]S33084: a novel, selective and potent radioligand at cloned human dopamine D3 receptors. Namyn Sehmiedebergs Arch. Pharmacol. 361 (5), 569-572.

Di Cara, B., Maggio, R., Aloisi, G., Rivet, J.M., Lundius, E.G., Yoshitake, T., Svenningsson, P., Brocco, M., Gobert, A., De Groote, L., Cistarelli, L., Veiga, S., De Montrion, C., Rodriguez, M., Galizzi, J.P., Lockhart, B.P., Cogé, F., Boutin, J.A., Vayer, P., Verdouw, P.M., Groenink, L., Millan, M.J., 2011. Genetic deletion of trace amine 1 receptors reveals their role in auto-inhibiting the actions of ecstasy (MDMA). J. Neurosci. 31 (47), 16928-16940.

Fiorentini, C., Busi, C., Spano, P., Missale, C., 2010. Dimerization of dopamine D1 and D3 receptors in the regulation of striatal function. Curr. Opin. Pharmacol. 10 (1), 87-92.

Gross, G., Drescher, K., 2012. The role of dopamine D(3) receptors in antipsychotic activity and cognitive functions. Handb. Exp. Pharmacol. 213, 167-210.

Accepted manuscript

ACCEPTED MANUSCRIPT

Heidbreder, C.A., Newman, A.H., 2010. Current perspectives on selective dopamine D3 receptor antagonists as pharmacotherapeutics for addictions and related disorders. Ann. N.Y. Acad. Sci. 1187, 4-34.

Hudson, B.D., Ulven, T., Milligan, G., 2013. The therapeutic potential of allosteric ligands for free fatty acid sensitive GPCRs. Curr. Top. Med. Chem. 13 (1), 14-25.

Jakubík, J., El-Fakahany, E.E., Tucek, S., 2000. Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors. J. Biol. Chem. 275 (25), 18836-18844.

Jakubík, J., Zimčík, P., Randáková, A., Fuksová, K., El-Fakahany, E.E., Doležal, V., 2014. Molecular mechanisms of methoctramine binding and selectivity at muscarinic acetylcholine receptors. Mol. Pharmacol. 86 (2), 180–192.

Joyce, J.N., Millan, M.J., 2005. Dopamine D3 receptor antagonists as therapeutic agents. Drug Discov. Today. 10 (13), 917-925.

Karpa, K.D., Lin, R., Kabbani, N., Levenson, R., 2000. The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3-D3nf interaction causes mislocalization of D3 receptors. Mol. Pharmacol. 58 (4), 677-683.

Kumar, V., Moritz, A.E., Keck, T.M., Bonifazi, A., Ellenberger, M.P., Sibley, C.D., Free, R.B., Shi, L., Lane, J.R., Sibley, D.R., Newman, A.H. 2017. Synthesis and Pharmacological Characterization of Novel trans-Cyclopropylmethyl-Linked Bivalent Ligands That Exhibit Selectivity and Allosteric Pharmacology at the Dopamine D3 Receptor (D3R). J. Med. Chem. 60 (4), 1478-1494.

Lane, J.R., Donthamsetti, P., Shonberg, J., Draper-Joyce, C.J., Dentry, S., Michino, M., Shi, L., López, L., Scammells, P.J., Capuano, B., Sexton, P.M., Javitch, J.A., Christopoulos, A. 2014. A new mechanism of allostery in a G protein-coupled receptor dimer. Nat. Chem. Biol. 10 (9), 745-752.

Accepted manuscript

ACCEPTED MANUSCRIPT

Maggio, R., Barbier, P., Toso, A., Barletta, D., Corsini, G.U., 1995. Sodium nitroprusside induces internalization of muscarinic receptors stably expressed in Chinese Hamster Ovary cell lines. J. Neurochem. 65 (2), 943-946.

Maggio, R., Innamorati, G., Parenti, M., 2007. G protein-coupled receptor oligomerization provides the framework for signal discrimination. J. Neurochem. 103 (5), 1741-1752.

Maggio, R., Millan, M.J., 2010. Dopamine D2-D3 receptor heteromers: pharmacological properties and therapeutic significance. Curr. Opin. Pharmacol. 10 (1), 100-107.

Maggio, R., Scarselli, M., Capannolo, M., Millan, M.J., 2015. Novel dimensions of D3 receptor function: Focus on heterodimerisation, transactivation and allosteric modulation. Eur. Neuropsychopharmacol. 25 (9), 1470-1479.

Maggio, R., Scarselli, M., Novi, F., Millan, M.J., Corsini, G.U., 2003. Potent activation of dopamine D3/D2 heterodimers by the antiparkinsonian agents, S32504, pramipexole and ropinirole. J Neurochem 87 (3), 631-641.

May, L.T., Bridge, L.J., Stoddart, L.A., Briddon, S.J., Hill, S.J., 2011. Allosteric interactions across native adenosine-A3 receptor homodimers: quantification using single-cell ligand-binding kinetics. FASEB J. 25 (10), 3465-3476.

Marsango, S., Caltabiano, G., Jiménez-Rosés, M., Millan, M.J., Pediani, J.D., Ward, R.J., Milligan, G., 2017. A molecular basis for selective antagonist destabilization of dopamine D3 receptor quaternary organization. Sci. Rep. 7 (1), 2134.

Marsango, S., Caltabiano, G., Pou, C., Varela Liste, M.J., Milligan, G., 2015. Analysis of human dopamine D3 receptor quaternary structure. J. Biol. Chem. 290 (24), 15146-15146.

Millan, M.J., Andrieux, A., Bartzokis, G., Cadenhead, K., Dazzan, P., Fusar-Poli, P., Gallinat, J., Giedd, J., Grayson, D.R., Heinrichs, M., Kahn, R., Krebs, M.O., Leboyer, M., Lewis, D., Marin, O., Marin, P., Meyer-Lindenberg, A., McGorry, P., McGuire, P., Owen, M.J., Patterson, P., Sawa, A., Spedding, M., Uhlhaas, P., Vaccarino, F., Wahlestedt, C., Weinberger, D., 2016.

Accepted manuscript

ACCEPTED MANUSCRIPT

Altering the course of schizophrenia: progress and perspectives. Nat. Rev. Drug Discov. 15 (7), 485-515.

Millan, M.J., Dekeyne, A., Rivet, J.M., Dubuffet, T., Lavielle, G., Brocco, M., 2000a. S33084, a novel, potent, selective, and competitive antagonist at dopamine D(3)-receptors: II. Functional and behavioural profile compared with GR218,231 and L741,626. J. Pharmacol. Exp. Ther. 293 (3), 1063-1073.

Millan, M.J., Gobert, A., Newman-Tancredi, A., Lejeune, F., Cussac, D., Rivet, J.M., Audinot, V., Dubuffet, T., Lavielle, G., 2000b. S33084, a novel, potent, selective, and competitive antagonist at dopamine D(3)-receptors: I. Receptorial, electrophysiological and neurochemical profile compared with GR218,231 and L741,626. J. Pharmacol. Exp. Ther. 293 (3), 1048-1062.

Millan, M.J., Mannoury, la Cour, C., Novi, F., Maggio, R., Audinot, V., Newman-Tancredi, A., Cussac, D., Pasteau, V., Boutin, J.A., Dubuffet, T., Lavielle, G., 2008. S33138 [N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro[1]-benzopyrano[3,4-c]pyrrol-2(3H)-yl)-ethyl]phenylacetamide], a preferential dopamine D3 versus D2 receptor antagonist and potential antipsychotic agent: I. Receptor-binding profile and functional actions at G-protein-coupled receptors. J. Pharmacol. Exp. Ther. 324 (2), 587-599.

Mistry, S.N., Shonberg, J., Draper-Joyce, C.J., Klein, Herenbrink, C., Michino, M., Shi. L., Christopoulos, A., Capuano, B., Scammells, P.J., Lane, J.R., 2015. Discovery of a Novel Class of Negative Allosteric Modulator of the Dopamine D2 Receptor Through Fragmentation of a Bitopic Ligand. J. Med. Chem. 58 (17), 6819-6843.

Nimchinsky, E.A., Hof, P.R., Janssen, W.G., Morrison, J.H., Schmauss, C., 1997. Expression of dopamine D3 receptor dimers and tetramers in brain and in transfected cells. J. Biol. Chem. 272 (46), 29229-29237.

Novi, F., Scarselli, M., Corsini, G.U., Maggio, R., 2003. An unusual form of the association binding kinetics of N-[^{3}H]methylscopolamine to the split muscarinic M2trunk/M2tail receptor. J. Pharmacol. Exp. Ther. 305 (2), 786-795.

Accepted manuscript

ACCEPTED MANUSCRIPT

Pich, E.M., Collo, G., 2015. Pharmacological targeting of dopamine D3 receptors: Possible clinical applications of selective drugs. Eur. Neuropsychopharmacol. 25 (9), 1437-1447.

Picchietti, S., Belardinelli, M., Taddei, A.R., Fausto, A.M., Pellegrino, M., Maggio, R., Rossi, M., Giorgi, F., 2009. Thyroid disruptor 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) prevents internalization of TSH receptor. Cell Tissue Res. 336 (1), 31-40.

Pou, C., Mannoury, la Cour, C., Stoddart, L.A., Millan, M.J., Milligan, G., 2012. Functional homomers and heteromers of dopamine D2L and D3 receptors co-exist at the cell surface. J. Biol. Chem. 287 (12), 8864-8878.

Rossi, M., Fasciani, I., Marampon, F., Maggio, R., Scarselli, M., 2017. The first negative allosteric modulator for dopamine D2 and D3 receptors SB269652 may lead to a new generation of antipsychotic drugs. Mol. Pharmacol. 91 (6), 586-594.

Scarselli, M., Annibale, P., Gerace, C., Radenovic, A., 2013. Enlightening G-protein-coupled receptors on the plasma membrane using super-resolution photoactivated localization microscopy. Biochem. Soc. Trans. 41 (1), 191-196.

Scarselli, M., Annibale, P., McCormick, P.J., Kolachalam, S., Aringhieri, S., Radenovic, A., Corsini, G.U., Maggio, R., 2016. Revealing G-protein-coupled receptor oligomerization at the single-molecule level through a nanoscopic lens: methods, dynamics and biological function. FEBS J. 283 (7), 1197-217.

Scarselli, M., Armogida, M., Chiacchio, S., DeMontis, M.G., Colzi, A., Corsini, G.U., Maggio, R., 2000. Reconstitution of functional dopamine D(2s) receptor by co-expression of amino- and carboxyl-terminal receptor fragments. Eur. J. Pharmacol. 397 (2-3), 291-296.

Scarselli, M., Novi, F., Corsini, G.U., Maggio, R., 2003. Functional rescue of the inactive splice variant of the dopamine D3 receptor D3nf. Brain Res. 987 (2), 244-247.

Scarselli, M., Novi, F., Schallmach, E., Lin, R., Baragli, A., Colzi, A., Griffon, N., Corsini, G.U., Sokoloff, P., Levenson, R., Vogel, Z., Maggio, R., 2001. D2/D3 dopamine receptor heterodimers exhibit unique functional properties. J. Biol. Chem. 276 (32), 30308-30314.

Accepted manuscript

ACCEPTED MANUSCRIPT

Shivanandan A, Deschout H, Scarselli M, Radenovic A., 2014. Challenges in quantitative single molecule localization microscopy. FEBS Lett. 588 (19), 3595-602.

Shonberg, J., Draper-Joyce, C., Mistry, S.N., Christopoulos, A., Scammells, P.J., Lane, J.R., Capuano, B., 2015. Structure-activity study of N-((trans)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1H)-yl)ethyl)cyclohexyl)-1H-indole-2-carboxamide (SB269652), a bitopic ligand that acts as a negative allosteric modulator of the dopamine D2 receptor. J. Med. Chem. 58 (13), 5287-5307.

Silvano, E., Millan, M.J., Mannoury, la Cour, C., Han, Y., Duan, L., Griffin, S.A., Luedtke, R.R., Aloisi, G., Rossi, M., Zazzeroni, F., Javitch, J.A., Maggio, R., 2010. The tetrahydroisoquinoline derivative SB269,652 is an allosteric antagonist at dopamine D3 and D2 receptors. Mol. Pharmacol. 78 (5), 925-934.

Smith, N.J., Milligan, G., 2010. Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes. Pharmacol. Rev. 62 (4), 701-725.

Sokoloff, P., Andrieux, M., Besancon, R., Pilon, C., Martres, M.P., Giros, B., Schwartz, J.C., 1992. Pharmacology of human dopamine D3 receptor expressed in a mammalian cell line: comparison with D2 receptor. Eur. J. Pharmacol. 225 (4), 331-337.

Sokoloff, P., Le Foll, B., 2017. The dopamine D3 receptor, a quarter century later. Eur. J. Neurosci. 45 (1), 2-19.

Sukalovic, V., Soskic, V., Kostic-Rajacic, S., 2015. Modeling of Dopamine D2 Receptor - Overview of 35-Year Evolution. Curr. Med. Chem. 22 (25), 2972-2990.

Vaidehi, N., Bhattacharya, S., Larsen, A.B., 2014. Structure and dynamics of G-protein coupled receptors. Adv. Exp. Med. Biol. 796, 37-54.

Accepted manuscript

ACCEPTED MANUSCRIPT

Tables

Table 1. B_{max} values of [^{3}H]SB269,652, [^{3}H]Nemonapride and [^{3}H]SCH23390 for wild type and dopamine receptor mutants stably and transiently expressed in CHO and COS-7 cells. B_{max} values of N-[^{3}H]metylscopolamine for the wild type muscarinic M_4 receptor expressed in CHO cells.

Receptor	**[^{3}H]SB269,652 (B_{max} fmol/mg)**	**[^{3}H]Nemonapride (B_{max} fmol/mg)**	**[^{3}H]SCH23390 (B_{max} fmol/mg)**
CHO-D_3	5,204 ± 138 (3)	5,456 ± 120 (3)	
CHO-D_{2L}	N.D. (4)	3312 ± 227 (3)	
CHO-D_1	N.D. (3)		1739 ± 273 (3)
COS-7 – D_4	N.D. (4)	902 ± 130 (3)	
COS-7 – D_3D110A	N.D. (7)	N.D. (4)	
COS-7 – D_3-trunk	N.D. (2)	N.D. (2)	
COS-7 – D_2-trunk	N.D. (2)	N.D. (2)	
COS-7 – D_3D110A + D_3-trunk	71.3 ± 13.4 (4)	87.5 ± 11.7 (4)	
COS-7 – D_3D110A + D_2-trunk	N.D. (4)	63.4 ± 7.91 (4)	
CHO-M_4	89.5 ± 13.7[a] (3)	[^{3}H]NMS (B_{max} fmol/mg) = 571 ± 22.3	

[a]This value represent the amount of specific [^{3}H]SB269,652 binding at a concentration of 10 nM. Next to this value is reported the B_{max} of CHO-M_4 cells calculated with [^{3}H]NMS. Positive values represent the mean ± S.E.M. of three or four replicates. The number of experiments per condition is shown in round brackets. N.D. = not detectable binding; [^{3}H]NMS = N-[^{3}H]methylscopolamine

Accepted manuscript

ACCEPTED MANUSCRIPT

Table 2. Binding parameters of inhibition isotherm of [^{3}H]SB269,652 binding to CHO-D_3 cell membranes. Values are expressed as means ± S.E.M. of three experiments. Competition curves were analyzed by a non-linear regression according to models assuming a single or a double class of binding sites. The best fit of two sites as compared to one site model was evaluated by F-test, utilizing normalized data from three experiments.

Radioligand	**[^{3}H]SB269,652**
S33084 $IC_{50}1$ [nM]	0.27 ± 0.06
S33084 $IC_{50}2$ [nM]	44.5 ± 26.4
$B_{max}1$ % = 100 - $B_{max}2$ %	43.6
$B_{max}2$ %	56.4 ± 4.69
Hill Coefficient	0.58 ± 0.06
$F_{2,42} = 38.19 \rightarrow P < 0.001$	
Haloperidol $IC_{50}1$ [nM]	3.4 ± 1.68
Haloperidol $IC_{50}2$ [nM]	151 ± 57.4
$B_{max}1$ % = 100 - $B_{max}2$ %	43.7
$B_{max}2$ %	56.4 ± 9.15
Hill Coefficient (one site)	0.64 ± 0.06
$F_{2,39} = 10.09 \rightarrow P < 0.001$	
Dopamine $IC_{50}1$ [µM]	0.55 ± 0.2
Dopamine $IC_{50}2$ [µM]	10,7 ± 9,39
$B_{max}1$ % = 100 - $B_{max}2$ %	51.4
$B_{max}2$ %	48.6 ± 14.5
Hill Coefficient (one site)	0.78 ± 0.05
$F_{2,30} = 8.13 \rightarrow P < 0.01$	

Accepted manuscript

ACCEPTED MANUSCRIPT

Table 3. Binding parameters of [^{3}H]SB269,652 kinetics at CHO-D_3 cell membranes. Values are expressed as means ± S.E.M. of three experiments. Association and dissociation time courses were best fitted by a non-linear regression analysis according to a biexponential model. The best fit of the biexponential as compared to the monoexponential model was evaluated by F-test, utilizing normalized data from three experiments.

Kinetic parameters of [^{3}H]SB269,652 on CHO-D_3 cells		**Binding (%)**
K_{off} [min^{-1}] (dilution)	0.03 ± 0.002	
$K_{off}1$ [min^{-1}] (dilution + excess haloperidol)	0.24 ± 0.09	19.7
$K_{off}2$ [min^{-1}] (dilution + excess haloperidol)	0.02 ± 0.001	80.3
$F_{2,27} = 14.06 \rightarrow P < 0.0001$		
$K_{off}1$ [min^{-1}] (dilution + excess dopamine)	0.42 ± 0.09	41
$K_{off}2$ [min^{-1}] (dilution + excess dopamine)	0.03 ± 0.003	59
$F_{2,27} = 33.43 \rightarrow P < 0.0001$		
$K_{obb}1$ [min^{-1}] (in presence of 1.6 nM [^{3}H]SB269,652)	0.55 ± 0.41	24.2
$K_{obb}2$ [min^{-1}] (in presence of 1.6 nM [^{3}H]SB269,652)	0.04 ± 0.01	75.8
$F_{2,30} = 10.99 \rightarrow P = 0.0003$		

Accepted manuscript

ACCEPTED MANUSCRIPT

Table 4. pIC_{50} values of SB269,652 at several human GPCRs stably expressed in recombinant cell lines.

Receptor	**Cell line**	**Radioligand (concentration)**	**pIC_{50}**
Human adenosine A_1	CHO	[^{3}H]DPCPX (1 nM)	<5
Human adenosine A_{2A}	HEK-293	[^{3}H]CGS-21680 (6 nM)	<5
Human adrenergic α_{1A}	CHO	[^{3}H]Prazosin (0.3 nM)	<5
Human adrenergic α_{1B}	CHO	[^{3}H]Prazosin (0.3 nM)	<5
Human adrenergic α_{1D}	CHO	[^{3}H]Prazosin (0.2 nM)	<5
Human adrenergic α_{2A}	CHO	[^{3}H]RX821,002 (0.8 nM)	<5
Human adrenergic α_{2B}	CHO	[^{3}H]RX821,002 (4.0 nM)	<5
Human adrenergic α_{2C}	CHO	[^{3}H]-RX821,002 (0.6 nM)	<5
Human adrenergic β_1	Sf9	[^{3}H]CGP12,177 (0.15 nM)	<5
Human adrenergic β_2	Sf9	[^{3}H]CGP12,177 (0.15 nM)	<5
Human histamine H_1	HEK-293	[^{3}H]pyrilamine (1 nM)	<5
Human histamine H_2	CHO	[^{125}I]APT (0.075 nM)	<5
Human serotonin $5HT_{1A}$	CHO	[^{3}H]8-OH-DPAT (0.4 nM)	5.44
Human serotonin $5HT_{1B}$	CHO	[^{3}H]GR125,743 (1.0 nM)	<5
Human serotonin $5HT_{1D}$	CHO	[^{3}H]GR125,743 (1.0 nM)	<5
Human serotonin $5HT_{2A}$	CHO	[^{3}H]Ketanserin (0.5 nM)	<5
Human serotonin $5HT_{2B}$	CHO	[^{3}H]Mesulergine (1.0 nM)	<5
Human serotonin $5HT_{2C}$	CHO	[^{3}H]Mesulergine (1.0 nM)	<5
Human serotonin $5HT_4$	CHO	[^{3}H]GR113,808 (0.1 nM)	<5
Human serotonin $5HT_{5A}$	HEK-293	[^{3}H]LSD (1.0 nM)	<5
Human muscarinic M_1	CHO	[^{3}H]Pirenzepine (2.0 nM)	<5

Accepted manuscript

ACCEPTED MANUSCRIPT

Human muscarinic M_2	CHO	$[^{3}H]$AF-DX 384 (2.0 nM)	6.67
Human muscarinic M_3	CHO	$[^{3}H]$4-DAMP (0.2 nM)	5.8
Human muscarinic M_4	CHO	$[^{3}H]$4-DAMP (0.2 nM)	7.22
Human muscarinic M_5	CHO	$[^{3}H]$4-DAMP (0.3 nM)	5.77
Human dopamine D_1	CHO	$[^{3}H]$SCH23,390 (0.3 nM)	<5
Human dopamine D_{2L}	CHO	$[^{3}H]$Spiperone (0.5 nM)	6.43
Human dopamine D_3	CHO	$[^{3}H]$Spiperone (0.5 nM)	8.63
Human dopamine D_4	CHO	$[^{3}H]$Spiperone (0.4 nM)	<5
Human dopamine D_5	GH4	$[^{3}H]$SCH23,390 (0.3 nM)	<5

Accepted manuscript

Figure legends

Fig. 1. (A) saturation and (B, C and D) inhibition binding isotherms of [^{3}H]SB269,652 to membranes form CHO-D$_3$ cells. Concentrations of [^{3}H]SB269,652 in saturation experiments ranged from 0.05 nM to 5.5 nM (from 2860 to 314600 dpm for sample), while its concentration in competition experiments was 1.6 nM (91520 dpm per sample). In the saturation experiment, at 5.5 nM concentration of [^{3}H]SB269,652, the total binding was 5419 dpm while nonspecific binding was 1244 dpm, and the amount of proteins added in each sample was ~15 μg. Competition curves were fitted by a non-linear regression according to models assuming a single (dashed line) or two (solid line) binding sites. Graphs are representative of a single experiment out of three, each performed in triplicate (bars represent the S.D. of each triplicate determination). Spec. and Unsp. refer to specific and unspecific binding in the absence and presence of 2 mM dopamine set at 100% and 0%, respectively.

Fig. 2. Dissociation (A, B and C) and association (D) binding kinetics of [^{3}H]SB269,652 to dopamine D$_3$ receptor. For calculation of dissociation rate constants, 20 μl of membranes were preloaded with 5 nM [^{3}H]SB269,652 for 2 h and then diluted 100 times in the absence or in the presence of 100 μM haloperidol or 2 mM dopamine. The apparent association-rate constant (K_{obb}) was calculated in the presence of 1.6 nM [^{3}H]SB269,652. Data were fitted by a non-linear regression according to models assuming a monoexponential (dashed line) or biexponential (solid line) kinetics. Graphs are representative of a single experiment our of three, each performed in triplicate (bars represent the S.D. of each triplicate determination).

Fig. 3. Saturation binding of [^{3}H]SB269,652 to membranes from striatal synaptosomes of wild type and dopamine D$_3$ receptor (D$_3$–/–) knockout mice. Concentrations of [^{3}H]SB269,652 in saturation experiments ranged from 0.038 to 3.08 nM (from 2174 to 176176 dpm per sample). At 3.08 nM concentration of [^{3}H]SB269,652, the total binding was 5828 dpm while nonspecific

Accepted manuscript

ACCEPTED MANUSCRIPT

binding was 5118 dpm, and the amount of proteins added in each sample was ~190 µg. Data were fitted by a non-linear regression according to models assuming a single population of binding sites. The graph is representative of a single experiment out of four, each performed in triplicate (bars represent the S.D. of each triplicate determination).

Fig. 4. Influence of SB269,652 and SB277,011A upon quinpirole-induced inhibition of forskolin-stimulated ACVI activity at dopamine D_2 receptor and co-transfected dopamine D_2/D_3 receptor.

COS-7 cells were single transfected or co-transfected with dopamine D_2 and D_3 receptors together with ACVI. The amount of plasmids co-transfected in µg/dish were: a) D_2/ACVI (0.5/1); b) D_3/ACVI (2.5/1); c) D_2/D_3/ACVI (0.5/2.5/1). In a and b the amount of transfected plasmids was made up to 4 µg with empty pcDNA. In panel A, quinpirole inhibited forskolin stimulated ACVI only in dopamine D_2 receptor and co-transfected dopamine D_2/D_3 receptor cells, while no effect was observed in dopamine D_3 receptor transfected cells. In panel B, SB269,652 inhibited only moderately quinpirole-induced inhibition of forskolin-stimulated ACVI activity in dopamine D_2 receptor transfected cells, while it gained efficiency and potency in presence of dopamine D_2 and dopamine D_3 receptors. In panel C, SB277,011A completely inhibited quinpirole-induced inhibition of forskolin-stimulated ACVI activity in both cells transfected with dopamine D_2 receptor and co-transfected with dopamine D_2 and D_3 receptors.

Fig. 5. Schematic representation of the binding of SB269,652 at dopamine D_3 receptor monomers and dimers. SB269,652 is represented with its three main moities, the 7CN-THIQ group (blue), the transcyclohexylene spacer in the middle and the indole-2-carboxamide tail (yellow). In the upper left part of the schema, SB269,652 is shown to bind allosterically to a ligand pre-occupied monomer thus preventing the dissociation of the orthosteric ligand (dopamine; red) or, upper right part, to bind in a bitopic mode to the orthosteric (Orth) and allosteric (All 1) sites, and

Accepted manuscript

ACCEPTED MANUSCRIPT

competing with the orthosteric ligand in the monomer. Binding to the sole allosteric site in the pre-occupied receptor monomer has probably very low affinity and is not detectable in radioligand binding, as demonstrated by the lack of [^{3}H]SB269,652 binding of the dopamine D_3D110A receptor mutant. Nevertheless, binding to the sole allosteric site in pre-occupied receptors can be inferred by our previous experiment with cold SB269,652, where we demonstrated that this compound decreases the dissociation speed of [^{3}H]nemonapride from dopamine D_3 receptor (Silvano et al., 2010). This binding mode would be unfavourable respect to the bitopic binding mode, and it would occur only when the receptor is pre-occupied and at high concentrations of SB269,652. This would justify why we did not see any binding of [^{3}H]SB269,652 to the D3D110A mutant. Furthermore, it is possible that this configuration could be stabilized by the 7CN-THIQ group engaging another allosteric site (All 2). In the lower part of the cartoon, SB269,652 is shown to bind in a bitopic mode to one protomer of the dopamine dimer, and to exert an allosteric effect across dimer on the orthosteric ligand bound to the other protomer (Lane et al., 2014). Since the negative allosteric effect between the two compounds localised on the two different protomers of the dimer is reciprocal, the orthosteric ligand would increase the dissociation of SB269,652 through the dimer. Recognition of a single population of binding sites by [^{3}H]SB269,652 in saturation experiments and dissociation kinetics (when dissociation was started by dilution), together with comparable B_{max} values of [^{3}H]SB269,652 and [3H]nemonapride at dopamine D_3 receptor, suggest that binding of SB269,652 to one protomer of a dimer would not alter the binding of other molecules of SB269,652 to the other protomer.

ACCEPTED MANUSCRIPT

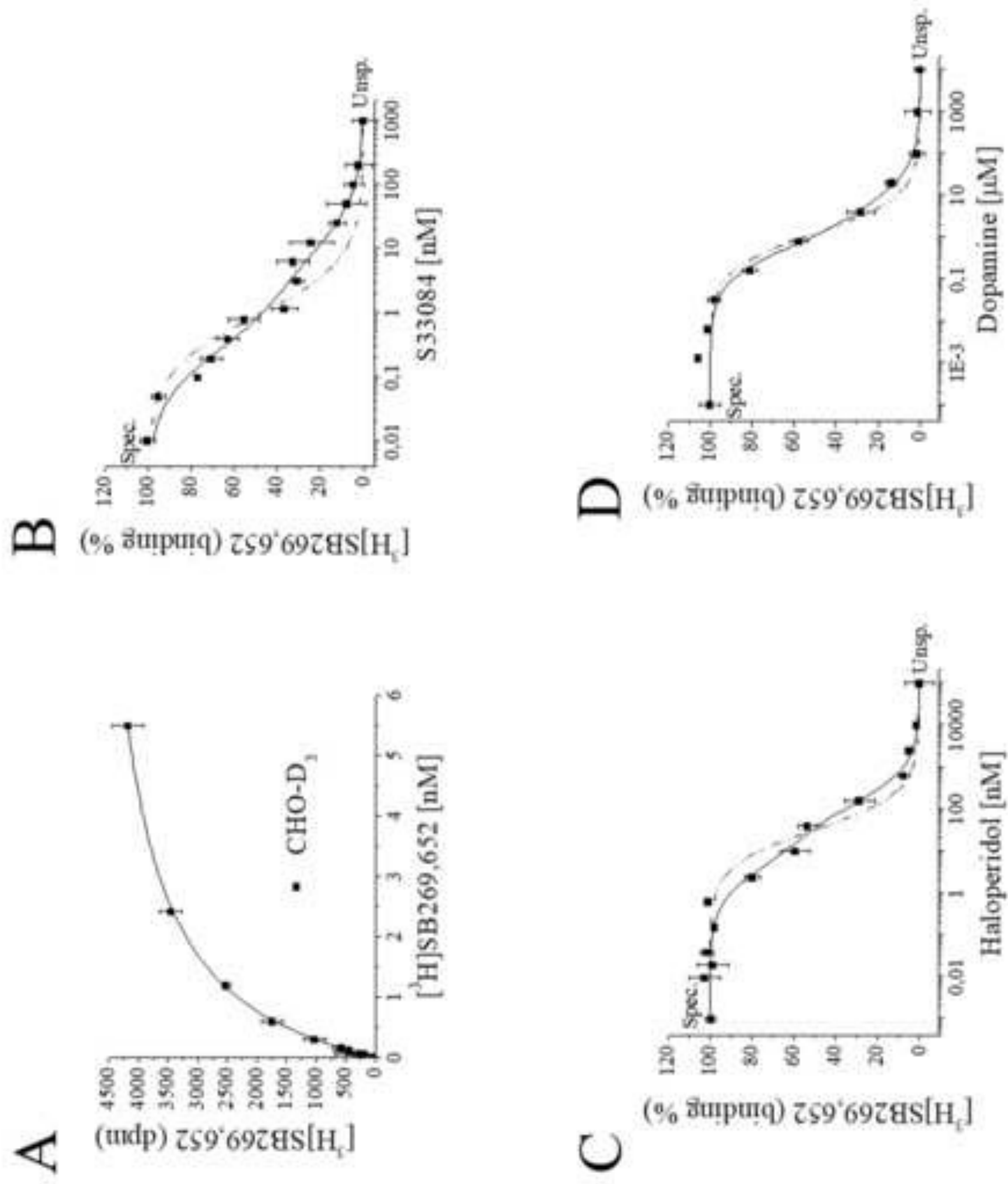

Figure 1

ACCEPTED MANUSCRIPT

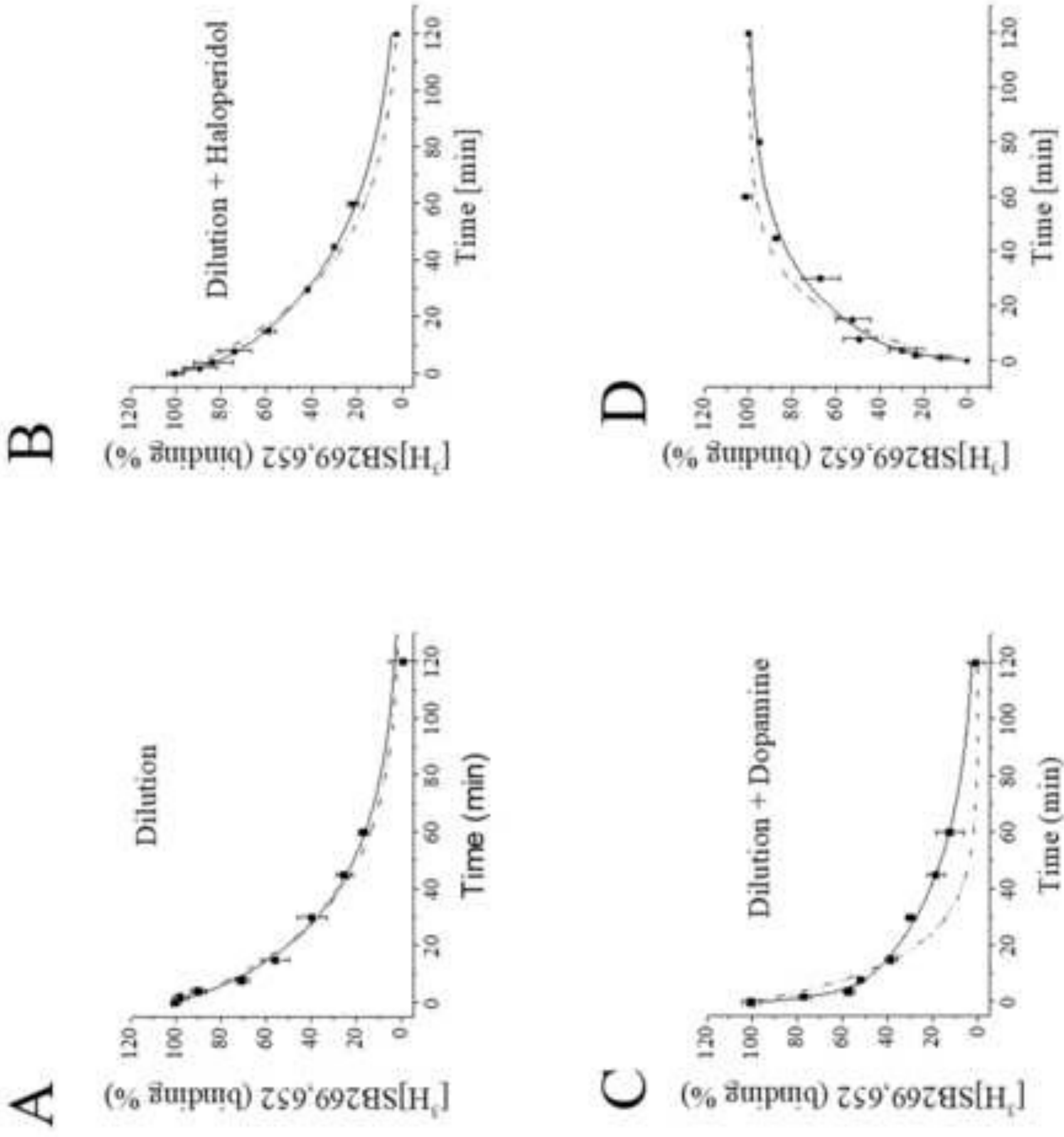

Figure 2

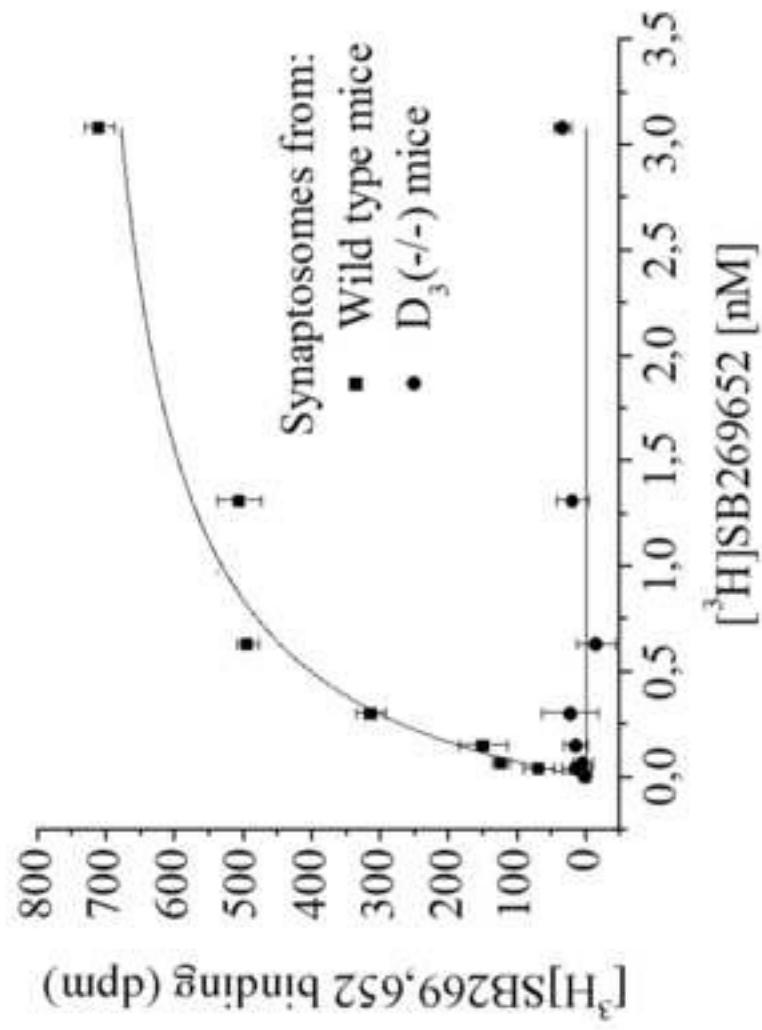

Figure 3

Figure 4

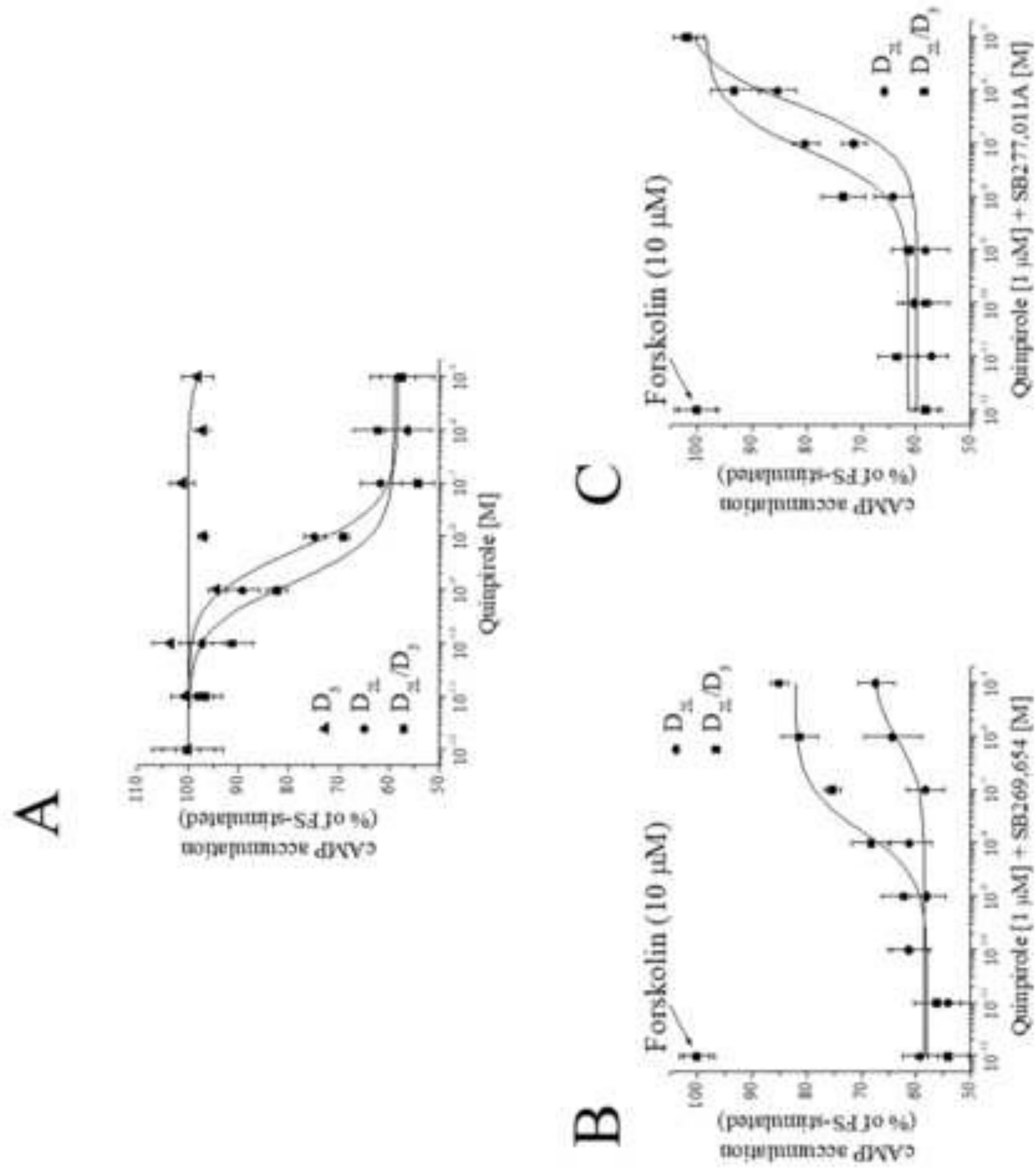

Figure 5