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The clinical efficacy of aripiprazole in the treatment of psychosis relies on a partial agonism at D2 receptors. As the expression of this receptor differs physiologically between pre- and post-synaptic sites and is affected by pathological conditions or pharmacological treatments, it appears difficult to predict the clinical response to partial agonists. In addition, the response to this novel antipsychotic was shown to depend on the cell-line and the pathway analyzed, suggesting a functional selective profile at the D2 receptor. This study aims at examining the influence of receptor density and ionic environment on the pharmacological properties of aripiprazole. A cell line was developed in which the expression of the recombinant D2 receptor can be tightly manipulated using doxycycline and sodium butyrate. The potency and efficacy of aripiprazole and other reference D2 receptor ligands were examined in [35S]GTPγS binding assays, in buffers containing either NaCl or N-methyl-D-gluc...
Increasing the density of the D$_2$L receptor and manipulating the receptor environment are required to evidence the partial agonist properties of aripiprazole

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Abstract

The clinical efficacy of aripiprazole in the treatment of psychosis relies on a partial agonism at D$_2$ receptors. As the expression of this receptor differs physiologically between pre- and post-synaptic sites and is affected by pathological conditions or pharmacological treatments, it appears difficult to predict the clinical response to partial agonists. In addition, the response to this novel antipsychotic was shown to depend on the cell-line and the pathway analyzed, suggesting a functional selective profile at the D$_2$ receptor. This study aims at examining the influence of receptor density and ionic environment on the pharmacological properties of aripiprazole. A cell line was developed in which the expression of the recombinant D$_2$ receptor can be tightly manipulated using doxycycline and sodium butyrate. The potency and efficacy of aripiprazole and other reference D$_2$ receptor ligands were examined in [35S]GTP$^\gamma$S binding assays, in buffers containing either NaCl or N-methyl-D-glucamine (NMDG) which is proposed to enhance G protein coupling. Increasing the density of D$_2$ receptors considerably enhanced the [35S]GTP$^\gamma$S binding induced by dopamine and the full agonist NPA. In maximally induced cells, the agonist properties of the partial agonist (−)-3-PPP was revealed in a buffer containing NaCl, whereas the response to aripiprazole was not evidenced. Substituting NMDG for NaCl promoted the response to dopamine and (−)-3-PPP and was proven efficient to reveal the partial agonist profile of aripiprazole. While NMDG substitution for NaCl strongly enhanced receptor-G protein coupling, these ionic manipulations are likely to influence receptor conformations, thereby modulating the activation of signaling pathways. Our data obtained with partial agonists acting at the D$_2$ receptor suggest that these changes in the experimental conditions could contribute to reveal the functional selective profile of GPCR ligands. They also emphasize that the properties of functional selective ligands do not only depend on receptor density but also on the surrounding environment which likely differs between brain structures.

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1. Introduction

The inhibition of dopamine transmission, through antagonism at D$_2$ receptors, is a key aspect in the pharmacological management of psychosis (Creese et al., 1976; Seeman et al., 1976). Side effects resulting from to this blockade can be balanced by either 5-HT$_2$A antagonism but also by a rapid dissociation from the D$_2$ receptor (Kapur and Seeman, 2001), two properties shared by atypical antipsychotics and that account for their atypical properties (Kapur and Seeman, 2001), among which aripiprazole, launched in 2002, is considered as the first member of this new class of antipsychotics (Burris et al., 2002). Nevertheless, even though the fine-tuning of dopamine transmission by these compounds is an attractive hypothesis to explain their clinical efficacy (Tammenga and Carlsson, 2002), the ideal intrinsic activity of these partial agonists remains unclear. Hence, the partial agonism of aripiprazole at dopamine receptors in native brain tissue remains a matter of debate (Jordan et al., 2007; Koener et al., 2011).

It is established that the intrinsic activity of a given partial agonist varies, depending on the density of targeted receptors in the tissue (Hermans et al., 1999; Watts et al., 1995). Accordingly, when focusing on the modulation of second messengers such as the inhibition of cAMP accumulation in cells expressing low levels of the D$_2$ receptor, aripiprazole and (S)-(-)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride ((−)-3-PPP) behave as antagonists whereas an agonist profile is clearly evidenced in models where the receptor density is high (Burris et al., 2002; Lawler et al., 1999; Tadori et al., 2005, 2009). In in vivo studies, aripiprazole was shown to display partial agonism at the presynaptic receptor and antagonism at the postsynaptic site (Kikuchi et al., 1995; Kiss et al., 2010; Momiyama et al., 1996; Semba et al., 1995), where high and low receptor reserves are commonly

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evidenced, respectively (Meller et al., 1986). In addition to the question of receptor density, the partial agonism of aripiprazole at dopamine receptors also depend on the cell line used and the functional response examined (Burris et al., 2002; Jordan et al., 2007; Shapiro et al., 2003; Urban et al., 2007). Thus, when examining the modulation of independent signaling pathways in a given cell type, large differences in the estimated efficacy and potency of aripiprazole were reported, suggesting that this drug is endowed with functional selective properties (Shapiro et al., 2003; Urban et al., 2007). Thus, when examining the modulation of independent signaling pathways in a given cell type, large differences in the estimated efficacy and potency of aripiprazole were reported, suggesting that this drug is endowed with functional selective properties (Shapiro et al., 2003; Urban et al., 2007). There is indeed accumulating evidence for the multiplicity of couplings of the majority of GPCRs, including dopamine receptors, which can be manipulated by such functional selective ligands (Bosier and Hermans, 2007; Hermans, 2003; Kilts et al., 2002; Mottola et al., 2002). Guanylyl

![Fig. 1. Inducible expression of D2L receptors.](image)

**Fig. 1.** Inducible expression of D2L receptors. The specific binding of [3H]spiperone was measured on homogenates from Hela-Tet-On D2L cells in NaCl binding buffer. A. [3H]spiperone (1 nM) binding experiments were conducted on cells cultured for 48 h in the presence of increasing concentrations of doxycycline ranging from 0 to 3.16 μg/mL. Shown is a representation of means with s.e.mean from a single experiment repeated three times independently in triplicate. B. The specific binding of [3H]spiperone (2 nM) was determined on cells cultured in the absence (open bars) or in the presence (closed bars) of 2 μg/mL of doxycycline for 48 h, with or without the addition of NaBu (0, 0.1, 1, 2 and 5 mM) during the last 24 h. Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate.

**Table 1** Pharmacodynamic parameters derived from [3H]spiperone and [35S]GTPγS binding assays on cells cultured in the presence of increasing concentrations of doxycycline. Shown are Kmax and KD values from the binding saturation-curves performed in NaCl and NMDG containing buffer. pEC50 values and Emax values were derived from the dopamine- and NPA-induced [35S]GTPγS binding. Data are expressed as means±s.e.mean for 3 independent experiments, each performed in triplicate. At single concentrations of doxycycline, parameters measured in buffers containing NaCl or NMDG were compared using unpaired Student's t-test. * p<0.05, ** p<0.01 and *** p<0.001 denote differences between values obtained in the two buffers; N.D.: not determined.
nucleotide binding assays, focusing on the first step following receptor activation is an alternative to the measure of downstream responses which may involve complex signaling crosstalks. However, probably due to low signal-to-noise ratios, the agonist properties of low intrinsic activity partial agonists have hardly been demonstrated by this method (Jordan et al., 2007; Koener et al., 2011), and necessitate optimization of experimental protocols. The aim of this study is to characterize the pharmacodynamic properties of partial agonists such as \((-\)3-PPP and aripiprazole in comparison to reference agonists, in a tetracycline-inducible mammalian cell line expressing the D2L receptor. Using \[^{35}\text{S}\text{GTP}^\gamma\text{S}\] binding assays, we herein present evidence that enhancing the density of the D2L receptor and manipulating the chemical environment differentially affect the properties of partial agonists.

2. Material and methods

2.1. Materials

Cell culture medium (Dulbecco’s Modified Eagle Medium) and geneticin (50 mg/mL Stock) were purchased from Gibco-Invitrogen (Merelbeke, Belgium). Bovine serum was purchased from Thermo-scientific (Tournai, Belgium). Hygromycin B (50 mg/mL Stock) was purchased from Roche (Brussels, Belgium). Doxycycline was purchased from Clontech (Westburg BV, Leusden, The Netherlands). Guanosine 5'-O-(\(\gamma\)-[\(\text{35}\text{S}\text{]}\text{thiotriphosphate}\) (\[^{35}\text{S}\text{GTP}\gamma\text{S}\]) (spec. act. of at least 1000 Ci mmol\(^{-1}\)), \[^{3}\text{H}\]spiperone (specific activity of 15 Ci mmol\(^{-1}\)), 96-well plate adapted-GF/B glass fiber filters (Unifilter® GF/B), Pico Fluor and Microscint 20 were purchased from Perkin Elmer (Zaventem, Belgium). Deep 96-well plates used for binding experiments (Masterblock) were purchased from Greiner (Wemmel, Belgium). Dopamine hydrochloride, \(R(-)\)-10,11-dihydroxy-N-n-propylnorapomorphine hydrochloride (NPA), \((-)\)-3-PPP, guanosine diphosphate (GDP), 5'-guanylylimidodiphosphate (Gpp (NH) p), sodium butyrate (NaBu) and domperidone, were purchased from Sigma Aldrich (Bornem, Belgium). Aripiprazole was obtained from Toronto Research Chemicals (Toronto, Canada). Plastic tubes and Glass fiber filters (GF/B) were purchased from filter Service (Eupen, Belgium) and Whatman (Maidstone, United Kingdom) respectively.

2.2. Generation of the inducible system

The cDNA encoding the mouse D2L receptor, kindly provided by Dr. Emiliana Borrelli (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) (Guiramand et al., 1995), was cloned into the NotI and EcoRV restriction sites of the pTRE expression vector (Clontech) and the sequence was confirmed by double strand DNA sequencing. This construct was used in stable transfection of Hela Tet-On cells using Lipofectamine™ reagent (Invitrogen), according to manufacturer’s instructions. After selection with G418 (500 μg/mL) and hygromycin (100 μg/mL), individual clones were isolated and further experiments were conducted with a single clone. This clone (Hela Tet-On-pTRE-D2L) was chosen considering a low basal \[^{3}\text{H}\]spiperone binding when grown in the absence of doxycycline and a
substantial and reproducible specific binding after exposure to this inducer. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium, supplemented with fetal bovine serum (10% v/v), 2% penicillin–streptomycin (Invitrogen), G418 (250 μg/ml) and hygromycin (50 μg/ml), at 37 °C in 5% CO₂. For binding studies, cells were plated in Petri dishes and grown in the absence of the selection antibiotics G418 and hygromycin. When indicated, the cultured medium was supplemented with doxycycline (0.01 to 3.16 μg/ml) for 48 h and NaBu (0.01 to 5 mM) for 24 h before harvesting the cells.

2.3. Membrane preparation

Confluent cells grown with or without doxycycline and NaBu were washed and scraped from the culture dishes with ice-cold phosphate buffered saline (PBS). The cells were centrifuged and the pellet was frozen at −80 °C until use. The day of the experiment, the cell pellet was thawed and homogenized after 10 passages through a 26 gauge needle in 50 mM Tris–HCl (pH 7.4). The cell membranes were collected by centrifugation (49,000 g) at 4 °C and this homogenization/centrifugation procedure was repeated twice. The final pellet was resuspended in a minimal volume of 50 mM Tris–HCl (pH 7.4) and protein concentration was determined by the method of Bradford.

2.4. [3H]spiperone binding assay

Cell membranes (20–40 μg of protein) were incubated with [3H]spiperone (0.25 nM in competition studies or 0.0625 to 2 nM in saturation binding studies) in a binding buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 150 mM NaCl, 1 mM dithiotreitol, and 0.1% sodium metabisulfite. In some experiments, NaCl was replaced by 100 mM NMDG and sodium metabisulfite was omitted. Non-specific binding was determined in the presence of domperidone (1 μM). In competition experiments, the competitors (dopamine, aripiprazole, haloperidol and (−)-3-PPP) and the G protein disruptor Gpp (NH) p were added to [3H]spiperone in the wells, before initiating the binding by the addition of the membranes.

Incubation was performed for 60 min at 30 °C, and was terminated by addition of ice-cold washing buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 150 mM NaCl or 100 mM NMDG) and rapid filtration followed by two washes. Binding assays were performed in a total volume of 500 μL in deep 96-well plates and bound radioactivity was measured after filtration through 96-well plate adapted-GF/B glass fiber filters. Microscint 20 (40 μL) was added to each well of the filter plates for saturation experiments while for the competition experiments, and counting was performed by a liquid scintillation counter (Topcount® Microplate scintillation and luminescence counter, Perkin Elmer).

2.5. [35S]GTPγS binding assay

The binding experiments were performed in deep 96-well plates. Each well contained 10–25 μg protein, resuspended in a final volume of 500 μL. Binding buffer contained 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 150 mM NaCl, 10 μM GDP, 1 mM dithiotreitol, and...
0.1% sodium metabisulfite. When indicated, NaCl was substituted by 100 mM NMDG and sodium metabisulfite was omitted. In all experiments, [35S]GTPγS, at the final concentration of 0.1 nM was added to each well, before initiating the binding by addition of the membrane suspension. Incubation was performed at 30 °C for 40 min and terminated by the addition of 1 mL ice-cold washing buffer (Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl2, with either 150 mM NaCl, or 100 mM NMDG). The non-specific binding was measured in the presence of 0.1 mM Gpp (NH)p. The suspension was immediately filtered through 96-well plate adapted-GF/B glass fiber filters, and washed three times with the washing buffer. After overnight drying, Microscint 20 (40 μL) was added to each well and after 24 h, plates were counted with a Topcount® Microplate scintillation and luminescence counter.

2.6. Data analysis

Data were analyzed by non-linear regression, using the curve-fitting software GraphPad Prism (GraphPad Software, CA, USA). Values derived from concentration–response curves where compared using one-way ANOVA followed by Tukey’s test for multiple comparison when comparing all data sets, or by Dunnett’s test for multiple comparison when comparing data to a control value. Unpaired Student’s t-test was used when comparing two single values.

3. Results

3.1. Inducible expression of D2L receptors determined in [3H]spiperone binding

The expression of the D2L receptor was examined in cell membranes from Hela Tet-On-pTRE-D2L cells cultured for 48 h in medium supplemented with doxycycline. The binding of [3H]spiperone (1 nM) was barely detected in samples from non-induced cells and exposure to doxycycline resulted in a concentration-dependent increase in the radioligand specific binding (Fig. 1A) (significant induction with doxycycline concentrations equal or above 1 μg/mL). Saturation curves depicting [3H]spiperone specific binding (presented in Fig. 4A) revealed that increasing the receptor density was without significant influence on the estimated radioligand affinity (Table 1). At the highest concentration of inducer tested (3.16 μg/mL), Bmax value reached 12,130 ± 120 cpm/mg protein (corresponding to approximately 1 pmol/mg protein).

Combining doxycycline with the histone deacetylase inhibitor NaBu during the last 24 h of induction was previously shown to enhance the expression of the target gene in Tet-On inducible systems (Reeves et al., 2002) Additionally, addition of NaBu to the culture medium of Hela Tet-On-pTRE-D2L cells concentration-dependently enhanced the specific binding of [3H]spiperone induced by 2 μg/mL of doxycycline (Fig. 1B). While a robust effect was observed when combining NaBu with doxycycline (5 to 10 fold increased binding values as compared to values obtained with doxycycline alone), we noticed that at the highest concentration tested (5 mM, 24 h induction), NaBu alone significantly increased the binding of the radioligand (p<0.001).

3.2. Functional responses induced by D2 ligands in Hela Tet-On pTRE D2L cells

The pharmacological properties of agonists and partial agonists of the D2 receptor were examined in [35S]GTPγS binding assays performed on homogenates from cells expressing the receptor at different densities. The amplitude of the functional response induced by dopamine and NPA appeared closely correlated to the doxycycline concentration (Fig. 2A and B). Thus, when expressed as percentage above the basal nucleotide binding, the estimated maximal efficacy (Emax) of dopamine and NPA reached respectively from 7.9 to 77.2% and 7.7 to 61.7% in samples from cells exposed to 0.01 and 3.16 μg/mL doxycycline, respectively (Table 1). The estimated potency of both ligands was not influenced by the receptor expression level (Table 1). At variance with these reference agonists, the maximal efficacy of (−)-3-PPP and aripiprazole was unaffected by the manipulation of the receptor densities, as no significant induction of [35S]GTPγS binding by these ligands was observed at all doxycycline concentrations tested (Fig. 2C and D).

This characterization was pursued in cells where the receptor induction was further enhanced with NaBu. As expected, combining doxycycline and NaBu substantially amplified the response to dopamine (Fig. 3A). In these conditions, both the maximal response and the potency of dopamine were increased (Table 3). Thus at the highest concentration of NaBu tested, the estimated efficacy of dopamine reached 258.7±5.5% above basal and the potency (pEC50) was shifted to 6.12±0.06 (as compared to 5.29±0.12 in the absence of NaBu) (Table 3), likely reflecting the development of receptor reserve. In importance, in samples from cells where expression was boosted with NaBu, the partial agonist profile of (−)-3-PPP was revealed (Fig. 3C). Thus, addition of NaBu concentration-dependently induced the maximal response to this partial agonist, reaching 28.9±1.2% at the highest receptor density tested (Table 3). This reveals that the partial agonist profile of (−)-3-PPP depends on the density of the targeted receptor. Finally, even in these conditions combining doxycycline and NaBu, no significant functional response to aripiprazole was evidenced, thus failing to demonstrate the partial agonist properties of this drug. Hence, aripiprazole behaved similarly as the reference antagonist haloperidol of which properties were not affected in all conditions tested (Fig. 3D and B, respectively).

Fig. 4. Specific binding of [3H]spiperone on Hela-Tet-On-pTRE-D2L cells measured in NaCl and NMDG containing buffer. The specific binding of [3H]spiperone (0.05–2 nM) was measured on homogenates from Hela-Tet-On-pTRE-D2L cells in a buffer containing NaCl (A) and in buffer where NMDG was substituted for NaCl (B). Hela Tet-On D2L cells cultured for 48 h in the presence of increasing concentrations of doxycycline ranging from 0 to 3.16 μg/mL. Shown are representative of saturation binding curves (means with s.e.mean) from single experiments repeated three times independently in triplicate. The pharmacodynamic parameters derived from these curves are shown in Table 1.
3.3. Influence of NMDG on the functional responses induced by D2 ligands in Hela Tet-On pTRE D2L cells

Analysis of the pharmacodynamic parameters derived from [3H]spiperone saturation-binding curves revealed that the binding of this radiolabeled antagonist was not changed when NMDG was substituted for NaCl in the binding buffer (Fig. 4A and B). Thus, throughout the range of doxycycline concentrations tested, the Bmax values measured in the two buffers appeared not different (Table 1). Besides, a modest increase in the affinity of [3H]spiperone was measured in the NMDG containing buffer when examined on cells induced with high concentrations of doxycycline (Table 1).

As previously evidenced in rat striatal membranes (Koener et al., 2011), this change in buffer composition markedly increased the potency of dopamine in the [35S]GTPγS binding assay, an effect that was also observed for the other reference agonist NPA (Fig. 5 A and B). Such increase in the potency of the full agonists was not dependent on the density of receptors, as it was observed for all effective concentrations of doxycycline (Table 1). This effect was also evidenced when the expression of the receptor was further boosted by addition of NaBu during the induction phase (Fig. 6, Table 3).

At variance with the results obtained with the full agonists, switching to the NMDG containing buffer dramatically changed the apparent properties of the partial agonists (−)-3-PPP and aripiprazole (Fig. 5C and D). Thus, on samples from cells exposed to high concentrations of doxycycline (from 1 to 1.33 μg/mL and beyond) or further boosted with NaBu, both compounds efficiently promoted the specific binding of [35S]GTPγS (Tables 2 and 3). As documented for dopamine and NPA, increasing the receptor density was reflected in the estimated efficacy of aripiprazole and (−)-3-PPP (Fig. 6C and D, Table 3). At the highest density of receptors (doxycycline 2 μg/mL and NaBu 5 mM), the maximal responses of these two partial agonists reached approximately 70 and 160% above basal value for aripiprazole and (−)-3-PPP, respectively (a quarter or half of the response observed with dopamine). Finally, in these optimized conditions (high receptor density and NMDG containing buffer), no functional response to haloperidol was detected, confirming its antagonist profile (Fig. 6B).

3.4. Influence of NMDG on the displacement of [3H]spiperone binding by dopamine receptor ligands

The consequence of substituting NMDG for NaCl in the buffer on the properties of agonists, partial agonists and antagonists, was further examined in [3H]spiperone (0.25 nM) competition binding studies. These experiments were conducted on samples from cells induced with the combination of doxycycline (2 μg/mL) and NaBu (2 mM). The influence of G protein coupling on the ligand affinities was further examined using the stable GTP analog Gpp(NH)p, known to indirectly decrease the affinity of GPCR agonists, by disrupting G protein and the

Fig. 5. Characterization of the functional response induced by dopamine receptor ligands in NMDG containing buffer. The dopamine (A), NPA (B), (−)-3-PPP (C) and aripiprazole (D)-induced [35S]GTPγS binding was measured in homogenates of Hela-Tet-On-pTRE-D2L cells in a buffer where NMDG was substituted for NaCl. The experiment was conducted on sample from cells cultured for 48 h in the presence of different concentrations of doxycycline (0 to 3.16 μg/mL). Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. The pharmacodynamic parameters derived from these curves are detailed in Table 1 (for dopamine and NPA) and 2 (for (−)-3-PPP and aripiprazole).
subsequent ternary complex. In the absence of Gpp (NH)p, the curve depicting [3H]spiperone competition by dopamine in the NaCl containing buffer best fitted with a two-site model (Fig. 7A), with 35% of the receptors showing a high affinity (Table 4). In the NMDG containing buffer, this fraction was not changed significantly, but the calculated high and low affinities were substantially increased (Fig. 7B, Table 4). In the presence of Gpp (NH)p (100 μM), the competition curves for dopamine best fitted to a single-site model, in both NaCl and NMDG containing buffers, with an estimated pKi value corresponding to the low affinity sites (Table 4). Considering the above mentioned increased potency in the NMDG containing buffer, the K_i/EC_{50} ratio for dopamine was almost four times higher in this buffer as compared to the standard NaCl buffer (Table 4).

In accordance with its partial agonist properties validated in [35S]GTPγS binding studies in NMDG containing buffer, the curve for [3H]spiperone binding inhibition by (−)-3-PPP also best fitted with a two-site model (Fig. 7F, Table 4), with 17.26±6.57% of receptors in the high affinity state, and curves fitted to a one-binding site in the presence of Gpp (NH)p (Fig. 7F, Table 4). Consistent with the very modest partial agonist activity evidenced for (−)-3-PPP in [35S]GTPγS binding studies in NaCl buffer, the [3H]spiperone binding inhibition curve best fitted to a single-site model (Fig. 7E) in this buffer. It is however noteworthy that Gpp (NH)p slightly decreased the affinity of the ligand (Table 4). Additionally, as for dopamine, the K_i/EC_{50} ratio for (−)-3-PPP was substantially increased in NMDG compared to NaCl buffer.

As expected for an antagonist (Cordeaux et al., 2001; Neve, 1991), haloperidol displaced [3H]spiperone binding with concentration–inhibition curves fitting with a single-site model, both in the absence or in the presence of Gpp (NH)p (Fig. 7G and H, Table 4). Similarly, [3H]spiperone binding inhibition curves obtained with aripiprazole also best fitted with a single-site model in the absence or in the presence of Gpp (NH)p, in both NaCl and NMDG buffers (Fig. 7C and D), which contrasts with the partial agonist properties measured in [35S]GTPγS binding studies in NMDG buffer. The presence of Gpp (NH)p was without significant effect on the estimated affinity of aripiprazole (Table 4).

| Table 2 | Pharmacodynamic parameters derived from the concentration–response curves of (−)-3-PPP and aripiprazole in NMDG containing buffer. |
|----------------------|------------------------|------------------------|------------------------|------------------------|
| Dox (μg/mL) | pEC_{50} | E_{max} (% above basal) | pEC_{50} | E_{max} (% above basal) |
| CTRL | N.D. | N.D. | N.D. | N.D. |
| 0.01 | N.D. | N.D. | N.D. | N.D. |
| 0.10 | N.D. | N.D. | N.D. | N.D. |
| 0.21 | N.D. | N.D. | N.D. | N.D. |
| 0.46 | N.D. | 5.18±0.28 | N.D. | 23.5±3.0 |
| 1.00 | N.D. | 5.40±0.22 | N.D. | 25.9±2.5 |
| 1.33 | N.D. | 4.88±0.30 | N.D. | 31.7±4.8 |
| 1.78 | 7.76±0.28 | 5.77±0.23 | 13.7±1.6 | 27.5±2.5 |
| 2.37 | 8.15±0.37 | 5.13±0.27 | 9.6±1.5 | 25.5±3.0 |

Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. N.D.: not determined.
### 4. Discussion

It is well documented that the properties of GPCR agonists are dependent on the density of targeted receptor (Gazi et al., 1999; Hermans et al., 1999; Kenakian, 1997; Watts et al., 1995). Hence, the partial agonist profile of several D2 receptor ligands has been studied on cellular models expressing different densities of receptors. Thus, in cells expressing low levels of D2 receptors, aripiprazole and (−)-3-PPP behave as antagonists (Burris et al., 2002; Tadori et al., 2005, 2009), whereas when receptor density is high, both compounds efficiently decrease cAMP accumulation, at variance with antagonists which remain silent (Tadori et al., 2009). These observations encouraged us to develop a Tet-On based inducible system in which the expression of the D2 receptor can be tightly manipulated. Thus, increasing the concentration of the chemical inducer doxycycline in the culture medium of this cell model efficiently promoted the expression of the D2 receptor up to approximately 1100 fmol/mg protein, and addition of NaBu (Cook et al., 2008; Jerman et al., 2001) further boosted this induction by up to 6–7 folds. Focusing on the activation of G proteins, this study first emphasizes the receptor reserve dependent properties of the partial agonist (−)-3-PPP. Thus, in classical assay conditions (buffer containing NaCl), the agonist properties of (−)-3-PPP could not be detected on cells expressing low receptor levels, whereas partial agonism was clearly evidenced when examined on cells where expression was robustly induced with the combination of doxycycline and NaBu. Such increase in the relative efficacy of a partial agonist, as well as the increased potency of dopamine in maximally induced conditions is consistent with the theoretical concepts related to receptor reserve (Burris et al., 2002; Hermans et al., 1999; Jerman et al., 2001; Tadori et al., 2005, 2009). However, at variance with previous observations (Burris et al., 2002; Kenakian, 1997; Tadori et al., 2005, 2009), no significant response was evidenced for aripiprazole even at maximal receptor density. In these conditions, aripiprazole thus behaved like the reference antagonist haloperidol, indicating that increasing the receptor reserve is not sufficient to reveal its agonist profile.

The omission of sodium and its substitution by NMDG dramatically changed the response profile of all agonists tested and in particular revealed the partial agonist properties of aripiprazole. Thus, in these conditions, while aripiprazole did not elicit guanylyl nucleotide exchange in non-induced cells, its efficacy tended to increase with the receptor density. Also, substituting NMDG for NaCl noticeably modified the response profile of (−)-3-PPP, which behaved as a high-intrinsic activity agonist at high receptor density. As a mean to replace sodium in assay buffers, the use of NMDG in the functional evaluation of GPCR ligands has been already reported, including for dopamine receptors (Lin et al., 2006). However, the mechanism supporting the facilitation of the detection of G protein coupling in these conditions remains largely unknown. Herein, the omission of sodium from the buffer does not only promoted the efficacy of partial agonist, but also markedely affected the pharmacodynamic parameters determined for full agonists, as indicated by a leftward shift in the concentration–response curves systematically observed for dopamine and NPA. Through interaction with a highly conserved aspartate residue within the second transmembrane domain of the several GPCRs, sodium ions act as putative allosteric modulators influencing the affinity of orthosteric ligands endowed with an agonist profile (Emmerson et al., 2004; Neve, 1991; Neve et al., 2001; Pihlaviasto et al., 1998; Selent et al., 2010). Accordingly, the estimated affinity of dopamine was increased when omitting sodium ions in the assay buffer, an effect that was however not observed for (−)-3-PPP and aripiprazole. This putative allosteric influence of sodium ions on agonist binding appears insufficient to explain the leftward shift in concentration-curves of full agonists or the increased efficacy of partial agonists in the absence of sodium, as the K/EC50 ratio of both dopamine and (−)-3-PPP was significantly higher when assays where performed in the NMDG containing buffer. The K/EC50 ratio was previously referred as an index of receptor/G protein coupling (Cordeaux et al., 2001; Gardner et al., 1996; Lin et al., 2006). Hence, corroborating previous reports indicating that sodium ions decrease the G protein coupling of dopamine receptors (Schnell and Seifert, 2010; Selley et al., 2000), the present data obtained for dopamine and (−)-3-PPP indicate that omitting sodium ions reinforces this coupling, an effect that coincides with an apparent increase in the receptor reserve.

Despite the abundant literature related to the role of sodium ions on GPCR activation, the mechanism supporting this ionic influence remains largely debated. Hence, halides such as NaCl are thought to support the high affinity of GDP for certain subtypes of G proteins, partially impeding their activation, especially for compounds with a low intrinsic activity profile (Schnell and Seifert, 2010). This could explain how NMDG substitution for NaCl facilitates G protein activation by the partial agonists (−)-3-PPP and aripiprazole and increases the potency of full agonists, as already proposed (Lin et al., 2006). However, such increase in receptor/G protein coupling would also implicate an increased proportion of receptors in a high affinity state, an effect that was not evidenced for all ligands in [3H]spiperone binding inhibition studies. Although competition curves for (−)-3-PPP showed the occurrence of two agonist binding sites in NMDG compared to NaCl, the percentage of receptors in the high affinity state was equivalent in both buffers when using dopamine as competitor.

### Table 3

<table>
<thead>
<tr>
<th>Dopamine</th>
<th>(−)-3-PPP</th>
<th>Aripiprazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC50</td>
<td>Emax (% above basal)</td>
<td>pEC50</td>
</tr>
<tr>
<td>NaCl</td>
<td>NMDG</td>
<td>NaCl</td>
</tr>
<tr>
<td>Dox + NaBu 5 mM</td>
<td>6.12 ± 0.06**</td>
<td>258.7 ± 5.5***</td>
</tr>
<tr>
<td>Dox + NaBu 2 mM</td>
<td>5.82 ± 0.15**</td>
<td>180.5 ± 9.8**</td>
</tr>
<tr>
<td>Dox + NMDG 1 mM</td>
<td>5.98 ± 0.10**</td>
<td>127.3 ± 45.5**</td>
</tr>
<tr>
<td>Dox + NMDG 0.1 mM</td>
<td>5.77 ± 0.20**</td>
<td>48.2 ± 3.6</td>
</tr>
<tr>
<td>Dox + NMDG 2 μg/mL</td>
<td>5.29 ± 0.12</td>
<td>6.49 ± 0.34</td>
</tr>
<tr>
<td>CTRL</td>
<td>4.16 ± 0.87</td>
<td>4.95 ± 1.52</td>
</tr>
</tbody>
</table>

Shown are pEC50 and Emax values from the dopamine, (−)-3-PPP- and aripiprazole-induced [35S]GTPγS binding curves. Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. * p < 0.05 and *** p < 0.001 denote significant differences with values obtained on cells cultured with doxycycline alone (2 μg/mL) (one-way ANOVA, followed by Dunnett's test for multiple comparison). N.D. = Not determined.
Fig. 7. Effects of NMDG substitution for NaCl on the inhibition of [3H]spiperone binding by dopamine receptor ligands. [3H]spiperone (0.25 nM) binding was performed on homogenates of Hela-Tet-On-pTRE-D2L cells cultured for 48 h in the presence of doxycycline (2 μg/mL) and NaBu (2 mM) for the last 24 h. Competition curves for dopamine, aripiprazole, (−)-3-PPP and haloperidol, in NaCl buffer are represented respectively in A, C, E, G, and in NMDG in B, D, F, H. These experiments were performed in the absence (squares and full lines) or in the presence (triangle and dotted line) of Gpp (NH)₃ (100 μM). Data are shown as means with s.e.mean for 3 (4 for (−)-3-PPP) separate experiments performed in triplicate. Pharmacodynamic parameters derived from the curves are represented in Table 4.
Furthermore, despite its efficacy evidenced in the NMDG containing buffer, the $K_{i}/EC_{50}$ ratio for aripiprazole was particularly low and curves depicting $[^{3}H]$sipiperone binding competition best fitted to a single-binding site model in all conditions tested. Finally, despite an increase in basal $[^{35}S]$GTPγS binding in NMDG compared to NaCl (data not shown) indicating an enhancement in receptor/G-protein coupling, compounds with partial agonist activity as (−)-3-PPP should switch from being partial agonists to inverse agonists (so-called protean agonists), which was not the case. Together, these observations clearly imply that another biochemical mechanism supports the effects of substitution of NaCl by NMDG on the response to certain D$_2$ partial agonists. Beside changing the affinity of agonists, the interaction of sodium ions with the conserved aspartate residue within the transmembrane core of several GPCRs is likely to influence receptor conformations, thereby modulating the activation of selected signaling pathways (Schnell and Seifert, 2010; Selent et al., 2010). Thus, changing the assay buffer could indirectly alter the coupling of the receptor with certain G proteins while facilitating the interaction with alternative subtypes upon activation with appropriate ligands. Consistent with the concepts of multiplicity of coupling (Hermans, 2003) and functional selectivity (Bosier and Hermans, 2007), the D$_{2L}$ and D$_{25}$ receptors have been shown to activate distinct G proteins in an agonist dependent fashion (Cordeau et al., 2001; Gazi et al., 1999; Lane et al., 2007, 2008; Nickolls and Strange, 2003, 2004). While both receptor variants interact with G$_{oA}$, G$_{oB}$, G$_{oC}$, and G$_{oD}$ proteins, the long isoform shows preferential coupling to the G$_{oA}$ and G$_{oB}$ protein subtypes (Cordeau et al., 2001; Gazi et al., 1999; Guiramand et al., 1995). Indeed, some compounds including dopamine and (+)-3-PPP appeared to be more potent when the D$_{2L}$ receptor was co-expressed in Sf9 cells with G$_{oA}$ protein, as compared to combination with G$_{o}$ protein subtypes (Cordeau et al., 2001), an effect that was similarly observed when fusing the D$_{2L}$ Receptor with G$_{oA}$ protein compared to G$_{o}$ subtypes (Lane et al., 2007; Nickolls and Strange, 2004). It is noteworthy that a similar difference in the potency of dopamine was observed in this previous report when examining the coupling to either G$_{oA}$ and G$_{o}$ protein subtypes or in the present study when examining guanylyl nucleotide exchange in NMDG or NaCl containing buffers. Additionally, in Sf9 cells co-expressing G-proteins, or in models of protein fusion, (−)-3-PPP behaved as a partial agonist when combining the D$_{2L}$ receptor with G$_{oA}$ or sometimes G$_{oC}$ protein subtypes, whereas no functional response was elicited with G$_{oB}$ or G$_{oD}$ proteins (Gazi et al., 1999; Lane et al., 2007). This overview of the literature data strongly suggests that the presence of NMDG or the omission of NaCl in our experiments influences the balance of G$_{oA}$ and G$_{o}$ protein couplings of the D$_{2L}$ receptor or at least that changing the experimental conditions may facilitate the detection of discrete couplings. On that basis, our working hypothesis is that aripiprazole behaves as a functional selective ligand with an agonist profile toward a subset of G proteins interacting with the D$_2$ receptor. Evidencing this agonism in guanylyl nucleotide binding assays requires the use of experimental conditions that maximize such coupling or that minimize the coupling to other G proteins. Although, aripiprazole has never been studied on cells combining the expression of the D$_2$ receptor and a specific subtype of G protein, there is accumulating evidence that aripiprazole acts as a functional selective compound activating distinct signaling pathways with different efficacies and/or potencies (Berris et al., 2002; Shapiro et al., 2003; Urban et al., 2007). Considering that dopamine, NPA and (−)-3-PPP elicited responses in both NaCl and NMDG containing buffers, this hypothesis implies that these ligands concomitantly activate diverse couplings. However, the modifications in the potency or efficacy of these ligands evidenced when tested in the NMDG buffer suggest that these compounds also display some functional selective properties. Our data may suggest that substituting NMDG for NaCl could constitute a simple and complementary method to reveal the functional selective profile of GPCR ligands, in particular those with low intrinsic activity.

5. Conclusion

This study underlines that the type of assay used is crucial in order to establish the properties of such low intrinsic activity compounds (Jordan et al., 2007). This work also emphasizes that the receptor environment, including the subtype of protein partners, the ionic environment and the cell type used are extrinsic factors that can strongly modify the response to partial agonists of the D$_{2}$ receptors. Recombinant systems show several limitations, as they do not recapitulate the heterogeneity of native tissue, in which scaffolding elements, receptor clustering, receptor dynamic regulations and protein partners will differ. However, the physiological relevance of this study using an inducible model relies first on the existence of distinct levels of D$_{2}$ receptor in vivo, as receptor reserve is reported to be higher at the presynaptic (10 folds higher) than the postsynaptic sites (Meller et al., 1986; Yokoo et al., 1988). Additionally, pathological and pharmacological treatments (Geurts et al., 1999; Koener et al., 2011; Seeman, 2011) can affect D$_{2}$ receptor expression and sensitivity and may thereby influence the response to drug acting as partial agonists. Secondly, the environment dependent properties of these compounds suggest that their response profile differs between cerebral dopamine pathways. The understanding of the effects in different brain structures should bring further information on the multiple symptoms that are mainly targeted by these novel antipsychotics. Finally, our observation that the agonist properties of aripiprazole and (−)-3-PPP are largely influenced by NaCl suggests that the presence of NMDG or the omission of NaCl in our experiments influences the balance of G$_{oA}$ and G$_{o}$ protein couplings of the D$_{2L}$ receptor or at least that changing the experimental conditions may facilitate the detection of discrete couplings.
that their pharmacodynamic profile may depend upon neuronal resting or active status. In fact, intracellular neuronal levels of NaCl are low except during depolarization conditions. At low NaCl concentrations, the receptor will be in a highly-responsive state, a situation analogous to the experiments conducted with NMDG. When depolarization occurs, the influx of sodium would “switch off” the receptor and suppress agonist responses, a situation analogous to the conditions used in this study with NaCl (Selent et al., 2010). However, this hypothesis suggests that intracellular sodium concentrations change the ability of the receptor to signal. Whereas intra- or extracellular sodium concentrations influence GPCRs binding and signaling still remains a matter of debate (Selent et al., 2010). Further investigations need to be performed in order to understand the importance of sodium ions, in vivo, in the ability of GPCR to signal.

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