Inverse agonism at dopamine D\textsubscript{2} receptors: a receptor recalcitrant to high levels of constitutive activation

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Abstract

Neuroleptic drugs have been suggested to act as inverse agonists at the dopamine D\textsubscript{2} receptor. Nevertheless, the capacity with which inverse agonism at this receptor subtype can be resolved is limited. Modulation of the constitutive activation of the D\textsubscript{2} receptor was investigated in different cellular systems by monitoring either \[^{35}\text{S}\]GTP\textsubscript{yS} binding responses at mutant Thr\textsuperscript{343}Ser D\textsubscript{2short} receptor or inositol phosphates formation mediated by a chimeric D\textsubscript{2}/\alpha\textsubscript{1B} ICL receptor. A weak (about $-20\%$ vs. basal \[^{35}\text{S}\]GTP\textsubscript{yS} binding response) inverse agonist activity of putative dopamine antagonists (i.e., nemonapride, haloperidol or (+)-butaclamol) was observed with digitonin-permeabilized Chinese hamster ovary (CHO)-K\textsubscript{1} cells stably expressing a mutant Thr\textsuperscript{343}Ser D\textsubscript{2short} receptor only if a high (150 mM) KCl concentration was present in the binding buffer. No ligand-mediated decrease in basal \[^{35}\text{S}\]GTP\textsubscript{yS} binding was observed on membrane preparations of the same cells. Markedly increased inverse agonist responses were obtained with a series of dopamine antagonists by exchange of the D\textsubscript{2s} J10 receptor's 3ICL by that of the \alpha\textsubscript{1B}-adrenoceptor and incorporation of an activating mutation (Ala\textsuperscript{279}Glu) in the distal BBXXB motif of its 3ICL and by co-expression with a \text{Go}_{11} protein. This chimeric D\textsubscript{2}/\alpha\textsubscript{1B} receptor construct displayed a ligand binding profile comparable to that of the wt D\textsubscript{2short} receptor and an effector activation profile close to that of the wt \alpha\textsubscript{1P}-adrenoceptor. Most of the putative dopamine antagonists attenuated by $-54\%$ to $-59\%$ basal inositol phosphates (IP) formation, thus clearly acting as inverse agonists. Ziprasidone behaved virtually as a silent antagonist ($+5\%$ vs. basal IP level) and antagonized both dopamine (pK\textsubscript{B}: 7.61)-

Abbreviations: AR, adrenoceptor; CHO, Chinese hamster ovary; DA, dopamine; ICL, intracellular loop; IP, inositol phosphates; NPA, norpropylapomorphine; PLC, phospholipase C; (+)-UH 232, cis-(+)-5-methoxy-1-methyl-2-(di-n-propylamino)tetrinal; S 14066, 3-(1-(benzocyclobutan-1-ylmethyl)piperidin-4-yl)-6-fluoro-1,2-benzisoxazole; TEA, triethanol amine; TM, trans-membrane domain.

\textsuperscript{*} Some data presented in this paper were taken from Wurch et al. [1].

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and tropapride (pKᵦ: 8.52)-mediated IP responses. Clozapine, olanzapine and raclopride displayed partial inverse agonist properties (−31%, −67% and −71% vs. tropapride 1 µM, respectively), whereas bromerguride (+63%) and (+)-UH 232 (+88%) demonstrated positive agonism.

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**Keywords:** Inverse agonism; [³⁵S]GTPγS binding assay; Chimeric receptor; Inositol phosphates response; Intrinsic activity; Dopamine antagonist

### 1. Introduction

Determination of the intrinsic activity of receptor ligands remains a major issue in the pharmacological characterization of the activity profile of a compound. The resolving capacity by which compounds with various degrees of intrinsic activity can be differentiated is co-determined by the receptor/G protein coupling state. Fine-tuning of the assay system is often necessary to measure the entire window of pharmacological activities ranging from efficacious agonism via neutral antagonism to inverse agonism. Detection of inverse agonism implies that a receptor is constitutively active, a condition which is often achieved either at high receptor expression levels and/or upon introduction of activating receptor mutations [2,3]. Several of such mutations have been described like Asp¹⁴²Thr, Arg¹⁴³Lys in the second intracellular loop (2ICL) and Ala²⁹³Glu in the 3ICL of the α₁B-adrenoceptor (α₁B AR) [4-6]; Thr³⁷³Lys in the 3ICL of the α₂A AR [7,8]; and Leu²⁷²Ala in the 3ICL of the β₂ AR [9].

The dopamine D₂ receptor is of particular interest because it represents the major receptor target for neuroleptic drugs [10]. These compounds cover a broad spectrum of chemical structures classified as antagonists at D₂-like receptors and which act as antischizophrenic agents [10]. Former studies suggested haloperidol to act as an inverse agonist by increasing prolactin release from GH₄C₁ pituitary cells expressing a D₂short receptor [11,12] or by decreasing basal arachidonic acid release from a CHO pro cell line stably expressing a D₂long receptor isoform [13]. More recent reports also suggested that most of these antagonists may act as inverse agonists at either wt or mutant D₂ receptors [14—16]. Surprisingly, the amplitude of inverse agonism by these compounds seems to be comparable. The putative absence of a difference in the ligands’ amplitude of inverse agonism may perhaps be due to the low resolving capacity of the investigated assay systems. Thus, it is of interest to develop more sensitive assay systems that would be able to identify putative differences in the magnitude of inverse agonism at D₂ receptors.

In the present study, [³⁵S]GTPγS binding experiments were performed in a first attempt on CHO-K1 cells stably expressing a wild-type (wt) or mutant Thr³⁴³Ser D₂short receptor. Either a membrane preparation or digitonin-permeabilized cells were evaluated in different buffer formulations (i.e., in the presence of either Na⁺ or K⁺ ions) since the nature of these ions in the assay buffer has been shown to modulate [³⁵S]GTPγS binding responses for δ-opioid receptor and α₂A AR [17,18]. Detection of inverse agonism at the mutant Thr³⁴³Ser D₂short receptor was achieved with nemonapride, haloperidol and (+)-butaclamol. Nevertheless, the amplitude of the inverse agonist response was weak (about 20%) and apparently similar for these compounds. In order to enhance the magnitude of the inverse
agonist response, a chimeric receptor obtained by exchange of the 3ICL of the D₂ receptor for that of an α₁B AR and containing an activating mutation (Ala²⁷⁹Glu equivalent to the Ala²⁸₃ position in α₁B AR and noted D₂/α₁B Ala²⁷⁹Glu 3ICL) in its 3ICL was constructed. This construct displayed a large amplitude (about 60%) of inverse agonism in the presence of a Gα₁₁ protein. The intrinsic activity of different agonists and putative antagonists was evaluated at the chimeric D₂/α₁B Ala²⁷⁹Glu 3ICL receptor construct using both kinetic Ca²⁺ and inositol phosphates (IP) responses.

2. Materials and methods

2.1. Cell culture and transfection procedures

Cos-7 and Chinese hamster ovary (CHO)-K₁ cell lines were cultured, respectively, in Dulbecco’s Modified Eagle Medium and Ham’s F12 medium, each one supplemented with 10% heat-inactivated fetal calf serum. Transfection was performed by electroporation [Bio-Rad Gene pulser apparatus (250 V, 250 μF)] using 10 μg of indicated receptor plasmid in either the presence or absence of 10 μg of recombinant Gα protein plasmid. The culture was prolonged in complete culture medium containing 1% dimethylsulfoxide under conditions dependent on the monitored response. CHO-K₁ cell lines stably expressing either a wt or mutant Thr³⁴³Ser D₂ short receptor were generated upon dilution of transfected cells (10- to 1000-fold) and selection in complete Ham’s F12 medium containing 1.25 mg geneticin/ml. The selected wt and mutant Thr³⁴³Ser D₂ short receptor cell lines showed 8.31 ± 0.05 and 5.01 ± 0.04 pmol/mg protein, respectively, of specific [³H] nemonapride binding sites on their cellular membranes.

2.2. Construction of chimeric dopamine D₂ receptor constructs

The short splice form of the human wt dopamine D₂ receptor (R.C. 2.1.DA.02, GenBank accession number: S69899) was modified by exchanging its 3ICL by the equivalent portion of the human α₁B-adrenoceptor (α₁B AR, R.C. 2.1.ADR.A1B, GenBank accession number: U03865). A modified overlap extension PCR approach was applied by using overlapping complementary oligonucleotide primers allowing the junction of the D₂ receptor and α₁B AR portions without addition of restriction sites [19]. Incorporation of the Ala²⁷⁹Glu mutation was performed by using a QuickChange site-directed mutagenesis kit as described by the supplier (Stratagene, La Jolla, USA). The chimeric D₂/α₁B 3ICL receptor constructs were inserted into a pCR3.1 mammalian expression vector and fully sequenced on an ABI Prism 310 Genetic analyzer using a Big Dye Terminator Cycle Sequencing Ready Reaction kit, confirming the respective sequences.

2.3. [³⁵S]GTPγS binding assay to cellular membrane preparations

CHO-K₁ cells stably expressing a wt or mutant Thr³⁴³Ser D₂ short receptor were grown in complete Ham’s F12 medium containing 1.25 mg/ml geneticin. Cells were scraped mechanically in 10 mM Tris–HCl, 0.1 M EDTA (pH 7.5) and centrifuged for 10 min at 45 000 × g. The pellet was homogenised in the same buffer and centrifuged under similar
conditions. Membrane preparations were diluted in different types of buffers as indicated. Buffer formulations consisted in 20 mM HEPES, 100 mM of either NaCl or KCl, 3 mM MgCl$_2$, 0.2 mM ascorbic acid, 30 μM GDP (pH 7.4) or in 50 mM triethanolamine (TEA)–HCl, 1 mM MgCl$_2$, 150 mM KCl, 30 μM GDP (pH 7.4). Incubation was performed at 25 °C for 30 min, followed by the addition of 0.5 nM [³⁵S]GTPγS either in the absence or presence of compound. The reactions were stopped after 30-min incubation at 25 °C by rapid filtration over Whatman GF/B glass fiber filters using a Brandel harvester, washed and radioactivity was counted.

2.4. [³⁵S]GTPγS binding assay to digitonin-permeabilized intact cells

The [³⁵S]GTPγS binding procedure on permeabilized CHO-K1 cells was adapted from Wieland et al. [20]. Briefly, the above-described CHO-K1 cells were kept in permeation buffer [50 mM triethanolamine–HCl, 5 mM MgCl$_2$, 1 mM EDTA, 150 mM NaCl, 10 μM digitonin, pH 7.4] for 20 min at 37 °C. Upon removal of the permeation buffer, GTPγS binding buffer was applied [50 mM TEA-HCl, 1 mM MgCl$_2$, 150 mM of either NaCl or KCl, 30 μM GDP, 0.2 nM [³⁵S]GTPγS, pH 7.4] either in the absence or presence of compound and cells were incubated for 30 min at 37 °C. Cells were washed twice with ice-cold Hank’s balanced salt solution. Five-hundred microliters of scintillation liquid was added to extract radioactivity and scintillation counting was performed on a TopCount microplate reader (Packard Instruments).

2.5. Receptor binding assay

Membrane preparations described above were diluted in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, (pH 7.4). Incubation mixtures consisted of 0.4 ml membrane preparations (50 to 150 μg protein), 0.05 ml [³H]nemonapride and 0.05 ml of compound for inhibition or 10 μM of (+)-butaclamol to determine nonspecific binding. The reactions were stopped after 1-h incubation at 25 °C by the addition of 3.0 ml of ice-cold 50 mM Tris–HCl (pH 7.7) and rapid filtration over Whatman GF/B glass fiber filters using a Brandel harvester, washed and radioactivity was counted [21]. Scatchard analysis was performed as described [21] using concentrations of radioligand ranging from 3 pM to 3 nM. The protein level was estimated with a dye-binding assay using a Bio-Rad kit; bovine serum albumin was used as a standard [22].

2.6. Measurement of inositol phosphates formation

Cos-7 cells expressing the indicated receptor construct and Ga protein (for cloning details, see Pauwels et al. [23]) combination were loaded with [³H]myoinositol (4 μCi/well of a 24-well plate) for 48 h in DMEM supplemented with 2% dialyzed fetal calf serum. Cells were washed with 1.0 ml controlled-salt solution (CSS), and incubated for 1.5 h at 37 °C in 1.0 ml CSS containing 10 mM LiCl either in the presence or absence of compound. The reaction was stopped by the addition of 0.25 ml of sample buffer (30 mM Na$_2$B$_4$O$_7$, 3 mM EDTA) and the fraction of total [³H]IP was separated from the other [³H]derivatives by chromatography on an anion exchange AG1-X8 resin as described [24].
pIC$_{50}$ and pEC$_{50}$ values were defined as the concentration of compound at which 50% of its own maximal inhibitory, respectively, stimulatory effect was obtained.

2.7. Measurement of intracellular Ca$^{2+}$ responses

CHO-K1 cells expressing the indicated receptor construct and G$_{a}$ protein combination were assayed for Ca$^{2+}$ responses at 24- to 48-h post-transfection upon an 1-h loading with the Ca$^{2+}$ indicator dye Fluo-3 (2 μM). Dopaminergic ligands were assayed for their Ca$^{2+}$ response as previously described [21]. Data were expressed in arbitrary fluorescence units (AFU) and were not converted into Ca$^{2+}$ concentrations. Fluorescent readings were made every 2 s for a 3-min time period using a fluorometric image plate reader (FLIPR, Molecular Devices). $E_{\text{max}}$ values were defined as the ligand’s maximal high-magnitude response vs. that obtained with 10 μM DA. pEC$_{50}$ values correspond to a ligand concentration at which 50% of its own $E_{\text{max}}$ value was measured.

2.8. Statistical analysis

Statistical significance of the data was determined by comparisons performed for the [$^{3}$H] nemonapride binding data (chimeric D$_{2}$/α$_{1}$B Ala$^{279}$Glu 3ICL receptor construct vs. wt D$_{2short}$ receptor), [$^{35}$S]GTPγS binding data (ligand-mediated vs. basal [$^{35}$S]GTPγS binding level) and IP formation data (ligand-mediated vs. basal IP level) as performed with a two-tailed Student’s t-test.

2.9. Materials

The ABI Prism 310 Genetic Analyzer and Big Dye Terminator Cycle Sequencing Ready Reaction kit were from Applied Biosystems (Forster City, USA). The pCR3.1 expression vector was purchased from InVitrogen (San Diego, USA). QuickChange site-directed mutagenesis kit was from Stratagene. Cos-7 and CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, USA). [$^{35}$S]GTPγS was from Amersham Bioscience (Les Ulis, France). [$^{3}$H] Nemonapride (85 Ci/mmol) and [2-$^{3}$H](M)-myo-inositol (20 Ci/mol) were obtained from New England Nuclear (Les Ulis, France). DA, raclopride, haloperidol, chlorpromazine, (+)-NPA, risperidone, (+)-butaclamol, olanzapine, (−)-adrenaline, prazosin and clonidine were from Sigma-RBI (St. Louis, USA). cis-(+) 5-Methoxy-1-methyl-2-(di-n-propylaminotetralin) [(+)UH 232] was from Tocris (Ballwin, USA). Bromerguride was from Schering (Berlin, Germany). Nemonapride, tropapride, olanzapine and ziprasidone were synthesized at the Centre de Recherche Pierre Fabre.

3. Results

3.1. Inverse agonism at wild-type and mutant Thr$^{343}$Ser dopamine D$_{2short}$ receptors

In a first set of experiments, [$^{35}$S]GTPγS binding responses were monitored at a membrane preparation of CHO-K1 cells, stably expressing either a wt or a mutant D$_{2short}$
Table 1
Influence of buffer composition on \([35S]GTP^\gamma S\) binding responses to membrane preparations of CHO-K1 cells stably expressing either a wt or mutant Thr\(^{343}\)Ser \(D_{2short}\) receptor

<table>
<thead>
<tr>
<th>Buffer</th>
<th>wt (D_{2short})</th>
<th>wt (D_{2short})</th>
<th>Thr(^{343})Ser (D_{2short})</th>
<th>Thr(^{343})Ser (D_{2short})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>153 ± 13</td>
<td>163 ± 2</td>
<td>394 ± 3</td>
<td>434 ± 12</td>
</tr>
<tr>
<td>Nemonapride</td>
<td>103 ± 1</td>
<td>101 ± 1</td>
<td>97 ± 5</td>
<td>99 ± 12</td>
</tr>
<tr>
<td>(+)-UH 232</td>
<td>95 ± 2</td>
<td>107 ± 3</td>
<td>102 ± 4</td>
<td>118 ± 6</td>
</tr>
</tbody>
</table>

Membrane preparation, labelling procedures and buffer composition were described in Materials and methods. Basal \([35S]GTP^\gamma S\) binding levels set at 100% corresponded to 114 ± 3 and 177 ± 3 fmol/mg protein in Hepes/NaCl and Hepes/KCl buffer, respectively, for the wt \(D_{2short}\) receptor and to 140 ± 6, 185 ± 5 and 36 ± 1 fmol/mg protein in Hepes/NaCl, Hepes/KCl and TEA/KCl buffer, respectively, for the mutant Thr\(^{343}\)Ser \(D_{2short}\) receptor. Results are expressed as a percentage of the basal \([35S]GTP^\gamma S\) binding response measured in the absence of compound and correspond to mean values of one experiment performed in triplicate.

Table 2
Influence of buffer composition on \([35S]GTP^\gamma S\) binding responses to digitonin-permeabilized CHO-K1 cells stably expressing either a wt or mutant Thr\(^{343}\)Ser \(D_{2short}\) receptor

<table>
<thead>
<tr>
<th>Buffer</th>
<th>wt (D_{2short})</th>
<th>Thr(^{343})Ser (D_{2short})</th>
<th>Thr(^{343})Ser (D_{2short})</th>
<th>Thr(^{343})Ser (D_{2short})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX</td>
<td>TEA/KCl</td>
<td>TEA/NaCl</td>
<td>TEA/KCl</td>
<td>TEA/KCl</td>
</tr>
<tr>
<td>DA</td>
<td>301 ± 59*</td>
<td>581 ± 80*</td>
<td>524 ± 46*</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>(+)-UH 232</td>
<td>96 ± 3</td>
<td>91 ± 10</td>
<td>139 ± 8*</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>Nemonapride</td>
<td>89 ± 7</td>
<td>73 ± 6*</td>
<td>81 ± 4*</td>
<td>93 ± 10</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>95 ± 4</td>
<td>88 ± 8*</td>
<td>84 ± 8*</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>(+)-Butaclamol</td>
<td>93 ± 5</td>
<td>83 ± 10*</td>
<td>85 ± 8*</td>
<td>96 ± 7</td>
</tr>
<tr>
<td>(−)-Butaclamol</td>
<td>100 ± 9</td>
<td>97 ± 12</td>
<td>98 ± 7</td>
<td>100 ± 6</td>
</tr>
</tbody>
</table>

Cell permeation, labelling procedures and buffer composition were described in Materials and methods. Basal \([35S]GTP^\gamma S\) binding levels set at 100% corresponded to 30 ± 2 fmol/mg protein for the wt \(D_{2short}\) receptor in TEA/KCl buffer and to 34 ± 9 and 36 ± 4 fmol/mg protein in TEA/NaCl and TEA/KCl buffer, respectively, for the mutant Thr\(^{343}\)Ser \(D_{2short}\) receptor. Results are expressed as a percentage of the basal \([35S]GTP^\gamma S\) binding response measured in the absence of compound and correspond to mean values ± S.E.M. of three to nine independent experiments, each experimental data point performed in triplicate.

* \(p<0.05\) vs. basal \([35S]GTP^\gamma S\) binding response, Student's \(t\) test.
[^35]S]GTPγS binding at the mutant Thr^{343}Ser D_{2\text{short}} receptor but only in the presence of KCl (Table 1).

In a second step, [^35]S]GTPγS binding was performed on intact CHO-K1 cells upon digitonin (10 μM) permeation in order to better preserve receptor/G protein interactions. DA (10 μM) strongly stimulated (+201 to +481% vs. basal) the binding of [^35]S]GTPγS at both wt and mutant Thr^{343}Ser D_{2\text{short}} receptors. The putative antagonists nemonapride, haloperidol and (+)-butaclamol significantly reduced at a 1 μM concentration basal [^35]S]GTPγS binding by 15% to 19% (Table 2) but exclusively at the mutant Thr^{343}Ser D_{2\text{short}} receptor. The magnitude of inverse agonism was similar for TEA buffer in the presence of either NaCl or KCl (Table 2). In contrast, (+)-UH 232 (1 μM) significantly stimulated the [^35]S]GTPγS binding response by 39% at the mutant Thr^{343}Arg D_{2\text{short}} receptor but only in the presence of KCl (Table 2). Both positive and negative [^35]S]GTPγS binding responses were fully blocked upon pre-treatment with the ADP-ribosylating agent pertussis toxin (Table 2). Co-expression of a G_{α}\text{to} γ

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK_{i} values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D_{2/α_{1B}} Ala^{279}Glu 3ICL</td>
</tr>
<tr>
<td>Nemonapride</td>
<td>10.07 ± 0.06</td>
</tr>
<tr>
<td>Tropapride</td>
<td>9.82 ± 0.10</td>
</tr>
<tr>
<td>Bromerguride</td>
<td>9.29 ± 0.12*</td>
</tr>
<tr>
<td>(−)-NPA</td>
<td>8.75 ± 0.01** [7.54–7.43]</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>8.75 ± 0.09</td>
</tr>
<tr>
<td>Risperidone</td>
<td>8.48 ± 0.01</td>
</tr>
<tr>
<td>Raclopride</td>
<td>8.42 ± 0.02</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>8.26 ± 0.07</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>7.56 ± 0.08</td>
</tr>
<tr>
<td>(+)-UH 232</td>
<td>7.62 ± 0.07</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>7.65 ± 0.12</td>
</tr>
<tr>
<td>Clozapine</td>
<td>6.82 ± 0.08</td>
</tr>
<tr>
<td>Dopamine</td>
<td>6.46 ± 0.05** [5.45–5.41]</td>
</tr>
<tr>
<td>(−)-Adrenaline</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Prazosin</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Clonidine</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

pK_{i} values were determined on Cos-7 cellular membranes transiently expressing either the chimeric D_{2/α_{1B}} Ala^{279}Glu 3ICL receptor construct or the wt D_{2\text{short}} receptor using [^3]H nemonapride (0.14 nM) as described in Materials and methods. Values between brackets correspond to the chimeric D_{2/α_{1B}} 3ICL receptor construct. Monophasic Scatchard analysis yielded dissociation constants (46.0 ± 1.42 and 47.1 ± 0.9 pM) and maximal [^3]H nemonapride binding capacity (3.69 ± 0.73 and 1.48 ± 0.07 pmol/mg protein) for chimeric D_{2/α_{1B}} Ala^{279}Glu 3ICL and wt D_{2\text{short}} receptor constructs, respectively. Data are the mean ± S.E.M. of three independent experiments, each experimental data point performed in duplicate. Statistical analysis was performed for comparison of the ligands’ pK_{i} values between the D_{2/α_{1B}} Ala^{279}Glu 3ICL and wt D_{2\text{short}} receptor constructs using a Student’s t-test.

*p < 0.01.

**p < 0.001.
protein did not enhance the $[^{35}S]GTP\gamma S$ binding responses of the herein investigated compounds at the CHO-Thr$^{343}$Ser D2short cell line (not shown).

### 3.2. Receptor binding and functional properties of the chimeric D2/α1B Ala$^{279}$Glu 3ICL receptor construct

A chimeric receptor was constructed by exchanging the entire 3ICL of the dopamine D2short receptor by the equivalent portion of the α1B AR (noted D2/α1B 3ICL). Introduction of a facilitating Ala$^{279}$Glu mutation in the distal portion of this 3ICL was also performed. The binding profile of the chimeric D2/α1B Ala$^{279}$Glu 3ICL receptor construct for a series

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**Fig. 1.** Correlation between ligands' binding affinities at the chimeric D2/α1B Ala$^{279}$Glu 3ICL receptor construct as compared to the wt D2short receptor. Correlation was performed on the ligand's pK$_a$ values as determined on Cos-7 cellular membranes expressing either the D2/α1B Ala$^{279}$Glu 3ICL receptor construct or the wt D2short receptor as described in Table 3. Mean values were taken from Table 3 and correlation was calculated using a Pearson's correlation test.

**Fig. 2.** Time-dependent DA-mediated Ca$^{2+}$ responses at wt D2short, chimeric D2/α1B Ala$^{279}$Glu 3ICL receptor construct and wt α1B AR in either the absence or presence of a G$_{a11}$ or G$_{q10}$ protein in CHO-K1 cells. Co-transfection of 10 μg of either wt D2short receptor (A), chimeric D2/α1B Ala$^{279}$Glu 3ICL receptor construct (B) or wt α1B AR (C) plasmid and 10 μg of empty plasmid or plasmid containing a G$_{a11}$ or G$_{q10}$ protein was performed as described in Materials and methods. DA (10 μM) was applied at minute 0 and Ca$^{2+}$ responses were measured every 2 s for 3 min as described in Materials and methods. Curves illustrate a representative experiment out of a minimum of 12 independent experiments.
of putative dopamine antagonists was comparable to that of the wt D$_2$short receptor (Table 3). In contrast, an 11- and 22-fold increased binding affinity was observed, respectively, with the agonists DA and (-)-NPA at the chimeric D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct as compared to the wt D$_2$short receptor. Nevertheless, a strong correlation ($r^2$: 0.87, $p<0.001$) exists for the series of investigated dopamine ligands between their binding affinities at the chimeric D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct as compared to the wt D$_2$short receptor (Fig. 1). The higher agonist binding affinities were no longer observed with the chimeric D$_2$/oti$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct without the Ala$^{279}$Glu mutation (Table 3). Nevertheless, a strong correlation ($r^2$: 0.87, $p<0.001$) exists for the series of investigated dopamine ligands between their binding affinities at the chimeric D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct as compared to the wt D$_2$short receptor (Table 3).

At the level of effector activation, the chimeric D$_2$/a$_{1B}$ 3ICL and D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor constructs behaved like a G$_q$-coupled a$_{1B}$ AR. They were able to produce a DA (10 μM)-mediated Ca$^{2+}$ response in CHO-K1 cells in contrast to the wt D$_2$short receptor (Fig. 2). Nevertheless, the shape of the kinetic Ca$^{2+}$ response curves differed between the wt D$_2$short Receptor, wt a$_{1B}$ AR and the chimeric D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct (Fig. 2). In contrast to the wt D$_2$short receptor, DA (10 μM) stimulated inositol phosphates formation at both chimeric D$_2$/a$_{1B}$ 3ICL receptor constructs in the absence of recombinant G$_a$ proteins, as it is the case for (-)-adrenaline at the wt a$_{1B}$ AR (Table 4). These two Ca$^{2+}$ and IP parameters are related to the activation of the phospholipase C pathway by the chimeric D$_2$/a$_{1B}$ 3ICL receptor constructs.

3.3. Pharmacological Ca$^{2+}$ response by the chimeric D$_2$/a$_{1B}$ Ala$^{279}$Glu 3ICL receptor construct

Potencies of DA and (-)-adrenaline were modified by the presence and nature of the co-expressed G$_a$ protein subunit for the wt D$_2$short receptor with the following rank

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ca$^{2+}$ response (AFU)</th>
<th>IP formation (dpm/10$^5$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>CHO-K1</td>
<td>Cos-7</td>
</tr>
<tr>
<td>wt D$_2$short</td>
<td>896 ± 194</td>
<td>246 ± 419</td>
</tr>
<tr>
<td>wt a$_{1B}$ AR</td>
<td>12008 ± 371</td>
<td>132461 ± 12517</td>
</tr>
<tr>
<td>D$<em>2$/a$</em>{1B}$ 3ICL</td>
<td>14839 ± 1762</td>
<td>118216 ± 15324</td>
</tr>
<tr>
<td>D$<em>2$/a$</em>{1B}$ Ala$^{279}$Glu 3ICL</td>
<td>9749 ± 1386</td>
<td>86547 ± 13204</td>
</tr>
</tbody>
</table>

Dopamine (DA, 10 μM) or (-)-adrenaline (10 μM, for the wt a$_{1B}$ AR)-mediated Ca$^{2+}$ and inositol phosphates responses were measured in CHO-K1 and Cos-7 cells, respectively, as described in Materials and methods. Ca$^{2+}$ data are expressed in arbitrary fluorescence units (AFU) above basal Ca$^{2+}$ response, whereas inositol phosphates data correspond to dpm/10$^5$ cells above basal inositol phosphates level. Data correspond to mean ± S.E.M. values for a minimum of three independent transfection experiments.
order: \( G_{\text{eq/qo}} > G_{\alpha_{11}} > \) absence of \( G_{\alpha} \) protein. These parameters were weakly affected for the wt \( \alpha_{1B} \) AR and unaffected for the chimeric \( D_2/\alpha_{1B} \) Ala\(^{279}\)Glu 3ICL receptor construct (Fig. 3). DA and \((-\)\)-adrenaline yielded, respectively, a 12- to 19- and 45-

![Graphs showing Ca\(^{2+}\) response to dopamine and (-)-adrenaline](image)

Fig. 3. Comparison between DA- and \((-\)\)-adrenaline-mediated Ca\(^{2+}\) responses at wt \( D_2/\alpha_{1B} \) receptor construct and wt \( \alpha_{1B} \) AR in either the absence or presence of a \( G_{\alpha_{11}} \) or \( G_{\text{eq/qo}} \) protein in CHO-K1 cells. Co-transfection of 10 \( \mu \)g of either wt \( D_2/\alpha_{1B} \) receptor (A), chimeric \( D_2/\alpha_{1B} \) Ala\(^{279}\)Glu 3ICL receptor construct (B) or wt \( \alpha_{1B} \) AR (C) plasmid and 10 \( \mu \)g of empty plasmid (●) or plasmid containing a \( G_{\alpha_{11}} \) (O) or \( G_{\text{eq/qo}} \) (▲) protein was performed as described in Fig. 1. Curves were constructed using mean ± S.E.M. values for a minimum of three independent transfection experiments.
to 55-fold increased potency at the chimeric \( D_2/\alpha_{1B} \) Ala\(^{279}\)Glu 3ICL receptor construct as compared to the wt \( D_2\text{short} \) receptor either in the absence or presence of a \( G_{\alpha 11} \) protein; a weak enhancement was observed for DA in the co-presence of a \( G_{\alpha q0} \) protein (Fig. 3). The potency of DA at the wt \( \alpha_{1B} \) AR was slightly affected by the nature of the exogenous \( G_{\alpha} \) protein, and \((-\) -adrenaline was nine-fold more potent in the co-presence of a \( G_{\alpha 11} \) protein.

3.4. Inositol phosphates responses at the chimeric \( D_2/\alpha_{1B} \) Ala\(^{279}\)Glu 3ICL receptor construct

In the absence of recombinant \( G_{\alpha} \) proteins, DA (10 \( \mu \)M) stimulated (836 ± 65%) the formation of IP at the chimeric \( D_2/\alpha_{1B} \) Ala\(^{279}\)Glu 3ICL receptor construct, whereas tropapride (1 \( \mu \)M) did not significantly affect the basal signalling. Co-expression with various recombinant \( G_{\alpha} \) proteins (Fig. 4) demonstrated a significant enhancement (+86 to +226%) of the basal IP response with \( G_{\alpha 11}, G_{\alpha q} \) and \( G_{\alpha 15} \) proteins, a maximal effect being observed with the \( G_{\alpha 11} \) protein. Tropapride dose-dependently (pIC\(_{50} \): 9.10 ± 0.01) attenuated basal IP production by −59 ± 9% (Fig. 5A). Tropapride (0.1 \( \mu \)M) also antagonised the DA-mediated IP response in an insurmountable manner (Fig. 5B). A stereo-selective inverse agonist response was observed for butaclamol: the (+)-enantiomer was as efficacious as tropapride, whereas (−)-butaclamol (10 \( \mu \)M) did not affect

![Figure 4](image-url)
Fig. 5. Dose-dependent attenuation of basal and DA-mediated IP formation by tropapride and antagonism by ziprasidone using a chimeric D2/α1B Ala279Glu 3ICL receptor construct in the co-presence of a Gα11 protein. Cos-7 cells were co-transfected with 10 μg of chimeric D2/α1B Ala279Glu 3ICL receptor construct and 10 μg of Gα11 protein plasmid and assayed for IP formation as described in Materials and methods. (A) Attenuation of basal IP formation by tropapride in either the absence (●) or presence of ziprasidone (0.1 μM; ■). (B) Stimulation of IP formation by DA in either the absence (○) or presence of tropapride (0.1 μM; ●) or ziprasidone (0.1 μM; ■).

Data are calculated as a percentage of the basal IP response and expressed as the mean ± S.E.M. of three independent experiments, each experimental data point performed in triplicate.

The basal IP response (Fig. 6). Clozapine, olanzapine and raclopride (1 μM) behaved as less efficacious inverse agonists with a maximal attenuation of the IP response by −31 ± 5%, −67 ± 7% and −71 ± 3% (vs. maximal inhibition by tropapride 1 μM, Fig. 6). Other putative dopaminergic antagonists attenuated basal IP formation with a similar magnitude as tropapride in contrast to bromerguride and (+)-UH 232 which yielded efficacious positive agonism (respectively, 63% and 88% vs. 10 μM DA; Fig. 6). The bicyclic derivative ziprasidone did not affect the basal IP formation (+5 ± 3%, p>0.05, Student’s t-test). It potently and competitively antagonised both tropapride-
Fig. 6. Intrinsic activity of putative dopamine antagonists at chimeric D₂/α₁B Ala²⁷⁹Glu 3ICL receptor construct in the co-presence of a Gₐ₁₁ protein in Cos-7 cells. Cos-7 cells were transfected with 10 µg of chimeric D₂/α₁B Ala²⁷⁹Glu 3ICL receptor construct and 10 µg of Gₐ₁₁ protein plasmids and assayed for IP formation as described in Materials and methods. Data are calculated as a percentage of the tropapride (1 µM)-induced inhibition of basal IP formation or of the DA (10 µM)-mediated stimulation of basal IP formation (underlined for bromerguride and (+)-UH 232) and expressed as the mean ± S.E.M. value of three to eight independent experiments, each experimental data point performed in triplicate. Ligands were investigated at a concentration of 1 µM, a concentration effective for both antagonism of the DA-mediated high-magnitude and reversal of the low-magnitude Ca²⁺ responses (see Figs. 5 and 6 of Ref. [21]). Statistical analysis was performed on the ligand-induced vs. tropapride (1 µM)-induced IP level (based on data expressed in dpm/well) by using a Student’s t test. 

*p<0.01; **p<0.001.

(pKᵦ: 8.52 ± 0.27; Fig. 5A) and DA- (pKᵦ: 7.61 ± 0.27; Fig. 5B) mediated inhibition and stimulation of basal IP formation.

4. Discussion

Modulation of the constitutive activation level of the human dopamine D₂short receptor was investigated on either a membrane preparation or on permeabilized intact CHO-K1 cells stably expressing a mutant Thr³⁴³Ser D₂short receptor by measuring [³⁵S]GTPγS binding responses in different buffer formulations (i.e., NaCl, KCl, Heps, TEA). Indeed, both mathematical [26] and experimental [17,27] models demonstrated an inhibitory effect of sodium on receptor-mediated G protein activation. In the herein reported [³⁵S]GTPγS binding conditions, membrane preparations did not allow to detect inverse agonist activity of nemonapride neither in the presence of NaCl nor KCl. Maximal stimulation of [³⁵S]GTPγS binding by DA was also insensitive to the nature of monovalent cations although their presence was necessary (not shown). In contrast, in case of permeabilized
CHO-K1 cells expressing the mutant Thr$^{343}$Ser D$_{2\text{short}}$ receptor, inverse agonist activity was demonstrated for nemonapride, haloperidol and (+)-butaclamol, irrespective of the presence of sodium or potassium ions. These data contrast with studies performed on $\mu$-opioid receptors that indicated that sodium ions inhibited both agonist-independent and -dependent $\mu$-opioid receptor-mediated G protein activation in rat thalamic membranes and that KCl increased basal $[^{35}S]GTP\gamma S$ binding in receptor-expressing GH$_3$ cells [28,29]. These differences may be related to the investigated receptor or experimental conditions (i.e., GDP concentration, cell type). Interestingly, although detection of inverse agonist activity was insensitive to the nature of the monovalent cation present in the assay buffer, partial agonism of (+)-UH 232 was only detectable in the presence of KCl in CHO-K1 cells expressing the mutant Thr$^{343}$Ser D$_{2\text{short}}$ receptor. This result agrees with the mathematical modelling of Costa et al. [26], which predicted that relative agonist efficacy should be inversely proportional to the sodium concentration although we could not detect any modulation of basal $[^{35}S]GTP\gamma S$ binding level. Thus, although we established experimental conditions which increased constitutive activation of the mutant Thr$^{343}$Ser D$_{2\text{short}}$ receptor, we were not able to differentiate between the inverse agonist efficacy of the investigated putative dopamine antagonists.

Therefore, chimeric D$_2$/A$_{1\text{B}}$ receptor constructs were prepared to enhance constitutive receptor activation and to differentiate between the amplitude of inverse agonism. The construct D$_2$/A$_{1\text{B}}$ Ala$^{279}$Glu 3ICL displayed a large magnitude of constitutive activation and was highly sensitive to inverse agonism for most of the tested putative dopamine antagonists which behaved as efficacious inverse agonists. Two atypical neuroleptic drugs, olanzapine and clozapine as well as the benzamide-derivative raclopride displayed partial inverse agonist activities, whereas ziprasidone behaved as a silent antagonist. These results strongly suggest that the inverse agonist feature is common to most neuroleptic drugs. Two compounds, (+)-UH 232 and bromerguride, displayed positive agonism and have to be considered as partial agonists rather than antagonists.

By exchanging the 3ICL of the A$_{1\text{B}}$ AR in a D$_2$ receptor backbone and by introducing the activating mutation Ala$^{279}$Glu in the distal BBXXB motif, the D$_2$ receptor was switched to a more active conformation. Dynamic computer simulations performed on a wt A$_{1\text{B}}$ AR rhodopsin-based tridimensional model revealed that the distal portion of the A$_{1\text{B}}$ AR’s 3ICL folds into an $\alpha$-helix and faces the $\alpha$-helical N-terminal portion of the same loop [30]. Modelling of mutant Ala$^{293}$Glu A$_{1\text{B}}$ AR structure indicates that the Glu$^{293}$ residue is involved in hydrogen bonds with both Tyr$^{277}$ and Lys$^{231}$ at the N-terminal portion of the 3ICL [30] in contrast to the wt Ala$^{293}$ residue. It is likely that this particular intramolecular interaction is also present in the chimeric D$_2$/A$_{1\text{B}}$ Ala$^{279}$Glu 3ICL receptor construct as the entire A$_{1\text{B}}$ AR’s 3ICL was exchanged. One can further postulate a conformational link between the Glu$^{279}$ residue and the Asp-Arg-Tyr (DRY) motif in the D$_2$ receptor-derived 2ICL, as previously shown for the wt A$_{1\text{B}}$ AR [4]. The presence of the A$_{1\text{B}}$ AR’s 3ICL combined with the Ala$^{279}$Glu mutation in a D$_2$ receptor backbone is apparently sufficient to modify the overall conformation of the protein to generate constitutive activity as suggested by the increased binding affinities observed for dopamine agonists. The chimeric receptor construct retained comparable antagonist binding characteristics of a wt D$_{2\text{short}}$ receptor, suggesting that the investigated ligands are predominantly interacting with the receptor’s TMs rather than with the A$_{1\text{B}}$ AR-derived 3ICL.
The binding affinities for the agonists DA and (−)-NPA were increased at the chimeric DA/α1B Ala^{279}Glu 3ICL construct as compared to the wt D_{2short} receptor. The increased agonist binding affinity is probably due to the formation of a high-affinity chimeric D_{2}/α1B Ala^{279}Glu 3ICL receptor state (R*) generated by the Ala to Glu mutation since the chimeric D_{2}/α1B 3ICL receptor did not demonstrate modified binding affinities. A comparable increased binding affinity was also observed for DA at the mutant Thr^{343}Ser D_{2short} receptor [15], suggesting that this mutant receptor behaves as a constitutively active receptor as confirmed by the herein reported [35S]GTPγS binding responses. The chimeric D_{2}/α1B 3ICL receptor constructs displayed functional characteristics of phospholipase C activation, such as induction of Ca^{2+} responses and stimulation of IP formation. Co-expression with three G_{α} proteins of the G_{q} family indicated the following rank order of enhanced basal IP formation: G_{α(q1)}>G_{α(q2)}>G_{α(q3)}. This may suggest a preferential coupling of the chimeric D_{2}/α1B Ala^{279}Glu 3ICL receptor construct to a G_{α(q1)} protein. It cannot be excluded that a difference in the expression level between these G_{α} proteins may also explain this G_{α} protein effect.

By monitoring forskolin-stimulated cAMP formation, Hall and Strange [14] suggested (+)-UH 232 to be a weak partial inverse agonist at the stably transfected wt D_{2short} receptor rather than a truly neutral antagonist. Both enantiomers of UH 232 have been characterized as partial agonists by measuring the extracellular acidification rate at the D_{2long} receptor stably transfected in CHO-K1 cells [31]. We classified (+)-UH 232 rather as a partial agonist (this study). (+)-UH 232 also behaved as a partial agonist in CHO-K1 cells transiently co-expressing a related mutant Thr^{343}Arg D_{2short} receptor and a Ga_{0}Cys^{351}Ile protein [23]. It cannot be excluded that these differences in intrinsic activities for (+)-UH 232 reflect effector-dependent features. Clozapine displayed a partial inverse agonist response reaching about 30% to that of tropapride. This compound acted as an inverse agonist at the mutant Thr^{343}Arg D_{2short} with a tendency to be less efficacious than haloperidol [15]. In other experimental systems, such as sensitization of adenylate cyclase by mutant rat Thr^{344}Arg D_{2short} receptor [32] and potentiation of forskolin-stimulated cAMP accumulation by wt D_{2long} receptor [32], clozapine tended to behave as a sub-maximal inverse agonist. In contrast, clozapine exhibited full inverse agonist responses at a chimeric D_{1}/D_{2} [1−4,7] receptor construct (containing the [TMV-3ICL-TMVI] portion of a D_{1} receptor in a D_{2} receptor backbone) like (+)-butaclamol and haloperidol, although the ligand binding profile of this chimeric D_{1}/D_{2} receptor construct was modified as compared to the wt D_{2} receptor [32]. The benzamide derivative raclopride yielded as well partial inverse agonism (~70% vs. that of 1 µM tropapride) at the chimeric D_{2}/α1B Ala^{279}Glu 3ICL receptor construct. This compound has previously been reported as a silent antagonist by measuring the prolactin release response in GH_{4}C_{1} cells transfected with a D_{2short} receptor, while haloperidol displayed inverse agonism [12]. The magnitude of the inverse agonist response is mainly determined by the amount of ligand-independent activation of the receptor [33]. Hence, compounds that display weak inverse agonism may be better observable in an expression system in which the receptor displays a high level of constitutive activity (i.e., D_{2}/α1B Ala^{279}Gly 3ICL construct). It is possible that the detection of weaker inverse agonists by measuring basal prolactin release in GH_{4}C_{1} cells is limited.

In conclusion, increased isomerization in an active receptor state was achieved by the coupling of a dopamine D_{2} receptor to the phospholipase C pathway via the 3ICL
of an α_{1B} AR, the incorporation of an activating mutation (Ala^{279}Glu) in the distal BBXXB motif of its 3ICL and the co-expression with a G_{a11} protein. Under these experimental conditions, the chimeric D_{2}/α_{1B} receptor construct displayed a strongly enhanced basal IP formation. This could be reversed by a large series of dopamine antagonists acting as inverse agonists with the exception of ziprasidone which behaved as a silent antagonist. The herein described model makes it possible to differentiate between efficacious to partial inverse dopamine agonists as well as silent dopamine antagonists.

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References


Discussion 14

G. Milligan
Perhaps the next step in your experiments will be to move these compounds in a clinical setting.

T. Wurch
The only compound which is inactive in the clinic is the partial agonist UH 232, but only in one study. Now it would be nice to test aripiprazole, which is probably a promising compound in our system, because it is claimed to be a partial positive agonist. But many other compounds are active in clinic and they display differences in negative efficacy, so this is probably not related. For us the system is important, because what we ask for is to differentiate the compounds that our chemists are producing, and until we had this system, all the compounds behaved almost similar. So for us, it was difficult to say which chemical series were better than another. Now at least we have a trend to give an answer.

P. Strange
We are back to the same problem again, of defining efficacy and which system are you using, because we find most of those compounds to be full inverse agonists. But, for example, we have found ziprasidone to have lower efficacy in the fusion protein. And I don’t know to what extent the G protein that’s involved is affecting things. It’s very unclear what is going on here, I think.

T. Wurch
In this system, if we don’t over-express the G\alpha_{11} protein, we cannot really modulate the basal to a level enough to be able to detect inverse agonism.

P. Strange
I understand the problems, but we are back to definitions of efficacy and whether the system is the right one to look at. I’m not saying it’s the wrong one, but which is the right one?

C. Maack
When performing the mutation of a receptor at a certain point, and inducing the conformational change of this receptor, isn’t that in a two-state model, a shift of the equilibrium towards the activated state? and when achieving under these conditions inverse agonist activity, a shift of the equilibrium towards the inactivated state, does this really tell us that the agent that we are examining is an inverse agonist? or couldn’t it be that in reality, it’s a neutral antagonist, and that in a model of mutational receptor activation, this agent just shifts the equilibrium back to the place where it was in its native condition? And when putting it onto a normal receptor, it remains in the same condition where it is. So this is my understanding of receptor equilibrium, that any compound stabilises the receptor at a certain proportion of active state receptors, and that a neutral antagonist leaves it there where it is in its native, unliganded
condition. And if this equilibrium was shifted by a mutational increase of constitutive activity, then the compound itself is still a neutral antagonist, rather than an inverse agonist.

T. Wurch

In fact, these compounds behave in vivo as antagonists because the D$_2$ receptor is not activated in vivo. But to our concern, we had to do something to activate it, in order to be able to discriminate the compounds. Because all these antagonists behaved the same. Although there are different in vivo pharmacological properties, mainly in behaviour. So, this means that probably they are different, but they are antagonists.

C. Maack

But isn't that, in principle, a similar thing as antagonising a ligand-induced activation of the receptor? I'm not sure.

T. Wurch

We could differentiate these compounds just by saying that we could discriminate them in a dopamine-bound state. Unfortunately, when we correlated this data with the in vivo data, there was absolutely no correlation, so this means that the conditions in which these kinetic responses—calcium responses—were observed, are not really relevant to an in vivo situation. I don't say that this artificial model is related to an in vivo situation, but at least we can differentiate the compounds, and this was important for us.

M. Brann

Just to answer the question regarding the inverse agonist activity correlating with inverse agonism. When we look at mutations of muscarinic receptors, it covers the entire spectrum of the isomerisation constant, and inverse agonism, at least in that system, wasn't absolutely universal that you don't change inverse agonism, depending on how much constitutive activity you have. They always have essentially the same potency and they are inverse agonists, like atropine and drugs like that. The question I have is, to what extent have the laboratories tried to address the possibility that there is in vivo constitutive activity of the D$_2$ receptor, particularly in the context of neuro-chemical sorts of experiments, and particularly in the context of maybe having a neutral antagonist like ziprasidone. In theory, that makes possible to do those experiments, have you tried those experiments? Where's the state of the art in that?

T. Wurch

This is the reason why we had to modify so heavily the receptor, because the in vivo models, I think none of them are really addressing the inverse agonist question. Although we have to be able to discriminate between the compounds. So I hope there are in vivo tests which can do this.

M. Brann

There are bits and pieces of information in the literature with some effects of haloperidol on things like C-fos induction, which are the opposite of dopamine. That's the kind of thing and we have not done that. We have tried to interest electrophysiologists in doing some of these experiments, but so far have failed to get any interest. The other piece of information which suggests that these compounds are doing something is the observation in experimental animals and also in humans, where you get an increase in D$_2$ receptors when you chronically treat, but again I've tried to interest somebody to do experiments in which they would compare haloperidol with UH 232 in that paradigm, and
I have not yet succeeded. It’s not the sort of experiments that we can do in our labs. So that’s the answer, I’m afraid, at the moment.

T. Wurch

There also some data on up-regulation of RGS messenger RNA by treatment with haloperidol or clozapine, but is this related to the signalling pathways? I don’t know.

A. Newman-Tancredi

The problem with a lot of these drugs is that they are very “dirty drugs”, they are very multi-receptorial in their binding profiles, so ideally what we’d have is a very highly selective D_2 inverse agonist, and maybe these other kinds of models that can identify the drugs, and a neutral, very clean, D_2 antagonist. At the moment I don’t think we know of such drugs, so the in vivo data are very complicated to interpret.