

# Coupling of D<sub>2</sub> and D<sub>3</sub> dopamine receptors to G-proteins

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Recombinant cell lines expressing D<sub>2</sub> and D<sub>3</sub> dopamine receptor isoforms have been used to study coupling of these receptors to G-proteins from the effects of GTP to reduce agonist binding affinities in agonist/[<sup>3</sup>H]spiperone competition experiments. D<sub>2(short)</sub> expressed in Ltk<sup>-</sup> cells, D<sub>2(long)</sub> expressed in Ltk<sup>-</sup> or CHO cells and D<sub>3</sub> expressed in CHO cells all showed coupling to the endogenous G-proteins of the cells. The detailed agonist binding characteristics indicated that there were differences in receptor/G-protein coupling for the same receptor (D<sub>2(long)</sub>) expressed in two cell types or for different receptors (D<sub>2(short)</sub>, D<sub>2(long)</sub>) expressed in the same cell type.

D<sub>2</sub> dopamine receptor; D<sub>3</sub> dopamine receptor; G-protein; Agonist binding

## 1. INTRODUCTION

Whereas biochemical, pharmacological and physiological techniques suggested that there were two classes of dopamine receptor (D<sub>1</sub> and D<sub>2</sub>) [1], the application of molecular biological techniques has shown that these classes may be further subdivided into D<sub>1</sub>-like (cloned D<sub>1</sub>, D<sub>5</sub>) and D<sub>2</sub>-like (cloned D<sub>2</sub>, D<sub>3</sub>, D<sub>4</sub>) receptors [2,3]. On the basis of their amino acid sequences each of the receptor isoforms belongs to the super-family of receptors that couple to their effectors via G-proteins. Thus each receptor is thought to be composed of seven  $\alpha$ -helical transmembrane spanning regions linked by loops of varying size. Some intriguing observations have been made with respect to receptor/G-protein coupling for the D<sub>2</sub> and D<sub>3</sub> receptor isoforms.

The D<sub>2</sub> receptor has been found to exist in two forms (D<sub>2(short)</sub>, D<sub>2(long)</sub>) related by alternative splicing of a common gene [2,3]. D<sub>2(short)</sub> and D<sub>2(long)</sub> are identical except for a 29 amino acid insertion in the region of the receptor linking the fifth and sixth putative transmembrane spanning regions. This is a region of the receptor thought to be important for coupling to the G-protein so it has been hypothesised that perhaps D<sub>2(short)</sub> and D<sub>2(long)</sub> differ in their coupling to G-proteins. For the D<sub>3</sub> receptor, when expressed in a mammalian cell line, it was not possible to obtain any evidence of coupling to G-proteins for the rat receptor and for the human receptor coupling appeared rather weak [4].

In order to examine the coupling of D<sub>2(short)</sub>, D<sub>2(long)</sub> and D<sub>3</sub> receptors to G-proteins we have expressed the isoforms in mammalian cells from their cloned genes. This report describes the results of experiments de-

signed to examine receptor/G-protein coupling in these recombinant cell systems.

## 2. MATERIALS AND METHODS

### 2.1. Cell lines expressing dopamine receptor isoforms

Cell lines expressing rat D<sub>2(long)</sub> dopamine receptors (CHO6, Ltk59) were obtained as described in [5]. A cell line (LZRI) expressing rat D<sub>2(short)</sub> was obtained as a generous gift from Dr. O. Civelli, Oregon Health Sciences University. In order to express D<sub>3</sub>, a plasmid pSVD<sub>3</sub> including the coding region of rat D<sub>3</sub> and the dihydrofolate reductase gene (kindly supplied by Dr. P. Sokoloff, INSERM, Paris) was transfected into DUK cells (CHO cells lacking dihydrofolate reductase) using the calcium phosphate precipitation method [6]. Transfectants were selected by growth in medium containing 10% dialysed newborn calf serum and 0.05  $\mu$ M methotrexate and lacking hypoxanthine and thymidine and clones were isolated by dilution cloning. Clones were assessed for [<sup>3</sup>H]spiperone binding (see below) and highly expressing clones (DUK25) selected for further analysis.

### 2.2. Cell growth

LZRI, Ltk 59 and CHO6 cells were grown in 175 cm<sup>2</sup> tissue culture flasks in RPMI medium supplemented with 2 mM glutamine, 10% foetal bovine serum and 200  $\mu$ g/ml active geneticin in an atmosphere of 5% CO<sub>2</sub> at 37°C. For DUK25 cells the medium contained methotrexate (0.05  $\mu$ M) in place of geneticin and 10% dialysed newborn calf serum in place of foetal bovine serum.

### 2.3. Preparation of cell membranes

Cells were grown as above until confluent after which the medium was removed and replaced by a buffer containing 20 mM HEPES, 6 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, pH 7.4, 4°C. The cells were then scraped from the flasks and homogenised using 30 strokes of a Dounce homogeniser. The homogenate was centrifuged at 1,700  $\times$  g, 10 min, 4°C and the resulting supernatant was then centrifuged at 48,000  $\times$  g, 1 h, 4°C. The pellet from this centrifugation was resuspended in a buffer containing 20 mM HEPES, 6 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, pH 7.4, 4°C at a protein concentration of about 1 mg/ml and stored at -80°C in 1 ml aliquots.

### 2.4. Ligand binding assays for D<sub>2</sub> and D<sub>3</sub> dopamine receptors

Cell membrane preparation (30–50  $\mu$ g protein) was incubated in triplicate with [<sup>3</sup>H]spiperone (28 Ci/mmol, 0.25 nM for competition

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experiments with  $D_2$ , 0.5 nM for  $D_3$ , 40 pM–5 nM for saturation analyses) and other drugs where appropriate in a final volume of 1 ml assay buffer (20 mM HEPES, 6 mM  $MgCl_2$ , 1 mM EDTA, 1 mM EGTA, pH 7.4) at 25°C for 45 min. Specific [ $^3H$ ]spiperone binding was defined as that binding inhibited by 3  $\mu M$  (+)-butaclamol. Incubations were terminated by rapid filtration through GF/B glass fibre filters on a Brandel cell harvester with three washes of 3 ml of phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM  $KH_2PO_4$ , 5 mM  $Na_2HPO_4$ , pH 7.4). Filters were then soaked for at least 6 h in 2 ml of LKB optiphase 'Hisafe' 3 scintillation fluid before determination of radioactivity by liquid scintillation counting. Ligand-binding data were analysed using the EBDA and LIGAND computer programs.

### 3. RESULTS

The recombinant cell lines used in this study each expressed the respective dopamine receptor isoform at a high level (about 1 pmol/mg protein, about  $10^5$  receptors/cell) as judged by saturation binding analyses with [ $^3H$ ]spiperone (Table I). The affinities of the  $D_{2(short)}$  and  $D_{2(long)}$  receptors for [ $^3H$ ]spiperone were rather similar in the three cell lines whereas the  $D_3$  receptor showed a markedly lower affinity.

The coupling of the receptor isoforms to G-proteins in the different cell lines was assessed from the shape of agonist/[ $^3H$ ]spiperone competition curves in the absence of GTP and from the effects of GTP (100  $\mu M$ ) on the competition curve. For both agonists tested (apomorphine and dopamine) in each of the four cell lines in the absence of GTP the competition curves were characterised by Hill coefficients less than one and were described best by models with two binding sites of higher and lower affinity (Table II, Fig. 1). The effect of GTP (100  $\mu M$ ) was in all cases to shift the competition curve to higher agonist concentrations and in the presence of GTP a one binding site model fitted the data best.

For the  $D_{2(short)}$  and  $D_{2(long)}$  receptors the  $K_i$  values for

Table I  
Saturation binding analysis of [ $^3H$ ]spiperone binding to recombinant cell lines expressing  $D_2$  and  $D_3$  dopamine receptors

	LZRI (L- $D_{2(short)}$ )	Ltk59 (L- $D_{2(long)}$ )	CHO6 (CHO- $D_{2(long)}$ )	DUK25 (CHO- $D_3$ )
$K_d$ (pM)	42 $\pm$ 10	68 $\pm$ 6	51 $\pm$ 6	270 $\pm$ 70
$B_{max}$ (fmol/mg)	927 $\pm$ 44	1,185 $\pm$ 126	873 $\pm$ 73	1,497 $\pm$ 178

Data are expressed as mean  $\pm$  S.E.M. for three or more observations. Data for LZRI, Ltk59 and CHO6 are taken from [5].

the higher and lower affinity agonist binding sites derived from the two site model were in reasonable agreement in the three cell lines. The  $D_3$  receptor generally showed correspondingly higher agonist affinities. The  $K_i$  value for agonists in the presence of GTP agreed well with the  $K_i$  value for the lower affinity site seen in the absence of GTP for  $D_2$  and  $D_3$  receptor isoforms.

The proportions of the higher and lower affinity sites seen in the absence of GTP were different in the different cell lines expressing  $D_2$  receptor isoforms. There was a tendency to a greater proportion of higher affinity sites for  $D_{2(short)}$  compared with  $D_{2(long)}$  expressed in Ltk<sup>-</sup> cells and there was a similar tendency for  $D_{2(long)}$  expressed in CHO cells compared with  $D_{2(long)}$  expressed in Ltk<sup>-</sup> cells.

### 4. DISCUSSION

In this report we describe the use of four recombinant cell lines expressing dopamine receptor isoforms from their cloned genes in order to study the coupling of dopamine receptor isoforms with the endogenous G-proteins of the cells. The cell lines all express the recep-

Table II  
Agonist binding to recombinant cell lines expressing  $D_2$  and  $D_3$  dopamine receptors, effects of GTP (100  $\mu M$ )

	LZRI (L- $D_{2(short)}$ )	Ltk59 (L- $D_{2(long)}$ )	CHO6 (CHO- $D_{2(long)}$ )	DUK25 (CHO- $D_3$ )
Apomorphine				
Control $K_i^h$	0.75 $\pm$ 0.31 nM	0.36 $\pm$ 0.12 nM	1.17 $\pm$ 0.55 nM	0.22 $\pm$ 0.17 nM
$K_i^l$	41.7 $\pm$ 13.7 nM	76.1 $\pm$ 24.2 nM	61.5 $\pm$ 13.7 nM	12.2 $\pm$ 2.2 nM
%l	52 $\pm$ 8	70 $\pm$ 5	66 $\pm$ 4	84 $\pm$ 5
+GTP $K_i$	43.3 $\pm$ 8.8 nM	56.9 $\pm$ 19.1 nM	73.7 $\pm$ 14.2 nM	19.2 $\pm$ 3.2 nM
n	5	4	5	3
Dopamine				
Control $K_i^h$	7.7 $\pm$ 4 nM	8.0 $\pm$ 3.0 nM	29.5 $\pm$ 5.9 nM	2.5 $\pm$ 1.3 nM
$K_i^l$	750 $\pm$ 500 nM	960 $\pm$ 180 nM	3,480 $\pm$ 780 nM	68 $\pm$ 27 nM
%l	42 $\pm$ 7	75 $\pm$ 3	52 $\pm$ 6	60 $\pm$ 5
+GTP $K_i$	650 $\pm$ 130 nM	990 $\pm$ 230 nM	1,470 $\pm$ 120 nM	117 $\pm$ 67 nM
n	4	5	4	4

Agonist/[ $^3H$ ]spiperone competition data were obtained as described in section 2. Data were analysed by the computer programs EBDA and LIGAND and for all the control cases a two-site model was the best fit whereas in the presence of GTP (100  $\mu M$ ) a one site fit was best. The parameters shown are the  $K_i$  values for the higher and lower affinity sites and the % lower affinity sites for the two-site fit and for the one-site fit, the  $K_i$  value. Data are presented as mean  $\pm$  S.E.M. for  $n$  experiments performed in parallel (control/+GTP).

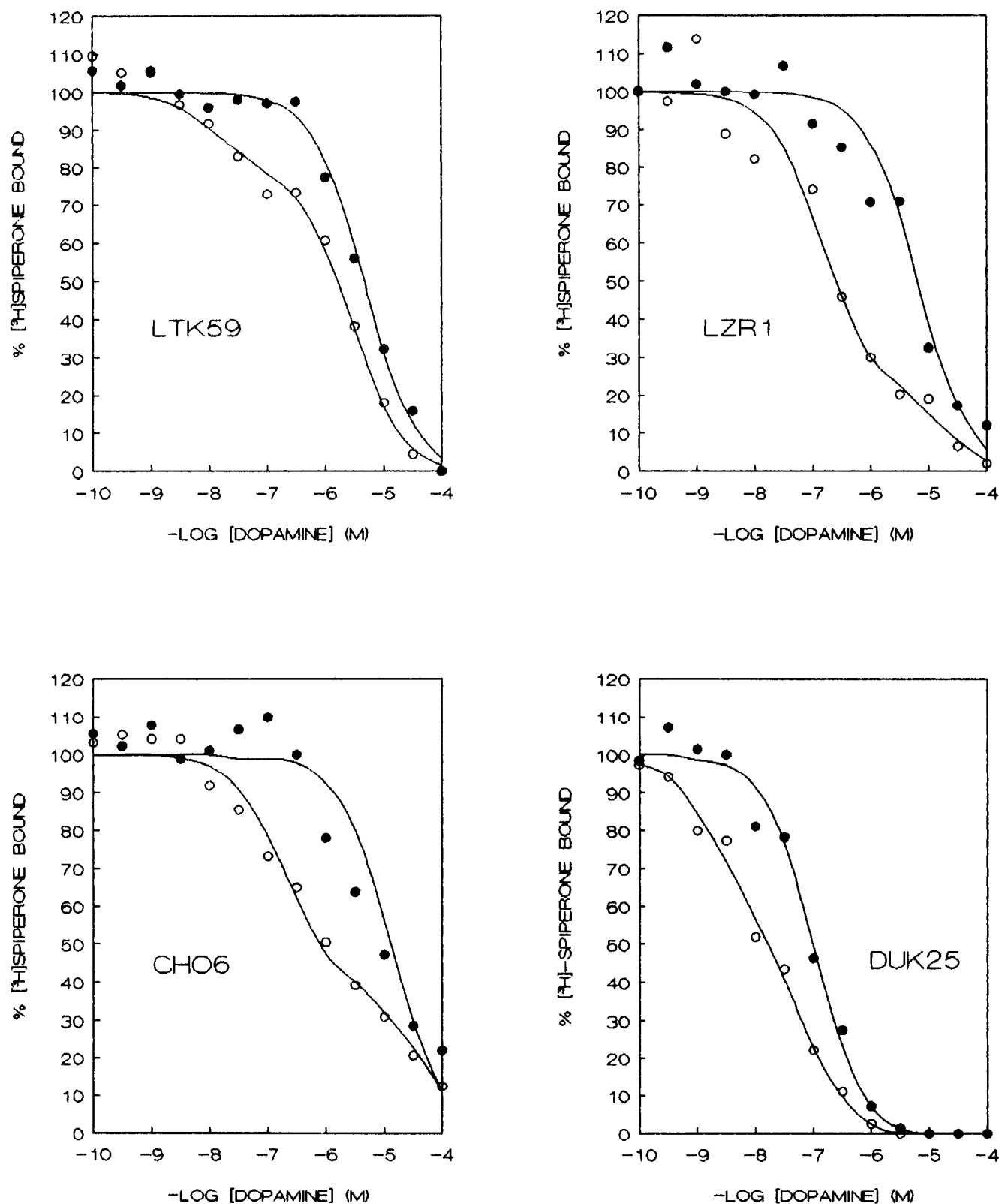


Fig. 1. Agonist binding to recombinant cell lines expressing  $D_2$  and  $D_3$  dopamine receptors, effect of GTP. Cell lines used were LTK59 ( $D_{2(long)}$ ), LZR1 ( $D_{2(short)}$ ), CHO6 ( $D_{2(long)}$ ) and DUK25 ( $D_3$ ). Dopamine/[<sup>3</sup>H]spiperone competition data were obtained in the absence (○) and presence (●) of 100  $\mu$ M GTP as described in section 2. The data are representative curves from experiments replicated as in Table II. The curves are the best fits to the data for two-site models (○) and one-site models (●).

tor isoforms at about the same level so that there should be no major effects of differences in receptor/G-protein ratio on the results observed when different isoforms are expressed in the same cell host. In each of the cell lines the dopamine receptor isoform ( $D_{2(\text{short})}$ ,  $D_{2(\text{long})}$ ,  $D_3$ ) appears to couple to the endogenous cellular G-proteins. This is based on the observations that agonist/ $[^3\text{H}]$ piperone competition curves are best fitted by two binding site models in the absence of guanine nucleotide and addition of GTP shifts the competition curve to higher agonist concentrations and agonists compete according to a one binding site model.

In order to assess the effect of cell host environment on the observed results  $D_{2(\text{long})}$  was expressed in CHO cells and  $\text{Ltk}^-$  cells. Differences in receptor/G-protein coupling are apparent between the two recombinant cell lines as indicated by the different proportions of higher and lower affinity agonist binding sites seen in the two cases. This presumably reflects interactions of  $D_{2(\text{long})}$  with different cellular populations or amounts of G-proteins in the two cell lines. Antagonist binding to  $D_{2(\text{long})}$  in the two cell lines is essentially identical [5] so that there is unlikely to be an effect of any difference in the cell membrane on the binding of agonists in the present study.

For  $D_{2(\text{short})}$  and  $D_{2(\text{long})}$  expressed separately in the same cell host ( $\text{Ltk}^-$  cells) again differences in apparent receptor/G-protein coupling were seen. This is based on the greater proportion of higher affinity agonist binding sites for  $D_{2(\text{short})}$  as compared with  $D_{2(\text{long})}$ . Although this difference cannot be interpreted at a molecular level it presumably reflects the difference in structure between  $D_{2(\text{short})}$  and  $D_{2(\text{long})}$  which resides in one part of the receptor thought to be of major importance to receptor/G-protein coupling, the putative third intracellular loop. Others have reported differences in agonist binding for  $D_{2(\text{short})}$  and  $D_{2(\text{long})}$  [7] in agreement with the present data with a tendency of  $D_{2(\text{short})}$  to show a greater proportion of higher affinity agonist binding sites. This may be associated with the greater apparent functional efficacy of  $D_{2(\text{short})}$  [7,8].

For  $D_{2(\text{short})}$  and  $D_{2(\text{long})}$  the  $K_i$  values for the higher and lower affinity agonist binding sites are rather similar in the three cell lines tested, it is the proportions of the sites that differ. This suggests that the free receptor and the receptor/G-protein complex have similar properties once formed but the proportions of these can be modulated by cellular populations of G-proteins ( $\text{L}-D_{2(\text{long})}$  versus  $\text{CHO}-D_{2(\text{long})}$ ) or the structure of the part of the receptor coupling to the G-protein ( $D_{2(\text{short})}$  versus  $D_{2(\text{long})}$ ).

For the  $D_3$  dopamine receptor clear evidence of cou-

pling to G-proteins was obtained from these studies. The effect was qualitatively similar to that seen for the  $D_2$  isoforms although agonist affinities tended to be higher at the  $D_3$  receptor as has been described previously [9]. The observation of coupling to G-proteins for the  $D_3$  receptor was unexpected as previous reports using CHO cells have indicated no coupling for the rat receptor [4]. Our results are quite clear so it is necessary to seek an explanation for the difference. One possibility is that in isolating the recombinant cell line a clone has been obtained which allows receptor/G-protein interaction whereas previous cell lines did not allow this. This could reflect the presence of particular G-proteins, other facilitatory proteins or the state of the receptor, for example its phosphorylation state.

Alternatively the difference could reflect the assay conditions. Other reports have used agonist/ $[^{125}\text{I}]$ iodosulpride competition and the buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , pH 7.5) and incubation conditions (30°C, 30 min) [9] are different to those used here. Given that our observations on  $D_2$  receptors are quite similar to those reported previously despite the different assay conditions [9,10] this would suggest a major difference between  $D_2$  and  $D_3$  dopamine receptors. This most likely reflects the requirement of the  $D_3$  receptor for coupling to a different G-protein.

In summary these data show that three  $D_2$ -like receptor isoforms ( $D_{2(\text{short})}$ ,  $D_{2(\text{long})}$  and  $D_3$ ) couple to G-proteins in the recombinant cell lines used here.

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