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Advantages of heterologous expression of human D2long dopamine receptors in human neuroblastoma SH-SY5Y over human embryonic kidney 293 cells

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1 The human D2long dopamine receptor when expressed heterologously in a human neuronal cell line, SH-SY5Y, produced more robust functional signals than when expressed in a human embryonic kidney cell line, HEK293. Quinpirole (agonist)-induced GTPγ^{35}S binding and high affinity sites were 3–4 fold greater in SH-SY5Y than in HEK293 cells.

2 N-type Ca^{2+} channel currents present in SH-SY5Y cells, but not HEK293 cells, were inhibited potently by quinpirole with a half-maximal inhibitory concentration of 0.15 ± 0.03 nM. Inhibition of adenylyl cyclases by agonists, on the other hand, was of similar potency and efficacy in the two cell lines.

3 GTPγ^{35}S-Bound Gα subunits from quinpirole-activated and solubilized membranes were monitored upon immobilization with various Gα-specific antibodies. Gα_i and Gα_o subunits were highly labelled with GTPγ^{35}S in SH-SY5Y cells, but only Gα_i subunits were labelled in HEK293 cells. The additional G_o coupling in SH-SY5Y cells could arise, at least in part, from the presence of G_o coupled-effectors, such as the N-type Ca^{2+} channel, and may contribute to robust agonist-induced GTPγ^{35}S binding, which is a reliable means for measuring ligand intrinsic efficacy.

4 It appears that expression of neuronal G protein-coupled receptors in neuronal environments could reveal additional functional characteristics that are absent in non-neuronal cell lines. This appears to be due to, at least in part, to the presence of neuron-specific effectors. These findings underscore the importance of the cellular environment in which drug actions are examined, particularly in the face of intensive efforts to develop drugs for G protein-coupled receptors of various origins.

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Keywords: Human D2long dopamine receptor; agonist-induced GTPγ^{35}S binding; immobilization of GTPγ^{35}S-bound Gα subunits; intrinsic efficacy; G protein-couplings; SH-SY5Y cells; human embryonic kidney 293 cells

Abbreviations: AC, adenylyl cyclase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate; EC_{50}, a half-maximal effective concentration; GPPNHP, 5′-guanylylimidodiphosphate; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IC_{50}, a half-maximal inhibitory concentration; K_D, dissociation constant; K_i, inhibition constant; PCR, polymerase chain reaction

Introduction

Heterologous expressions of neuronal catecholamine receptors have been frequently reported in diverse non-neuronal cell lines such as human embryonic kidney (HEK) 293 cells, African green monkey SV40 transformed cells and Chinese hamster ovary cells. Studies with such cloned receptors (e.g. dopamine receptors) have provided valuable information about their binding and functional characteristics (Civelli *et al*., 1993; Gardner *et al*., 1996; Seeman & Van Tol, 1994; Sokoloff *et al*., 1990; Tang *et al*., 1994; Missale *et al*., 1998). Signalling pathways in non-neuronal cells, however, could differ somewhat from those in neuronal cells, due to different target effectors and potentially different localizations of G proteins with respect to receptors. We have shown earlier that the human D3 dopamine receptor, when expressed heterologously in SH-SY5Y cells (a human neuroblastoma line), produced much more robust functional signals than when expressed in HEK293 cells (Zaworski *et al*., 1999). This could be attributed in part to the presence of D3-selective neuronal effectors such as adenylyl cyclase V (Robinson & Caron, 1997) and N-type Ca^{2+} channels in the neuroblastoma cell, but not in the epithelial cell (Zaworski *et al*., 1999). These findings led us to the current investigation of the D2long receptor. The D2long and D3 dopamine receptors belong to the same family of D2-like receptors, and share structural and functional features, e.g. a high identity in the primary sequence and inhibition of adenylyl cyclases (Civelli *et al*., 1993; Seeman & Van Tol, 1994; Sokoloff *et al*., 1990; Tang *et al*., 1994; Missale *et al*., 1998). In this study, we express heterologously the human D2long receptor in SH-SY5Y neuroblastoma and HEK293 cells, and show the benefit of expressing neuronal receptors in neuronal environments.

Methods

The human cDNA for the D2long dopamine receptor has been cloned from a human cDNA library using polymerase chain reactions (PCR), inserted into a PCI-Neo mammalian expression vector, and employed for transfection of HEK293 or SH-SY5Y cells, using Ca^{2+} phosphate precipitation techniques. Cells were selected for a month in the presence of G-418 (400 μg ml^{-1}). Membranes from the transfected cells were prepared by standard procedures including cell

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homogenization and differential centrifugation as described elsewhere (Pregenzer *et al*., 1993). Binding of radioactive ligands was measured in membranes expressing recombinant receptors, using filtration techniques as described elsewhere (Pregenzer *et al*., 1993). Briefly, [^{3}H]-raclopride binding was measured in a medium containing (mM) NaCl 100, $MgCl_2$ 2, EDTA 1, HEPES/Tris 20 (pH 7.4), the radioactive ligand at various concentrations (0.1–30 nM for typical binding profiles), and about 10 μg membrane protein, in a total volume of 500 μl at 23°C for 60 min. Reaction mixtures were filtered over Whatman GF/B filters under vacuum, and filters were washed three times with 4 ml of ice-cold buffer containing 50 mM Tris/HCl (pH 7.4). Non-specific binding was estimated in the presence of excess unlabelled raclopride (10 μM). All the stock solutions for ligands were prepared in 0.1% ascorbic acid. Displacement of [^{3}H]-raclopride by test compounds (competition assay) was carried out in the same assay buffer with the radioactive ligand at 2 nM, which yielded a near maximal ratio of specific to nonspecific binding.

GTPγ^{35}S binding was measured following the procedures reported earlier (Chabert *et al*., 1994; Pregenzer *et al*., 1997) in the medium containing (mM) HEPES 25, NaCl 100, EDTA 1, $MgCl_2$ 3, dithiothreitol 0.5, 0.003% digitonin, 2 nM GTPγ^{35}S ($5-3\times10^5$ c.p.m./assay), and about 10 μg membrane protein in a volume of 120 μl. We fixed the concentration of GTPγ^{35}S at 2 nM, which provided the maximal ratio of the agonist-induced to the basal GTPγ^{35}S binding, and is also close to the reported half-maximal effective concentration (EC_{50}) ranging from 0.7–2.5 nM at G_i/G_o coupled receptors. Test ligands were included at 10 μM, unless indicated otherwise. Membranes were pre-incubated with 100 μM 5′-adenylylimmidodiphosphate for 30 min at room temperature, and subsequently with 10 μM GDP for 10 min on ice. Reactions were initiated by adding treated membranes to the rest of reaction components, and continued at 30°C for 30 min. Reaction mixtures were filtered over Whatman GF/B filters under vacuum, and filters were washed three times with 4 ml of ice-cold buffer containing (mM) NaCl 100, Tris/HCl 20, $MgCl_2$ 25 (pH 8.0). Agonist-induced GTPγ^{35}S binding was obtained by subtracting that observed without agonists. Binding data were analysed using a nonlinear regression method (Sigma Plot).

Cellular changes in cyclic AMP were measured using a FlashPlate assay kit from NEN™ Life Science Products. Briefly, cells were grown in a 96-well plate to about 80% confluency, and then treated with forskolin at a submaximal concentration (typically 10 μM) with or without test ligands for 30 min. Cyclic AMP in cell lysates was measured using the competition between [^{125}I]-cyclic AMP and non-radioactive cyclic AMP for a fixed number of antibody binding sites in microplates coated with solid scintillant.

The whole-cell configuration of the patch clamp technique (Hamill *et al*., 1981) was used to record Ca^{2+}-channel currents. Patch pipettes were made of borosilicate glass (Kimax-51, Kimble Products, U.S.A.) and fire polished. Pipettes had a resistance of 5–10 MΩ (for whole-cell recording) in solutions described below. Currents were recorded by a current-voltage converter consisting of an Axopatch-1D amplifier and a CV-4 headstage (Axon Instrument Co., U.S.A.) and stored on diskettes in a PC computer using the 'pClamp' acquisition software (Axon Instrument Co.). BH-1 Bath headstage (Axon Instrument Co.) was utilized to compensate for bath potential drifts. The pipette solution contained (mM) N-methylglucamine 140, EGTA 10, glucose 5, GTP 0.3 and N-methylglucamine-HEPES 10, pH 7.2. External solution contained (mM) $BaCl_2$ 50, CsCl 35, tetraethylammonium chloride 25, glucose 25, N-methylglucamine-HEPES 10, pH 7.3 and 0.5 μM tetrodotoxin. All experiments were performed at room temperature (23°C).

GTPγ^{35}S binding to Gα subunits was monitored following the method described elsewhere (Okamoto *et al*., 1992; Zaworski *et al*., 1999) with the following modifications: Activation of receptor by quinpirole was performed in the presence of GTPγ^{35}S at 4 nM instead of 60 nM, followed by solubilization of membranes with detergents. At 60 nM, GTPγ^{35}S produced a background binding that was too high. Briefly, membranes were incubated in the presence of GTPγ^{35}S (4 nM) and quinpirole (10 μM) under conditions identical to those for GTPγ^{35}S binding as described above. Treated membranes were solubilized with an equal volume of a buffer containing (mM) Tris/HCl 100, pH 8.0, $MgCl_2$ 10, NaCl 100 and 3-[(3-cholamido)dimethylammonio]-1-propane sulphate (CHAPS) 0.6% for 30 min on ice, and were diluted to a final CHAPS concentration of 0.125%. Aliquots of the mixtures (typically 300 μl) were transferred to a 96 well plate which had been coated successively with goat anti-rabbit antibodies (1 : 100 dilution), bovine serum albumin (5 mg ml^{-1}) and one of the affinity-purified rabbit antibodies for various Gα subunits (1 : 200 dilution). After incubation at room temperature for 1 h, individual wells were washed and counted for ^{35}S, using a standard scintillation cocktail and a β-counter. The antibodies used are specific for $G\alpha_i$ (the C-terminal sequence) (Santa Cruz Biotechnology), $G\alpha_s$ (the C-terminal sequence, 385–394), $G\alpha_{q/11}$ (the common C-terminal sequence, QLNLKEYNLV) and $G\alpha_{13}$ (the sequence, 367–377) (Calbiochem). The mouse monoclonal antibody raised against bovine G_o protein was obtained from Chemicon. Agonist-induced GTPγ^{35}S binding was computed by subtracting that observed without test agonists.

GTPγ^{35}S and [^{3}H]-raclopride were obtained from NEN Life Sciences Products. CHAPS, 5′-guanylylimidodiphosphate (GPPNHP) and 5′-adenylylimmidodiphosphate were purchased from Sigma. Tetrodotoxin was obtained from Calbiochem. (5R)-5-(Methylamino)-5,6-dihydro-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (PNU-95666) and (5R)-5-(dipropylamino)-5,6-dihydro-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (PNU-86170) were synthesized at Pharmacia. (1R,2S)-5-Methoxy-1-methyl-dipropylaminotetralin (UH-232) and R(+)-7-hydroxy-dipropylaminotetralin (7-OH-DPAT) were obtained from the University of Gothenberg (Dr A. Carlsson), Sweden. 5-Chloro-2-methoxy-4-(methylamino)-N-[2-metyl-1-(phenylmethyl)-3-pyrrolidinyl]-benazmide (YM-9151-02) was obtained from Yamanouchi Co., Japan. All other chemicals were obtained from standard sources.

Results

We examined ligand binding properties of the human D2long dopamine receptor expressed in HEK293 and in SH-SY5Y cells. Binding data for [^{3}H]-raclopride at various concentrations fitted to a one-site binding model, and yielded dissociation constants (K_D) of 1.1 ± 0.1 and 1.4 ± 0.4 nM in HEK293 and SH-SY5Y cells, respectively, and maximal binding of 2.9 ± 0.3 and 3.2 ± 0.4 pmol mg^{-1} protein, respectively. The receptor density in HEK293 and SH-SY5Y cells, measured as [^{3}H]-raclopride binding, was stable up to at least 16 passages. The membranes were prepared from cells between passage 6 to 10, and used throughout this study. Competition binding experiments, using [^{3}H]-raclopride, were

carried out with standard dopaminergic ligands at various concentrations. Binding data for antagonists were analysed using a one-site binding model. Inhibition constants (K_i) for spiperone, chlorpromazine, YM-9151-02, UH-232 and haloperidol (Table 1), obtained using Cheng & Prusoff (1973) equation, were in good agreement between the two cell lines, and were also comparable with those reported in the literature (Civelli *et al*., 1993; Seeman & Van Tol, 1994). For agonists, as reported earlier (Civelli *et al*., 1993; Seeman & Van Tol, 1994; Missale *et al*., 1998), large affinity differences were observed between their high and low affinity sites (G protein-coupled and -uncoupled receptor phenotype, respectively).

Displacements of [^{3}H]-raclopride binding by quinpirole (a prototypic agonist) in SH-SY5Y cell membranes fitted to a two-site binding model (Figure 1) with a K_i of 19 ± 4 and 2336 ± 380 nM for the high and low affinity sites, respectively, and their relative proportion of 30 ± 4 and 70 ± 5% of the total binding sites, respectively. Addition of GPPNHP (10 μM) abolished high affinity sites for quinpirole, and displacement data fitted to a one-site binding model with a K_i of 2452 ± 290 nM. In HEK293 cell membranes, similar K_i values for the low and high affinity sites were observed, 19 and 2487 ± 285 nM, respectively, but the population of the high affinity sites amounted to only 10 ± 3% of the total binding sites.

Agonist-bound G protein-coupled receptors promote the exchange of GDP with GTP on G protein α subunits. This step can be monitored with GTPγ^{35}S, a slowly hydrolyzed GTP analogue. Quinpirole, in a concentration-dependent manner, enhanced GTPγ^{35}S binding in membranes from the SH-SY5Y cells, with an EC_{50} of 493 ± 53 nM and maximal binding of 369 ± 19 fmol mg^{-1} protein (Figure 1). In membranes from HEK293 cells, quinpirole enhanced

Table 1 Comparison of binding properties of antagonists for the human D2long dopamine receptor expressed in SH-SY5Y and HEK293 cells

Compounds	*SH-SY5Y* (K_i, nM)	*Hill slope*	*HEK293* (K_i, nM)	*Hill slope*
Raclopride	1.4 ± 0.4		1.1 ± 0.1	
Spiperone	0.05 ± 0.007	1.1 ± 0.2	0.05 ± 0.008	1.0 ± 0.1
Chlorpromazine	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.0 ± 0.2
YM-9151-02	0.039 ± 0.004	1.3 ± 0.2	0.033 ± 0.003	1.3 ± 0.2
UH-232	13.6 ± 0.6	0.9 ± 0.1	13.0 ± 0.9	0.9 ± 0.2
Haloperidol	0.89 ± 0.07	0.9 ± 0.1	0.89 ± 0.03	1.0 ± 0.1

The K_i for several antagonists were obtained from competition binding experiments using [^{3}H]-raclopride in the presence of test ligands at various concentrations. The K_D values were listed for raclopride. For the other compounds, the IC_{50} values from displacement profiles were converted to K_i using the Cheng & Prussoff equation. Experiments were carried out at room temperature in membranes from SH-SY5Y or HEK293 cells expressing the human D2long dopamine receptor. The maximal binding sites obtained as [^{3}H]-raclopride binding were 2.9 ± 0.3 and 3.2 ± 0.4 pmol mg^{-1} protein for HEK293 and SH-SY5Y cells, respectively. The data are mean$\pm$s.e. mean ($n=3$) from response profiles.

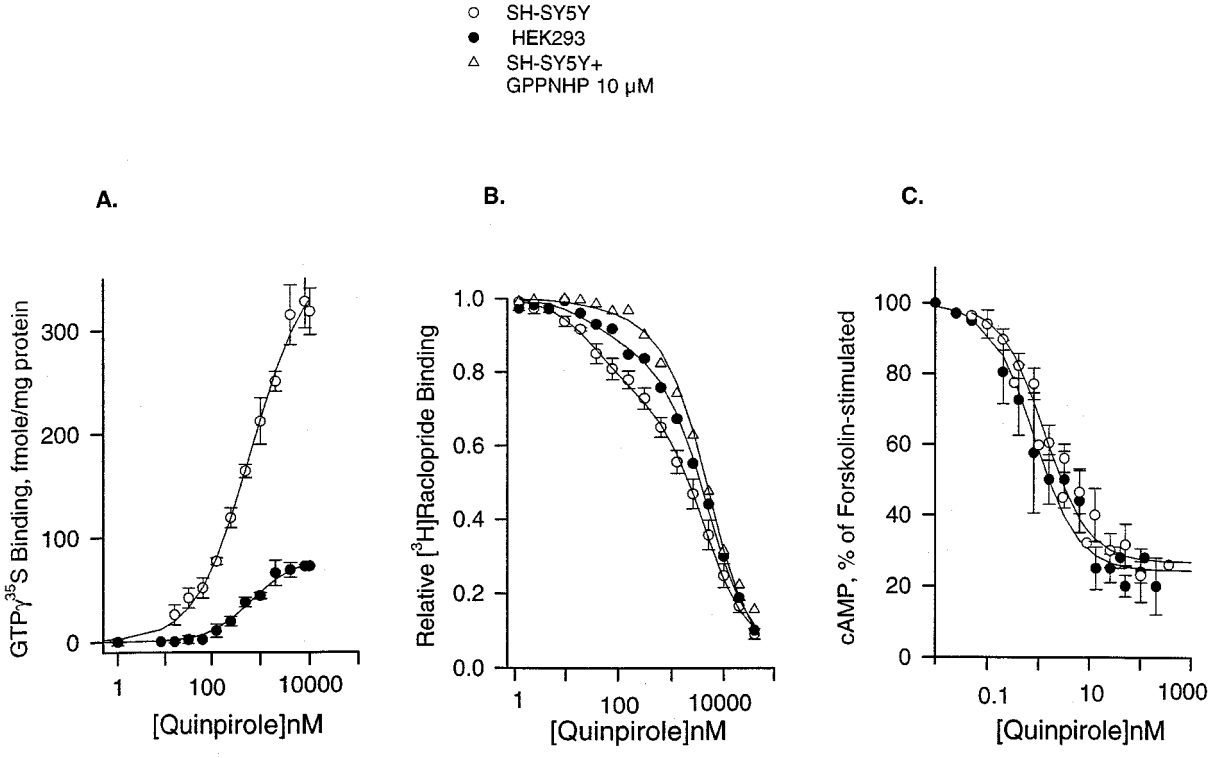

Figure 1 Comparison of functional properties of the human D2long receptor expressed in HEK293 and SH-SY5Y cells. (A) Enhancements of GTPγ^{35}S (2 nM) binding by quinpirole at various concentrations were measured in membranes from HEK293 and SH-SY5Y cells expressing the receptor. Amounts of agonist-dependent GTPγ^{35}S binding were obtained by subtracting that observed without the agonist. The solid lines represent data fitted to a single-site binding isotherm. (B) [^{3}H]-Raclopride (2 nM) binding to D2long receptors in HEK293 or SH-SY5Y cell membranes were biphasically displaced by quinpirole at various concentrations. The solid lines represent data fitted to a two-site binding model. High affinity sites for quinpirole in SH-SY5Y cells were completely abolished in the presence of 10 μM GPPNHP. (C) Quinpirole concentration-dependently inhibited forskolin (10 μM)-stimulated cyclic AMP formation in HEK293 and SH-SY5Y cells with similar potency and efficacy. The solid lines represent data fitted to a one-site model. The data are the mean$\pm$s.e.mean ($n=3$).

GTPγ^{35}S binding by only 22% of that observed in SH-SY5Y cell membranes, with an EC_{50} of 572 ± 78 nM and maximal binding of 80 ± 3 fmol mg^{-1} protein (Figure 1). Quinpirole-induced GTPγ^{35}S binding in SH-SY5Y and HEK293 cell membranes was blocked by haloperidol (10 μM) (data not shown). Haloperidol by itself reduced the basal GTPγ^{35}S binding by 9%, as normalized for that observed with quinpirole, probably by stabilizing receptors in inactive states.

The D2long receptor is known to mediate the inhibition of adenylyl cyclases (Civelli *et al*., 1993; Missale *et al*., 1998). In SH-SY5Y cells, quinpirole, in a concentration-dependent manner, reduced forskolin (10 μM)-stimulated cyclic AMP formation, with a half-maximal inhibitory concentration (IC_{50}) of 1.3 ± 0.2 nM and maximal inhibition of 74 ± 2% ($n=3$) (Figure 1). Similar potency and efficacy were observed with parallel assays in HEK293 cells, with an IC_{50} of 0.9 ± 0.2 nM and maximal inhibition of 79 ± 2% ($n=3$).

High threshold-activated Ca^{2+} channel currents in SH-SY5Y cells represent ω-conotoxin-sensitive N-type channels (Friederich *et al*., 1993; Reeve *et al*., 1995). Currents were measured in the whole cell configuration, with a pulse potential of 25 mV from a holding potential of -100 mV, using Ba^{2+} as the charge carrier. Quinpirole (10 nM) reversibly reduced currents by 70% in SH-SY5Y cells expressing the D2long receptor (Figure 2), but showed no effect in mock transfected cell (data not shown). The agonist, in a concentration-dependent manner, reduced the peak current amplitude with an IC_{50} of 0.15 ± 0.03 nM and maximal inhibition of 69 ± 3% ($n=3$) (Figure 2).

GTPγ^{35}S-Bound Gα subunits from quinpirole-activated and solubilized membranes of SH-SY5Y and HEK293 cells were monitored, upon immobilization with various Gα-specific antibodies (Okamoto *et al*., 1992) (Figure 3). In membranes prepared from SH-SY5Y cells, high levels of GTPγ^{35}S were associated with the Gα_i- and the Gα_o-specific antibodies. Typical values were 15734 ± 386 and 8895 ± 239 c.p.m. for the anti-Gα_i and the anti-Gα_o, respectively, with the background binding (no quinpirole) of 2791 ± 240 and 2808 ± 116 c.p.m., respectively. No appreciable agonist-induced GTPγ^{35}S association was observed with the other antibodies. Individual contributions of Gα_i, Gα_o, Gα_s, G$\alpha_{q/11}$ and G_{13} to the total binding in SH-SY5Y cell line were 65 ± 2, 31 ± 1, 4 ± 2, -1.3 ± 1.3 and -2.6 ± 1.3% ($n=3$), respectively. In membranes prepared from HEK293 cells, we also observed quinpirole-induced association of GTPγ^{35}S with the anti Gα_i (Figure 3), amounting to 29% of the corresponding value in SHSY5Y cell membranes. No appreciable binding was observed for the anti-Gα_o or the other antibodies. To underscore differences in the two cell lines, relative contributions of each Gα in HEK293 cell line were normalized for the

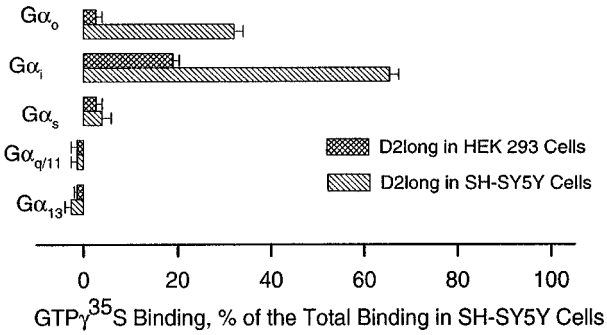

Figure 3 Immunoimmobilization of GTPγ^{35}S-bound Gα subunits from quinpirole-activated and solubilized membranes from HEK293 and SH-SY5Y cells expressing the human D2long dopamine receptor. High levels of GTPγ^{35}S radioactivity were observed with the antibodies specific for Gα_i and Gα_o in SH-SY5Y cell membranes and only Gα_i in HEK293 cell membranes. No appreciable levels of GTPγ^{35}S were observed with the antibodies specific for Gα_s, G$\alpha_{q/11}$ and Gα_{13}. Contributions of each Gα were normalized for the total binding in SH-SY5Y cell line; 65 ± 2, 31 ± 1, 4 ± 2, -1.3 ± 1.3 and -2.6 ± 1.3% for Gα_i, Gα_o, Gα_s, G$\alpha_{q/11}$ and G_{13}, respectively. To underscore differences in the epithelial and neuronal cell lines, contributions of each Gα in HEK293 cell line were also normalized for the total binding in SH-SY5Y cell line; 19 ± 2, 2.6 ± 1.3, 2.6 ± 1.3, -1.3 ± 1.2 and -2 ± 0.6% for Gα_i, Gα_o, Gα_s, G$\alpha_{q/11}$ and G_{13}, respectively. The data are mean$\pm$s.e.mean ($n=3$).

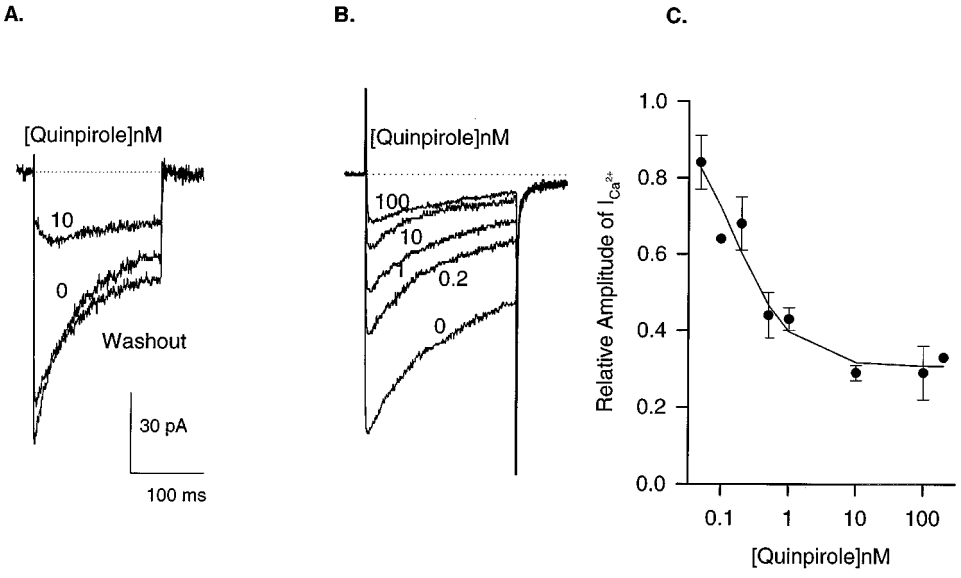

Figure 2 Inhibition of N-type Ca^{2+} channel currents by quinpirole in SH-SY5Y cells expressing the human dopamine D2long receptor. N-type Ca^{2+} channel currents were measured using whole cell patch clamp techniques. Currents (Ba^{2+} as charge carrier) were evoked by a pulse potential of 25 mV with duration of 500 ms from a holding potential of -100 mV. (A) Quinpirole at 10 nM reversibly blocked Ca^{2+} channel currents. (B) The agonist also concentration-dependently reduced currents. (C) The concentration-response profile fitted to a one-site model with an IC_{50} of 0.15 ± 0.03 nM and maximal inhibition of 69 ± 3% ($n=3$).

total binding in the SH-SY5Y cell line, and computed to be 19 ± 2, 2.6 ± 1.3, 2.6 ± 1.3, -1.3 ± 1.2 and $-2\pm0.6\%$ ($n=3$) for $G\alpha_i$, $G\alpha_o$, $G\alpha_s$, $G\alpha_{q/11}$ and G_{13}, respectively.

The human D2long receptor in SH-SY5Y cells, because of its robust functional coupling, could be useful for studying pharmacological properties of dopaminergic ligands, and here we examined six standard agonists (Figure 4). PNU-86150, PNU-95666, pramipexole, dopamine and 7-OH DPAT displaced [^{3}H]-raclopride binding in a biphasic manner, like quinpirole. Analysis of the data using a two-site binding model (Figure 3) showed the K_i ratio of low to high affinity sites being >100 for quinpirole, PNU-95666, pramipexole and dopamine, and 34 and 23 for PNU-86170 and 7-OH DPAT, respectively (Table 2). Terguride displaced [^{3}H]-raclopride binding in a monophasic manner, with a K_i of 0.89 ± 0.03 nM. The relative proportion of high affinity sites amounted to nearly 30% for quinpirole, PNU-86170, pramipexole and dopamine, but to only 20% for PNU-95666 and 7-OH DPAT (Table 2). For GTPγ^{35}S binding (Table 3), pramipexole, dopamine and PNU-86170 enhanced nucleotide binding as much as quinpirole, with EC_{50} values of 496 ± 36, 1460 ± 220 and 107 ± 10 nM, respectively. PNU-95666, 7-OH DPAT and terguride produced somewhat lower maximal GTPγ^{35}S binding, 76 ± 3, 74 ± 4 and $34\pm2\%$, respectively, as normalized to that of quinpirole, with EC_{50} values of 1612 ± 195, 106 ± 21 and 1.6 ± 0.4 nM, respectively. Note that the relative efficacies for PNU-95666 and 7-OH DPAT were appreciably less than that for quinpirole, as estimated from both GTPγ^{35}S binding and high affinity sites.

All agonists tested here also reduced forskolin (10 μM)-stimulated cyclic AMP formation in a concentration-dependent manner. Their maximal level of inhibition was similar (71 to 84%) (Figure 4 and Table 3), and their EC_{50} values were at nanomolar or subnanomolar ranges which are markedly smaller than the corresponding values obtained with GTPγ^{35}S binding (Table 3). The ratio of their EC_{50} values (GTPγ^{35}S/cyclic AMP assay) ranged from 384 to 669 for quinpirole, PNU-86170, pramipexole and dopamine, but 161, 185 and 10 for PNU-95666, 7-OH DPAT and terguride, respectively. This indicates that inhibition of adenylyl cyclases by dopamine D2 receptor agonists occurs at considerably lower receptor occupancy as compared with GTPγ^{35}S

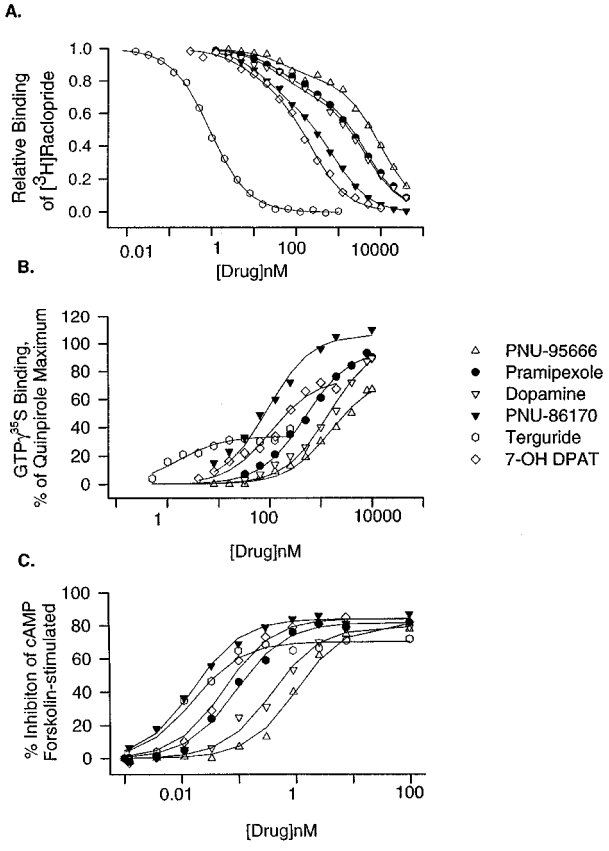

Figure 4 Standard dopaminergic agonists were examined for their intrinsic efficacy in SH-SY5Y cells expressing the human D2long dopamine receptor. PNU-95666, pramipexole, dopamine, PNU-86170, terguride and 7-OH DPAT were examined for their abilities (A) to produce high affinity sites as monitored with concentration-dependent displacement of [^{3}H]-raclopride binding, (B) to enhance GTPγ^{35}S binding, and (C) to inhibit forskolin-stimulated cyclic AMP production. Error bars are not shown to avoid cluttering, but ranged from 2 to 16% ($n=3$) except for those data points near zero baselines. The data are summarized in Tables 2 and 3.

Table 2 Characterization of agonist binding to the human D2long dopamine receptor expressed in SH-SY5Y cells

	K_i, nM			*% of high*
	High affinity	*Low affinity*	K_i *Ratio (low/high)*	*affinity sites*
Quinpirole	19 ± 4	2336 ± 380	123	30 ± 4
PNU-95666	24 ± 9	4750 ± 770	198	19 ± 3
Pramipexole	21 ± 5	2140 ± 260	102	28 ± 3
Dopamine	17 ± 4	2065 ± 329	121	29 ± 4
PNU-86170	9 ± 1	310 ± 81	34	30 ± 5
7-OH DPAT	4.9 ± 0.5	114 ± 32	23	20 ± 4
Terguride	0.89 ± 0.03	ND	ND	ND

Standard agonists at various concentrations were examined for their ability to inhibit [^{3}H]-raclopride binding to the D2 receptor. The binding data fitted to a two-site binding model, using non-linear regression analysis (Sigma plot). The IC_{50} values were converted to the K_i using the Cheng & Prusoff equation. The data are mean$\pm$s.e.mean ($n=3$) from response profiles.

Table 3 Comparison of the intrinsic efficacy of standard dopaminergic agonists at the human D2long dopamine receptor expressed in SH-SY5Y cells

	GTPγ^{35} binding		*Inhibition of adenylyl cyclase*	
Compounds	*EC_{50},* nM	*Maximum increase % of quinpirole*	*IC_{50},* nM	*Maximum inhibition %*
Quinpirole	709 ± 64	100	1.3 ± 0.2	72 ± 4
PNU-95666	1612 ± 195	76 ± 3	10.1 ± 1.1	82 ± 2
Pramipexole	496 ± 36	95 ± 2	0.89 ± 0.09	82 ± 4
Dopamine	1460 ± 220	100 ± 5	3.8 ± 0.6	79 ± 3
PNU-86170	107 ± 10	92 ± 19	0.16 ± 0.02	84 ± 3
7-OH	106 ± 21	74 ± 4	0.57 ± 0.07	84 ± 2
DPAT	1.6 ± 0.4	34 ± 2	0.16 ± 0.03	71 ± 2
Terguride				

Intrinsic efficacy of ligands was measured with GTPγ^{35}S binding and with inhibition of forskolin-stimulated cyclic AMP formation. GTPγ^{35}S binding was measured in SH-SY5Y cell membranes in the presence of test ligands at various concentrations. Agonist-induced level was computed by subtracting that observed in the absence of test ligands, and normalized for that observed with quinpirole (10 μM) in the same experiments. Changes in forskolin-stimulated cyclic AMP production were measured in intact cells upon treatment with forskolin (10 μM) with or without test ligands at various concentrations, using FlashPlate kits. The data fitted to a one-site model, and are mean$\pm$s.e.mean ($n=3$) from response profiles.

binding. Also, weak agonists at higher receptor occupancy may produce responses like full agonists.

Since D2long receptors in SH-SY5Y cells are coupled to the two G protein subtypes containing $G\alpha_i$ or $G\alpha_o$, we tested here if some agonists preferably activate one subtype over the other. Analysis of GTPγ^{35}S-bound Gα subunits, using the antibody immobilization assay, showed that the ratio of GTPγ^{35}S associated with $G\alpha_o$ to $G\alpha_i$ was fairly constant for all agonists tested; 0.47 ± 0.03, 0.46 ± 0.03, 0.43 ± 0.2, 0.46 ± 0.2, 0.45 ± 0.2, 0.47 ± 0.05 and 0.49 ± 0.08 ($n=3$) for quinpirole, PNU-95666, pramipexole, dopamine, PNU-86170, terguride and 7-OH DPAT, respectively.

Discussion

Various non-neuronal cell lines have been often used for heterologous expression of neuronal G protein-coupled receptors, because of their relatively high transfection efficiency, rapid growth rate and presumed lack of analogous endogenous receptors. Also such usage seemed to be justified by the ubiquity of most G proteins in mammalian cells and narrow scopes of earlier studies on receptor/G protein interactions. Recent studies, however, have revealed that signal transduction complexes for G protein-coupled receptors include not only receptors and G proteins, but also effectors, various cellular accessory and structural proteins (such as arrestins, caveolins, and small G proteins) (Klein *et al*., 1997; Chidiac, 1998; Mitchell *et al*., 1998; Okamoto *et al*., 1998). Such complexes, even though their molecular organizations remain to be defined, seem to dictate signalling efficiency and specificity of individual G protein-coupled receptors *via* their associated effectors and accessory proteins which are often expressed in a cell-line specific manner. These considerations clearly point out the importance of appropriate cell lines for heterologous expression of neuronal G protein-coupled receptors. In this study, we have shown that the human D2long receptor when expressed in a neuronal cell line, SH-SY5Y, displayed more robust and diverse functional responses than when expressed in an epithelial cell line, HEK293, despite similar levels of receptor expression, measured as [^{3}H]-raclopride binding. Agonist-induced GTPγ^{35}S binding and high affinity receptor binding sites, representing G protein-coupled receptor phenotypes, were 3- to 4- times greater in SH-SY5Y than in HEK293 cells. With respect to its target G proteins, both $G\alpha_o$ and $G\alpha_i$ subunits were heavily labelled with GTPγ^{35}S upon agonist activation in SH-SY5Y cells, but only $G\alpha_i$ subunits were labelled in HEK293 cells. The lack of $G\alpha_o$ coupling in HEK293 cells seems to be irrefutable, judging from the specificity of the $G\alpha_o$-selective antibody shown earlier (Zaworski *et al*., 1999). Among effectors, N-type Ca^{2+} channels, present in SH-SY5Y, but not HEK293 cells, were blocked potently by quinpirole, as a primarily G_o-coupled effector (Lledo *et al*., 1992; Friederich *et al*., 1993; Reeve *et al*., 1995) whereas adenylyl cyclases in the two cell lines were inhibited with similar potency and efficacy. Apparently, D2long receptors in neuronal environments expressed additional functional characteristics that were absent in non-neuronal cell lines, probably owing to the presence of their neuron-specific effectors (e.g. N-type Ca^{2+} channels), accessory and structural proteins yet to be identified.

The cell line-specific interaction of G_o subtypes with D2long is noteworthy because our earlier study demonstrated the abundance of $G\alpha_o$ subunits in the both SH-SY5Y and HEK293 cell lines (*via* reverse transcription-PCR and Western blots using $G\alpha_o$-specific antibody), and the same selective G_o coupling for the homologous D3 dopamine receptor (in SH-SY5Y, but not in HEK293 cells) (Zaworski *et al*., 1999). These coupling specificities further underscore the important role of signal transduction complexes in controlling signal efficiency and specificity. Moreover, such structurally restrained complexes prevent promiscuous interactions between receptors and various G proteins, as observed in isolated/reconstituted states, and thus prevent the futile cycle of agonist-induced activation of G proteins and subsequent GTP hydrolysis. This seems to be a measure necessary for cellular GTP economy.

The human D3 dopamine receptor, as a member of the D2-like dopamine receptor family, displays a high identity in its primary sequence with the D2long receptor, and also produced more robust functional signals in SH-SY5Y than in HEK293 cells (Zaworski *et al*., 1999). Nevertheless, the D3 receptor displayed several functional differences from the D2long receptor. (1) The D3 receptor couples with only G_o, but not G_i subtypes in SH-SY5Y cells, and with neither of these subtypes in HEK293 cells. (2) The D3 receptor-mediated inhibition of cyclic AMP formation was robust in SH-SY5Y, but only marginal in HEK293 cells, reflecting its selective modulation of adenylyl cyclase type V (ACV) (Robinson & Caron, 1997), which exists only in SH-SY5Y, but not in HEK cells (Zaworski *et al*., 1999). Thus, robust inhibition of cyclic AMP formation by the D2long receptor in HEK293 and SH-SY5Y cells indicates its modulation of one or more endogenous adenylyl cyclases found in HEK293 cells, such as ACII, III, IV and VII (Hellevuo *et al*., 1993). (3) Both D2long and D3 receptors mediated quinpirole-induced inhibition of N-type Ca^{2+} channel currents in SH-SY5Y cells, but with different potency. The IC_{50} for quinpirole at D3 (5.6 ± 1.5 nM) was about 30 fold greater than that at D2 (0.15 ± 0.3 nM) (Zaworski *et al*., 1999). This implies possible direct contributions of certain part(s) of the D2long receptor, not shared by the homologous D3 receptor, to inhibition of Ca^{2+} channel activity. We propose significant roles of the intracellular components of dopamine receptors, in selecting not only their G protein targets, but also their effectors and modes of interaction. Structural motifs responsible for such functions could be explored with various chimeras where intracellular components are interchanged.

In this study, we compared several standard D2 agonists for their abilities to induce high affinity sites and to enhance GTPγ^{35}S binding. In SH-SY5Y cell membranes, the K_i ratio of high and low affinity sites for most agonists was high enough to allow us to estimate reliably their relative populations, except for terguride, which displayed only one class of binding sites (Table 2). These two assays produced similar rank order of agonist efficacy (in decreasing order, quinpirole, dopamine, pramipexole, PNU-81670 > PNU-95666 and 7-OH DPAT > terguride), probably because both assays represent early steps of receptor/G protein activations, without intracellular amplification steps. Inhibition of cyclic AMP production, on the other hand, involves further downstream steps, and differed from the GTPγ^{35}S binding in two respects. (1) The IC_{50} values for cyclic AMP production were over 100-times less than the corresponding values observed for agonist-induced GTPγ^{35}S binding. This may indicate a greater receptor reserve for the cyclic AMP production than for the GTPγ^{35}S binding. (2) Partial agonists were not well differentiated in the cyclic AMP assay. Terguride, at higher receptor occupancy than full agonists, inhibited cyclic AMP production as much as full agonists. From this comparison, GTPγ^{35}S binding seems to be a

reliable assay for estimating ligand intrinsic efficacy, because it is solely dependent on the number of functionally activated receptor complexes, without downstream amplification or threshold steps.

In summary, the human D2long receptor when expressed heterologously in SH-SY5Y cells, a neuronal cell line, produced more robust and diverse functional signals through coupling to G_o and G_i subtypes of G proteins than when expressed in HEK293 cells where coupling is only to G_i subtypes. This could arise, at least in part, from the presence of neuronal target effectors such as N-type Ca^{2+} channels. These findings underscore the importance of the cellular environment in which drug actions are examined, particularly in the face of intensive efforts to develop drugs for G protein-coupled receptors of various origins.

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