Dopamine Receptors: Structure, Function, and Regulation

by

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CERTIFICATE OF APPROVAL

This is to certify that the Masters thesis of
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TABLE OF CONTENTS

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ABSTRACT

Two projects are discussed in this thesis. The first project was an investigation of mechanisms controlling receptor density. C_6 glioma cells expressing D_{2L} and D_{2S} receptors were treated with various ligands for extended periods of time. Overnight treatment with the agonist *N*-propylnorapomorphine (NPA) resulted in receptor proliferation of up to 130% above control. Smaller increases were found after treatment with the agonists quinpirole and dopamine (DA). Overnight treatment with the antagonist sulpiride increased receptor density by 60%. The antagonists epidepride and domperidone caused small increases in receptor density as well. In functional assays for the ability of drugs to inhibit activation of adenylate cyclase, there was a diminished response, or desensitization, after agonist treatment that was not observed after treatment with antagonists. Receptor proliferation after agonist treatment was not blocked by treatment with pertussis toxin, which inactivates the guanine nucleotide binding proteins (G proteins) G_i and G_o, thus ruling out the possibility that receptor interaction with these G proteins is responsible for this phenomenon. Treatments with the protein synthesis inhibitor cycloheximide decreased receptor density in cells treated with agonist or vehicle, but did not prevent a significant increase in receptor density compared to control cells.

The other area of focus of this thesis was in vitro mutagenesis of D_2 DA receptors. The most interesting results came from a deletion mutant in which the last 10 amino acid residues of the third intracellular loop (IC3), from serine 365 to glutamine 374, were deleted D_2(del 365-374). This region of the receptor has been implicated in G protein coupling for many G protein coupled receptors (GPCRs). Site specific mutations in this region have resulted in mutant receptors with impaired ability to interact with G proteins in some cases, and constitutively active receptors in others. The mutant D_2(del 365-374) exhibited several characteristics of constitutively active receptors. The D_2(del 365-374) receptor had higher affinity for agonists compared to the D_{2L} receptor, and high-affinity binding of agonists was resistant to the effects of GTP. Some antagonists acted as inverse agonists on cells expressing D_2(del 365-374) indicating a significant level of basal inhibition of adenylate cyclase by the mutant receptor. Other mutants of the D_{2L} receptor that were constructed, included phenylalanine 110 (F110) in transmembrane (TM) region 3 and serine 167 (S167) in TM 4. In competition binding studies with antagonists, F110A receptors had an unexpected increased affinity for the antagonist haloperidol but decreased

affinity for some other antagonists and agonists compared to wild type receptors. The density of S167A and D_2(del 365-374) receptors was dramatically increased after overnight treatment with agonist. The density of wild type receptors expressed in HEK 293 cells was significantly increased after overnight treatment with agonist plus forskolin.

PREFACE

In accordance with the guidelines set by Oregon Health Sciences University of Portland Oregon, School of Medicine Graduate Program, I have prepared my thesis consisting of a general introduction, two chapters of original data, and a conclusion chapter. Both data chapters include an abstract, introduction, materials and methods, results, discussion section. References are listed separately in alphabetical order. All work is original with the following exceptions. Dr. Kim Neve performed the cycloheximide studies in chapter 2. Dr. Laura Kozell performed the D1/D2 chimeric studies in chapter 2 as well as the inverse agonist studies in chapter 3. Medhane Cumbay performed the inverse agonist studies with the T373A mutant mentioned in the discussion.

I. INTRODUCTION

Why study DA receptors? One of the most compelling reasons to study DA receptors is that schizophrenia and Parkinson's disease may be related to abnormal levels of DA in the brain. Through better understanding of the actions of DA, as well as available synthetic analogs of DA and compounds that block or act to terminate the actions of DA, we may discover better treatments for patients afflicted with these diseases.

Disease states are attributed to dysfunction in some dopaminergic regions of the brain. Parkinson's disease, which involves the degradation of dopaminergic neurons projecting from the substantia nigra to the striatum of the brain, results in debilitating movement disorders. This disease is treated with DA replacement therapy agents including L-DOPA, the precursor to DA, or synthetic agonists such as apomorphine (Nutt, *et al.*, 1996). It is important to understand the underlying nature of the disease to develop better agents for treatment.

Schizophrenia may be due to a malfunction of D_2 DA receptors in the limbic regions of the brain. Patients with this disease are treated with neuroleptics which block D_2 DA receptors (Ohara *et al.*, 1996; Pickar *et al.*, 1996). Although these drugs ameliorate some of the symptoms of schizophrenia, treatment with neuroleptics or antipsychotics may result in extrapyramidal side-effects including Parkinsonian-like symptoms. Currently the D_4 selective antagonist clozapine has proven effective, and has different side effects than most other antipsychotic drugs which may be more beneficial to some schizophrenic patients (Seeman, 1992).

The dopamine receptors are a member of the superfamily of GPCRs. It is also important to study DA receptors as they belong to the superfamily of GPCRs. Members of this superfamily of receptors have seven transmembrane domains and transduce extracellular signals through interactions with G proteins inside the cell. GPCRs are involved in a variety of physiological functions and information gained about one member of this family can often be related to GPCRs in general (Dixon, 1988).

Classification of dopamine receptors as D_1-like or D_2-like. Dopamine receptors are classified as D_1-like or D_2-like according to pharmacology, homology, and signaling pathways (Kebabian and Calne, 1979).

Pharmacology of dopamine receptors. Pharmacologically D_1-like and D_2-like receptors can be differentiated by selective agonists and antagonists. Some D_1-selective agonists include

fenoldepam, 6-chloro-7,8-dihydroxy-3-allyl-2,3,4,4-phenyl-tetrahydro-1H-benzazepine (chloro-APB), and the partial agonist SKF-38393. SCH-23390 is a selective antagonist for D_1 receptors. D_2-selective agonists include NPA and bromocriptine. Spiperone and sulpiride are selective antagonists at D_2 receptors.

Sequence homology between receptor subtypes. There is highest homology between GPCRs in the putative transmembrane domains as this area is thought to form the binding pocket for small molecule ligands. There is 44% homology between the human D_1 and human D_2 amino acid sequences in the transmembrane regions, and a few percent less overall homology between the two receptor types (Zhou *et al.*, 1990). D_1-like receptors include the subtypes D_1 and D_5 which have 49% overall amino acid sequence homology (Grandy *et al.*, 1991). D_2-like receptors include the subtypes D_2 short (D_{2S}), D_2 long (D_{2L}), D_3, and D_4. D_{2S} and D_{2L} are splice variants with identical sequence except for 29 additional amino acids which are inserted in the third intracellular loop (IC3) of the longer form (Neve and Neve, 1997). D_3 amino acid sequence has 52% overall homology to D_2 and greater than 70% homology in the transmembrane regions (Sokoloff *et al.*, 1990). D_4 receptors have overall amino acid sequence homology of 41% with D_2 receptors (Van Tol *et al.*, 1991; Van Tol *et al.*, 1992). There are many allelic variants of the D_4 receptor, which contains a region of 16 amino acids which occur in varying numbers of repeats (Neve and Neve, 1997).

Signaling pathways of dopamine receptors. The most well documented signaling pathway used to distinguish between D_1-like and D_2-like receptors is the adenylate cyclase pathway. Though there are many types of adenylate cyclases which can be activated or inhibited by various agents (Marjamaki *et al.*, 1997), a simplified view involves activation of adenylate cyclase by D_1-like receptors and inhibition of enzymatic activity by D_2-like receptors. D_1-like receptors are coupled to the cholera toxin-sensitive G protein G_s. When activated, $G_{s\alpha}$ subunits stimulate the enzyme adenylate cyclase resulting in increases in cAMP levels. D_2-like receptors are coupled to the pertussis toxin sensitive G proteins G_i or G_o. Activated $G_{i\alpha}$ subunits inhibit adenylate cyclase activity and decrease production of cAMP. Other signaling pathways include modulation of phosphatidylinositol hydrolysis for both D_1 and D_2 (Huff, 1997; Vallar and Meldolesi, 1989). D_2 receptors are also implicated in the regulation of the Na^+/H^+ exchanger (Neve *et al.*, 1992),

potentiation of arachidonic acid release (Kanterman et al. (1990), changes in K^+ currents (Lledo *et al.*, 1992), and inhibition of Ca^{2+} channels (Lledo *et al.*, 1992; Malgaroli *et al.*, 1987).

Structural features of G_i and G_s coupled receptors. D_1-like receptors have a short third intracellular loop and long carboxyl tail which is characteristic of stimulatory G protein (G_s) -coupled receptors (Zhou, 1990). D_2-like receptors have a long third cytoplasmic loop and a short carboxy terminus which is characteristic of inhibitory G protein (G_i) -coupled receptors (Bunzow *et al.*, 1988).

Tissue distribution of D_1 and D_2 receptors The tissue distribution of D_1 and D_2 receptors is overlapping in some areas. The highest level of D_2 mRNA is found in the anterior basal ganglia and the neurointermediate lobe of the pituitary. Other areas with high D_2 message include the anterior lobe of the pituitary and the posterior basal ganglia (Bunzow *et al.*, 1988). The highest levels of D_1 mRNA transcripts are found in the anterior and posterior basal ganglia. High levels are also found in the septum and the amygdala. No D_1 transcripts are found in the pituitary (Zhou *et al.*, 1990).

Aims of this thesis: The aims of this thesis are to look at the mechanisms controlling receptor turnover in cultured cells expressing D_2 DA receptors and to test specific amino acids and regions of the D_{2L} DA receptor for their importance in ligand binding and receptor function.

C_6 rat glioma cells expressing D_{2L} and D_{2S} receptors were used to assess the effects of long-term treatment with ligands on changes in receptor density. Contrary to the compensatory model of receptor regulation, several groups have found increases in D_2 receptor density after overnight treatment with agonists (Ivins *et al.*, 1991; Filtz *et al.*, 1993; Zhang *et al.*, 1994).

Phenylalanine 110 and serine 167, which are conserved among catecholamine receptors (Trumpp-Kallmeyer et al, 1992), were suggested by molecular modeling studies to be important for ligand binding and receptor function. The carboxyl terminal region of the third intracellular loop was chosen as it has been implicated in G protein coupling and constitutive activation of other GPCRs (Burstein *et al.*, 1995; Shapiro *et al.*, 1993). This study sought to assess the effects of these mutations on ligand binding, receptor function, and regulation of receptor density.

II. Drug-Induced Up-Regulation of Dopamine D_2 Receptors on Cultured Cells

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ABSTRACT:

Ligand-induced proliferation of recombinant dopamine D_2 receptors was assessed using C_6 glioma cells stably expressing the short (D_{2S}) and long (D_{2L}) forms of the receptor. Overnight treatment of C_6-D_{2L} cells with *N*-propylnorapomorphine (NPA) caused a time- and concentration-dependent increase in the density of receptors, as assessed by the binding of radioligand to membranes prepared from the cells, with no change in the affinity of the receptors for the radioligand. The effect of 10 μM NPA was maximal after 10 h, at which time the density of D_{2L} receptor was more than doubled. The agonists dopamine and quinpirole also induced a proliferation of D_{2L} receptors. The receptor proliferation was not specific for agonists, since the antagonists epidepride, sulpiride, and domperidone caused smaller (30-60%) increases in receptor density. The magnitude of the receptor proliferation in each of four clonal lines of C_6-D_{2L} cells (mean increase = 80%) was greater than in all four lines of C_6 D_{2S} cells (33%). Inactivation of pertussis toxin-sensitive G proteins had no effect on the basal density of D_{2L} receptors or on the NPA-induced receptor proliferation. Treatment with 5 μg/ml of cycloheximide, on the other hand, decreased the basal density of receptors and attenuated, but did not prevent, the NPA-induced increase. Chimeric D_1/D_2 receptors were used to identify structural determinants of dopamine receptor regulation. Treatment with the D_1/D_2 agonist NPA decreased the density of D_1, CH4, and CH3 receptors. The latter two receptors have D_1 sequence from the amino terminus to the amino-terminal end of TM VII and VI, respectively. CH2, with D_1 sequence up to the amino-terminal end of TM V, and thus the third cytoplasmic loop of the D_2 receptor, was up-regulated by NPA or the D_2-selective agonist quinpirole. Quinpirole treatment decreased the density of CH3 and had no effect on CH4 or D_1 receptors. The different responses of CH2 and CH3 to agonist treatment suggest a role for TM V and the third cytoplasmic loop in the direction of receptor regulation.

INTRODUCTION:

One of the most thoroughly documented phenomena in pharmacology is the inverse relationship between the extent of stimulation of neurohormonal receptors and their responsiveness. When the neural afferent to an excitable tissue is removed or blocked, the tissue becomes supersensitive to the transmitter released by the afferent, a phenomenon that has been termed "the law of denervation" (Cannon and Rosenblueth, 1949). One component of supersensitivity is often a proliferation of receptors for the transmitter (Thesleff, 1974). Similarly, stimulation of a tissue causes desensitization, frequently due in part to a decreased density of receptors, so that the response to prolonged or repeated stimulation is attenuated (Harden, 1983).

The rat mesostriatal dopamine system has been used extensively as a CNS model of super- and subsensitivity. Destruction of the pathway results in behavioral supersensitivity (Ungerstedt, 1971), accompanied by an increase in the rate of synthesis of D_2 dopamine receptors (Creese *et al.*, 1977; Neve *et al.*, 1985; Neve *et al.*, 1991), whereas treatment with dopamine receptor agonists reverses supersensitivity (List and Seeman, 1979) and may induce subsensitivity of previously normosensitive dopamine receptors (Quik and Iversen, 1978; Chen *et al.*, 1993). Some characteristics of the regulation of dopamine receptors in vitro also fit a model in which overstimulation of the receptors causes a compensatory decrease in the density or responsiveness of the receptors. Treatment with an agonist decreases the responsiveness and density of D_1 dopamine receptors on cultured cells (Balmforth *et al.*, 1990; Barton and Sibley, 1990; Machida *et al.*, 1992), and also decreases the responsiveness of D_2 receptors (Agui *et al.*, 1988; Barton *et al.*, 1991; Bates *et al.*, 1991). There are, however, aspects of the regulation of dopamine receptors *in vivo* and *in vitro* that are apparently at variance with a compensatory model. Treating rats with an indirect agonist such as amphetamine or L-DOPA may increase the density of D_2 receptors (Klawans *et al.*, 1979; Wilner *et al.*, 1980). Furthermore, destruction of the mesotelencephalic dopamine system decreases the density of D_1 receptors in the neostriatum (Marshall *et al.*, 1989; Gerfen *et al.*, 1990), although not all studies are in agreement on this issue (Buonamici *et al.*, 1986; Porceddu *et al.*, 1987). Recently, there have been several reports that D_2 receptors on cultured cells are up-regulated by treatment with agonists (Ivins *et al.*, 1991; Filtz *et al.*, 1993; Zhang *et al.*, 1994). The experiments described below address the mechanisms of the paradoxical regulation of recombinant D_2 receptors expressed in C_6 glioma cells. We now report

that the receptor proliferation is only partially prevented by cycloheximide, and is unaffected by the inactivation of pertussis toxin-sensitive G proteins. Through the use of chimeric D_1/D_2 dopamine receptors, we have localized structural features that determine the direction (up or down) of regulation of the receptors to within the third cytoplasmic loop and TM V.

MATERIALS AND METHODS

Materials $Na^{125}I$ and [^{3}H]SCH 23390 were purchased from Dupont-New England Nuclear (Boston, MA), and [^{3}H]spiperone was purchased from Amersham (Arlington Heights, IL). Spiperone and domperidone (Janssen), quinpirole (Lilly), (S)-(-)-N-[(1-ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-n-butyltin)benzamide and epidepride (Dr. T. de Paulis, Vanderbilt University), and rat D_{2S} and D_{2L} cDNAs (Dr. O. Civelli, Oregon Health Sciences University) were generous gifts. Sulpiride and R(-)-propylnorapomorphine (NPA) were purchased from Research Biochemicals, Inc. (Natick, MA). Dopamine (3-hydroxytyramine) and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Expression of D_2 receptors in C_6 cells C_6 cells used for stable expression of D_2 receptor cDNAs were maintained as described previously (Cox *et al.*, 1992). Transfection of C_6 cells was carried out by calcium phosphate precipitation (Chen and Okayama, 1988) exactly as described previously (Cox *et al.*, 1992), combining either pRSV-D_{2S} or pRSV-D_{2L} cDNA (15 μg) with pBabe Puro (2 μg), to confer resistance to puromycin (Morgenstern and Land, 1990).

Drug-induced proliferation of D_2 receptors All drug solutions were freshly prepared and sterilized by filtration prior to use. Indicated drugs were diluted 100- or 1000-fold by addition to cells grown on 10 cm plates in DMEM. At the end of the incubation, cells were washed with calcium-, magnesium-free phosphate-buffered saline, re-fed with DMEM, and returned to the incubator. After ten minutes, cells were washed three times with growth medium and then harvested. To harvest, cells were lysed with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10-15 min, the cells were scraped from the plate and spun at 24,000 x *g* for 20 min. The resulting crude membrane fraction was resuspended in Tris-buffered saline with a Brinkman Polytron homogenizer at setting 6 for 10 sec. This membrane preparation was then spun again at 24,000 x *g* and resuspended. When assessing the time course of receptor proliferation, cells were frozen in hypotonic buffer at each time point and stored

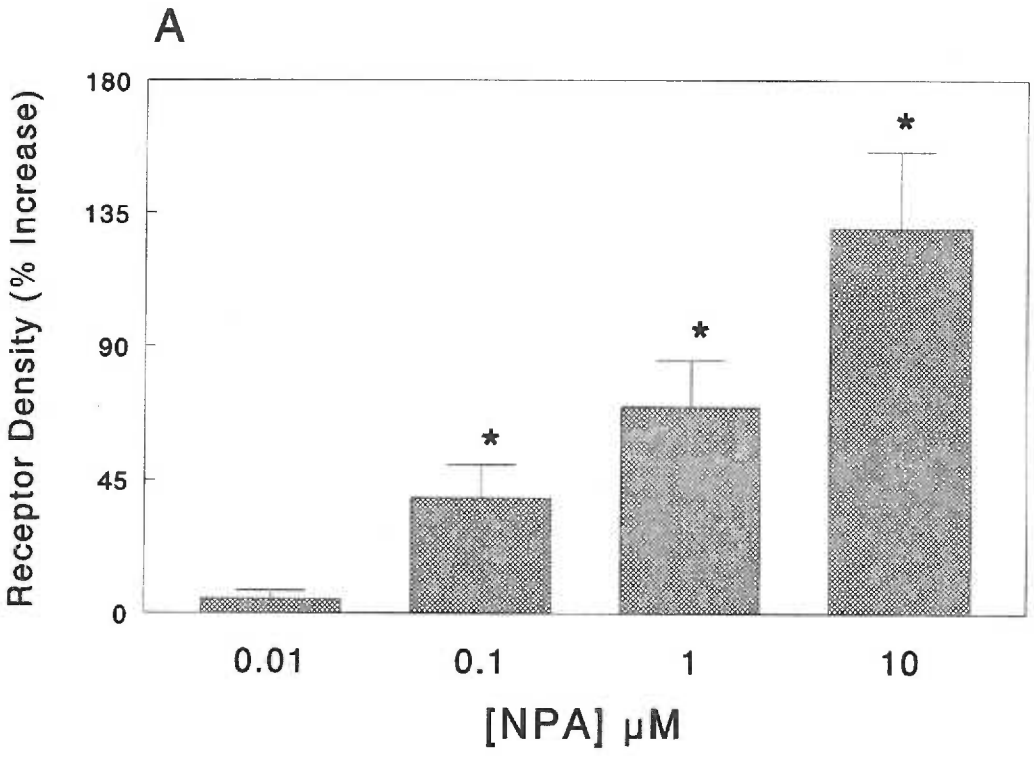
overnight at -20° C prior to centrifugation and quantification of receptors.

Radioligand binding assays [^{125}I]Epidepride (NCQ 219) was prepared from its tributyltin precursor using a modification (Neve, 1991) of the method of Clanton et al. (1991). The binding of [^{125}I]epidepride or [^{3}H]spiperone was assessed essentially as described (Bunzow *et al.*, 1988; Cox *et al.*, 1992). Aliquots of the membrane preparation (3-40 μg of protein) were added to duplicate assay tubes containing the following (final concentrations): 50 mM Tris-HCl, pH 7.4 with 155 mM NaCl (Tris-buffered saline), 0.001 % bovine serum albumin, radioligand, and appropriate drugs. Spiperone or (+)-butaclamol (2 μM) was used to define nonspecific binding of [^{125}I]epidepride or [^{3}H]spiperone, respectively. Incubations were carried out at 30° ([^{125}I]epidepride) or 37° C ([^{3}H]spiperone) for 60 min, in a volume of 0.5 and 1.0 ml, respectively. [^{3}H]SCH 23390 was used to quantify D_1 and chimeric receptors, exactly as described previously (Kozell *et al.*, 1994).

Data analysis Data were analyzed by nonlinear regression using the program GraphPAD. The free concentration of radioligand was calculated as the concentration added minus the concentration bound. Averages for K_D values are the geometric means [the antilogarithm of mean logarithms (Fleming *et al.*, 1972)].

RESULTS

Agonist-induced proliferation of D_{2L} receptors Overnight (14 h) treatment of C_6-D_{2L} cells with the D_2 receptor agonist NPA caused a concentration-dependent increase in receptor density, as determined by saturation analysis of the binding of [^{125}I]epidepride (Fig. 1A).



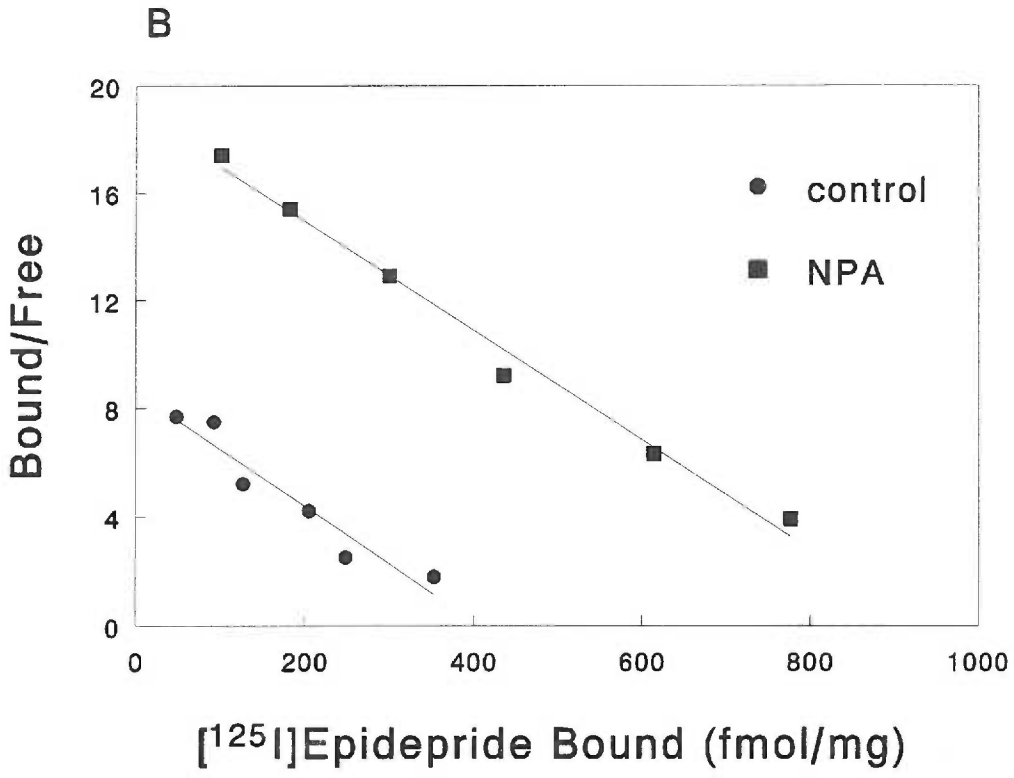


Fig. 1. A: Overnight treatment of C_6-D_{2L} cells with NPA caused concentration-dependent increases in receptor density. The density of D_2 receptors on membranes prepared from treated cells was determined by saturation analysis of the binding of [^{125}I]epidepride. Data shown are the mean ± SEM of 4 independent experiments. Results are plotted as receptor density, expressed as the percent increase over the control density of receptors, versus the concentration of NPA used for treatment.

B: The binding of [^{125}I]epidepride to membranes prepared from control C_6-D_{2L} cells or cells treated with 10 μM NPA overnight was characterized. Results shown are from a representative experiment in which the amount of radioligand specifically bound / the free radioligand concentration is plotted versus the amount specifically bound (fmol/mg of protein).

*$p < 0.05$ compared to the control density of receptors

A 39% increase was apparent after treatment with 0.1 μM NPA, and 10 μM NPA increased the density of D_{2L} receptors by 130%. Higher concentrations of NPA were not tested because they appeared to be toxic to the cells, and because of the difficulty of removing residual drug from the membranes before quantification of the density of receptors. As depicted in Fig. 1B, NPA treatment increased the density of binding sites without altering the affinity of the receptors for [^{125}I]epidepride.

The time-course of NPA-induced receptor proliferation was determined by harvesting C_6-D_{2L} cells after various periods of treatment with 10 μM NPA. The effect of NPA on the binding of [^{125}I]epidepride was maximal after 10-14 h (Fig. 2).

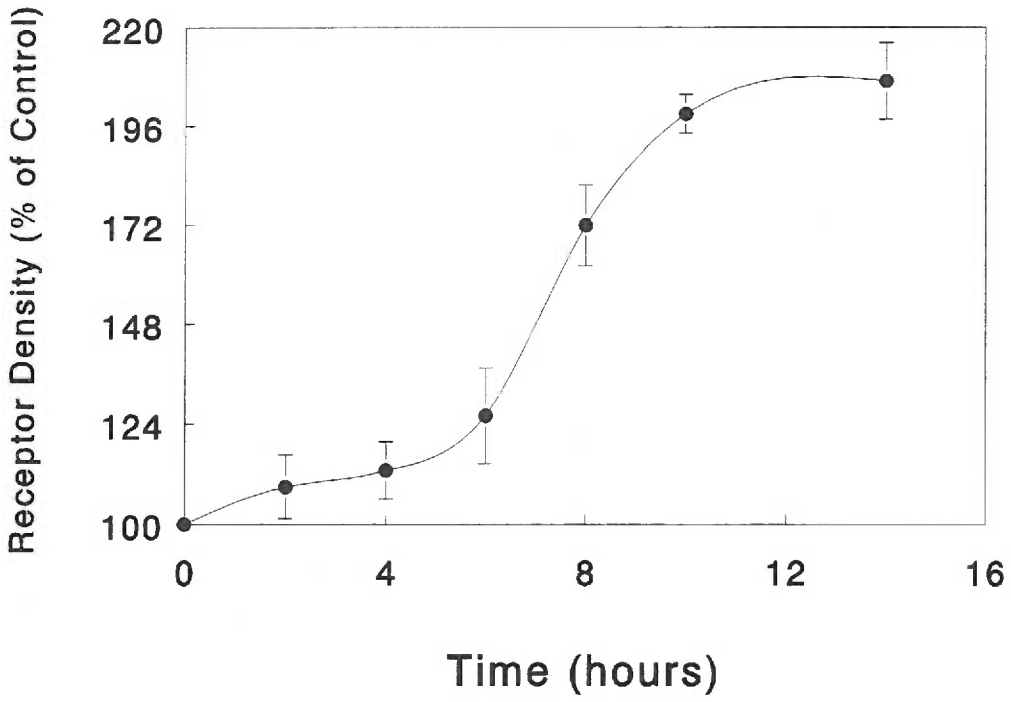


Fig. 2. The time-course of NPA-induced receptor proliferation was determined by saturation analysis of the binding of [^{125}I]epidepride to membranes prepared after various periods of treatment with 10 μM NPA. Data are plotted as the percentage of the control density of receptors versus the duration of treatment. The mean ± SEM of three independent experiments is shown.

Like NPA, the agonists dopamine and quinpirole increased the density of D_{2L} receptors (Table 1). Higher concentrations of these drugs were required, so that the density of receptors was only slightly increased by 1 μM quinpirole, and not significantly increased by 1 μM dopamine. Robust increases were observed after treatment with either agonist at 100 μM.

TABLE 1. Drug-induced up-regulation of D_{2L} receptors

Drug	Density (% increase)	Affinity (pM)
Dopamine (4)		
1 μM	102 ± 1	37 (35-40)
10 μM	101 ± 4	40 (30-52)
100 μM	147 ± 6*	42 (39-46)
Quinpirole (4)		
1 μM	106 ± 2	41 (40-42)
10 μM	131 ± 12*	41 (34-39)
100 μM	162 ± 6*	46 (42-54)
Epidepride		
10 nM (5)	111 ± 6.99	62 (55-70)
100 nM (5)	118 ± 12.63	74 (66-83)
1 μM (5)	126 ± 6.07*	87 (77-98)
Sulpiride		
100 nM (3)	126 ± 14.75	39 (34-45)
1 μM (3)	132 ± 8.49	40 (35-46)
10 μM (3)	160 ± 3.52*	46 (34-61)
Domperidone		
100 nM (3)	149 ± 15.49*	59 (39-87)

C_6- D_{2L} cells were treated with the indicated drugs for 14 h (overnight), then membranes were prepared and the density of D_2 receptors was determined as described in Materials and Methods. The number of independent experiments is in parentheses after each drug concentration, and K_D values for [^{125}I]epidepride, in pM, are the geometric means, followed by the 95% confidence limits of the mean in parentheses. In these experiments, the B_{max} and K_D of [^{125}I]epidepride in untreated cells were 986 ± 91 fmol/mg of protein and 49 pM (40-60 pM), respectively. The density of receptors is the mean ± SEM, expressed as a percentage of the control density of receptors. *$p < 0.05$, significant increase relative to control.

Antagonist-induced proliferation of D_{2L} receptors To ascertain if the drug-induced proliferation of receptors depended on occupation of the receptors by an agonist, C_6-D_{2L} cells were treated for 14 h with the antagonists epidepride, sulpiride, or domperidone prior to quantification of the density of receptors. Each of the antagonists significantly increased the density of D_{2L} receptors (Table 1).

Responsiveness of D_{2L} receptors after drug treatment C_6-D_{2L} cells were treated for 14 h with 10 uM NPA or 10 uM sulpiride, then rinsed three times with phosphate-buffered saline before quantification of dopamine-inhibited adenylyl cyclase activity (Fig. 3).

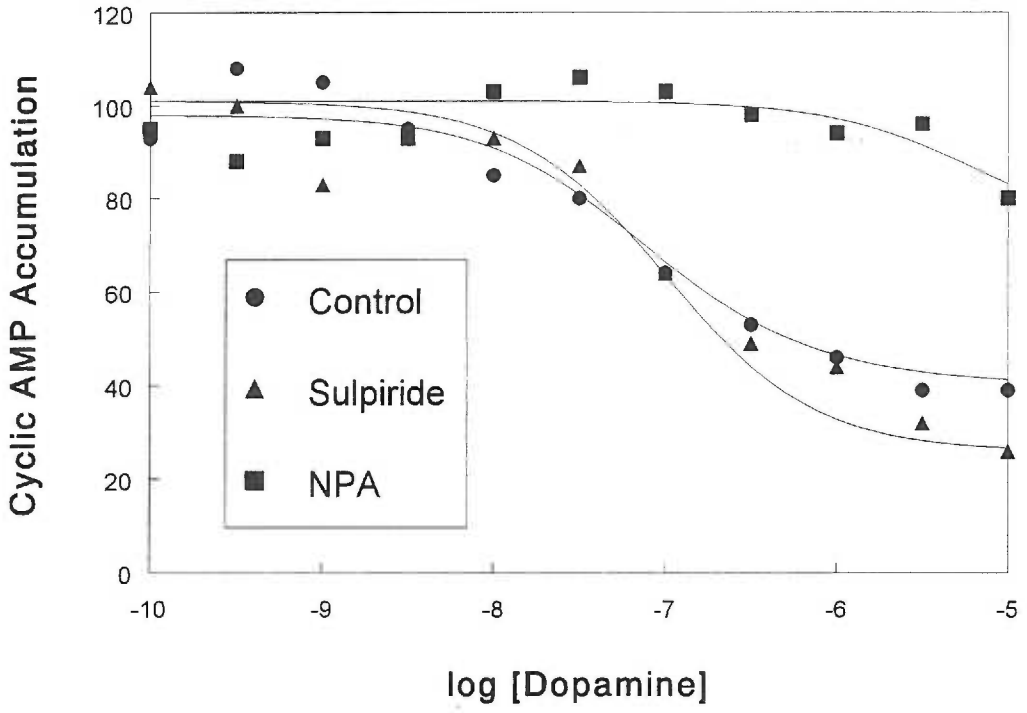


Fig. 3. Treatment of C_6-D_{2L} cells with NPA deceased and sulpiride treatment increased subsequent inhibition of adenylyl cyclase by dopamine. A representative experiment is shown in which cells were treated with 10 μM NPA, 10 μM sulpiride, or vehicle (control), followed by determination of the ability of dopamine to inhibit adenylyl cyclase. Results are expressed as a percentage of the total enzyme activity stimulated by 1 μM isoproterenol, which was 5.5% (control), 5.5% (NPA), and 5.9% (sulpiride) conversion of [^{3}H]ATP to [^{3}H]cyclic AMP.

Treatment with NPA caused an apparent desensitization of adenylyl cyclase. Although curves from two of four independent experiments with NPA-treated cells could not be fit to the data because of a failure to reach a plateau at 10 uM dopamine, the combined data from all four experiments were best fit by a curve in which the maximal inhibition of isoproterenol-stimulated adenylyl cyclase activity was 28% of total stimulated activity (EC_{50} = 0.5 uM), whereas the maximal inhibition in control (untreated) cells was 60% of the total (EC_{50} = 0.03 uM). In contrast, treatment with sulpiride increased the apparent efficacy of dopamine, as reflected in an increase in the maximal inhibition of adenylyl cyclase from 61 $\pm$ 3% in control cells to 77 $\pm$ 2 % in sulpiride-treated cells (n = 4, p, <0.01).

Comparison of D_{2L} and D_{2S} We compared the response of the alternatively spliced forms of D_2 receptors by treating C_6-D_{2L} and C_6-D_{2S} cells for 14 h with 10 μM NPA. To be sure that variations in the magnitude of the response were not due to unique characteristics of one clone, or due to differences in the initial density of receptors on a cell line, we tested several clones expressing each form of the receptor. In 4 clones of C_6-D_{2L} cells with control receptor densities of 186 ± 52 (n = 3), 187 ± 18 (n = 13), 295 ± 37 (n = 15), and 977 ± 165 fmol/mg of protein (n = 4), treatment with 10 μM NPA increased the density of receptors by 156%, 110%, 79%, and 66%, for a combined mean increase of 82 ± 10%. In 4 clones of C_6-D_{2S} cells with control receptor densities of 128 ± 19 (n = 6), 325 ± 30 (n = 5), 436 ± 72 (n = 4), and 605 ± 45 fmol/mg of protein (n = 16), treatment with 10 μM NPA increased the density of receptors by 54%, 33%, 40%, and 24%, respectively, for a combined mean increase of 33 ± 7%. Consistent with the reduced magnitude of the NPA-induced proliferation of D_{2S} receptors expressed in C_6 glioma cells, NPA (10 μM) had a small but significant effect on the density of D_2 receptors on LZR1 cells, mouse L cells that express D_{2S} (Bunzow *et al.*, 1988; Neve *et al.*, 1989), increasing the density of receptors 26% from 435 ± 51 to 549 ± 75 fmol/mg of protein.

Mechanism of up-regulation of D_{2L} The role of pertussis toxin-sensitive G proteins in the proliferation of D_2 receptors was assessed by addition of pertussis toxin to the culture medium at a final concentration of 100 ng/ml 16 h prior to treatment with 10 μM NPA. Exposure to the toxin continued throughout overnight treatment with NPA. This concentration of pertussis toxin was previously determined to abolish coupling of D_2 receptors to G proteins in intact C_6 cells

(Neve *et al.*, 1992). Treatment with pertussis toxin had no effect on the NPA-induced proliferation of D_{2L} receptors (Fig. 4).

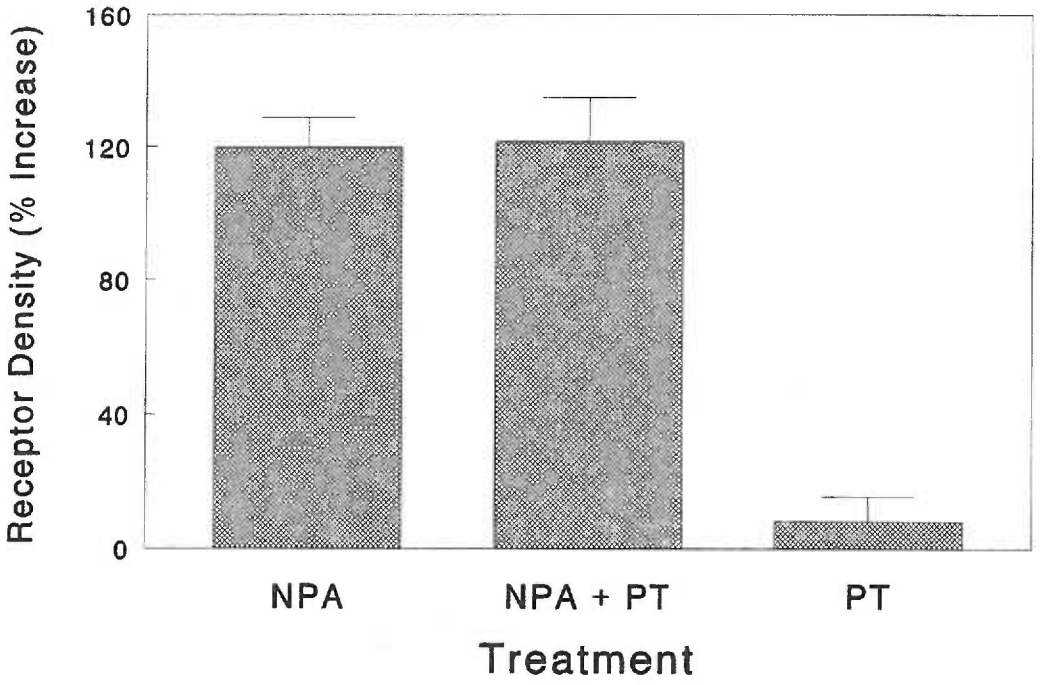


Fig. 4. Pertussis toxin had no effect on NPA-induced proliferation of D_{2L} receptors. C_6-D_{2L} cells were treated with 100 ng/ml of pertussis toxin for 16 h prior to treatment with 10 μM NPA. The density of receptors on membranes prepared from pertussis toxin-treated cells (PT), cells treated with only NPA (NPA), and cells treated with pertussis toxin and NPA (NPA + PT) was determined by saturation analysis of the binding of [^{125}I]epidepride. The data shown are the mean ± SEM of 4 independent experiments, expressed as the percent increase over the control density of receptors.

The basal density of receptors was 614 ± 47 (control) and 655 ± 31 (pertussis toxin) fmol/mg of protein, as assessed by the binding of [^{125}I]epidepride. Treatment with 10 μM NPA increased the density of receptors to 1346 ± 109 and 1345 ± 58 fmol/mg of protein in the absence or presence, respectively, of pertussis toxin.

In separate experiments, the effect of the protein synthesis inhibitor cycloheximide on NPA-induced receptor proliferation was assessed (Fig. 5).

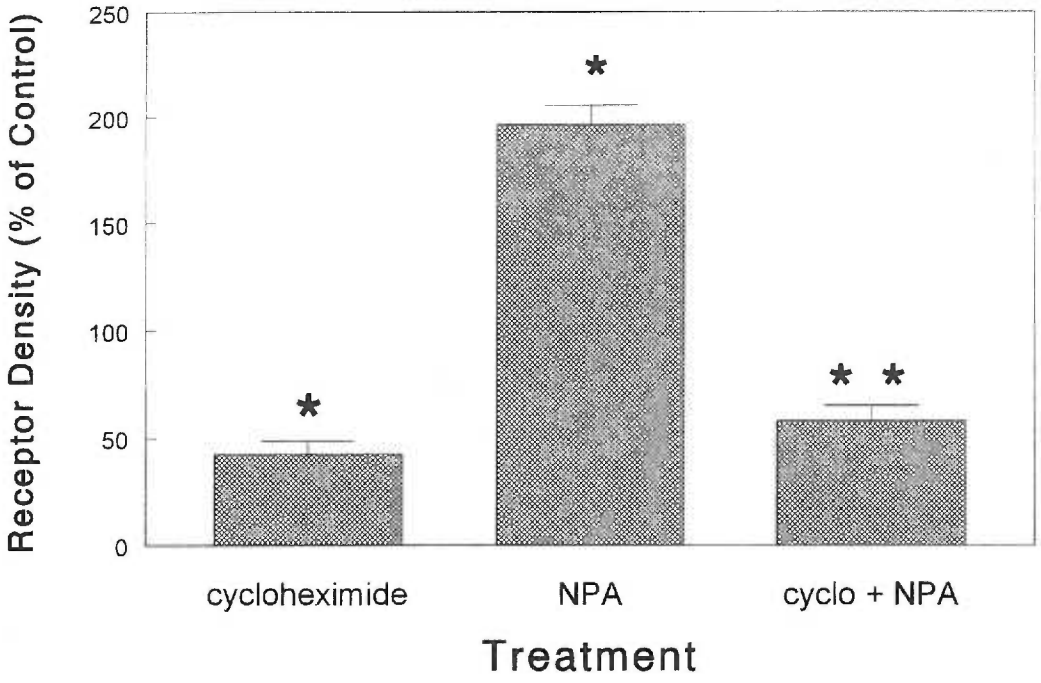


Fig. 5. The effect of cycloheximide on NPA-induced receptor proliferation was determined by saturation analysis of the binding of [^{3}H]spiperone. C_6-D_{2L} cells were treated with 5 μg/ml of cycloheximide, 10 μM NPA, or both (cyclo + NPA). The data shown are the mean ± SEM of 5 independent experiments, expressed as the percentage of the control density of receptors (926 fmol/mg of protein).
*significantly different from control, $p < 0.05$

Cycloheximide (5 μg/ml, a concentration that inhibits protein synthesis by >95% in C_6 cells; Ref. (Neve and Molinoff, 1986) was added to the cells simultaneously with the addition of 10 μM NPA, and the density of receptors on membranes prepared from the cells was determined by analysis of the binding of [^{3}H]spiperone. After treatment for 14 h, cycloheximide decreased the density of D_2 receptors by greater than 50%, from 926 to 394 fmol/mg of protein, whereas NPA almost doubled the density of receptors to 1796 fmol/mg of protein. Cycloheximide treatment reduced, but did not prevent, the NPA-induced receptor proliferation, as indicated by the 36% greater density of receptors in the NPA + cycloheximide cells (531 fmol/mg of protein), compared to cells treated with cycloheximide alone.

Structural determinants of dopamine receptor up- and down-regulation The effect of 10 μM NPA and 100 μM quinpirole on the density of chimeric D_1/D_2 receptors was assessed to permit the initial localization of receptor domains that determine the direction of receptor regulation. Fig. 6 illustrates that both the D_1/D_2 agonist NPA and the D_2 agonist quinpirole increased the density of D_2 receptors, whereas NPA, but not quinpirole, decreased the density of D_1 receptors by 35 ± 6%. Interestingly, CH2, which has D_2 sequence from the amino-terminal end of TM V to the carboxy terminus (Fig. 6, Inset), was up-regulated by both agonists even more than the wild type D_2 receptors.

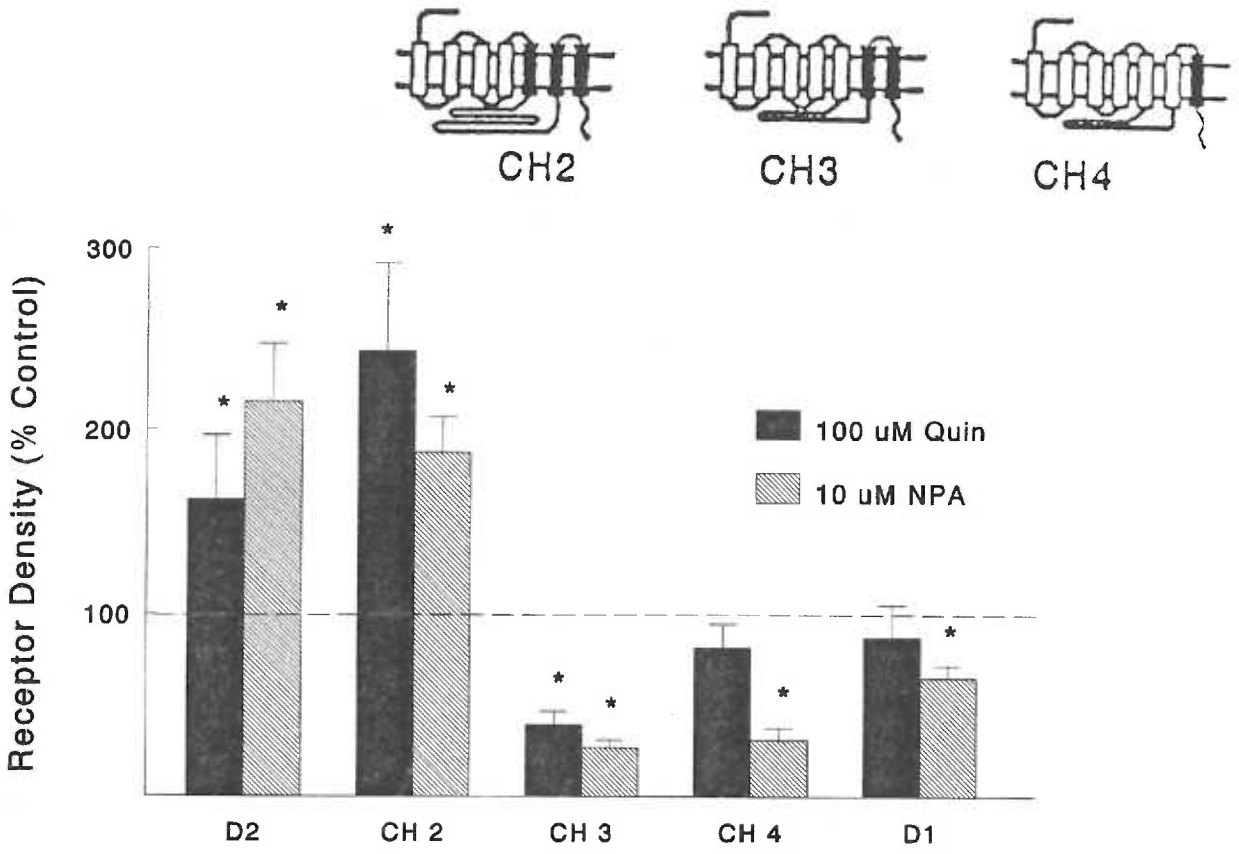


Fig. 6. C_6 cells expressing D_1, D_{2L}, or chimeric D_1/D_2 receptors were treated overnight with 10 μM NPA or 100 μM quinpirole. The density of D_{2L} receptors was determined by saturation analysis of the binding of [^{125}I]epidepride (NPA-treated and control cells) or [^{3}H]spiperone (quinpirole-treated and control cells). The density of CH2, CH3, CH4 and D_1 receptors was determined by saturation analysis of the binding of [^{3}H]SCH-23390. Data shown are the mean ± SEM of 3-5 independent experiments. Results are expressed as a percentage of the density of receptors on untreated cells. Inset: schematic representation of the structure of the chimeric receptors. D_1 sequence is denoted by open rectangles for putative TM domains and thick lines for intracellular and extracellular hydrophilic regions. D_2 sequence is denoted by filled rectangles for the putative TM domains and thin lines for hydrophilic regions.
*significantly different from control, $p < 0.05$

Treatment with 100 μM quinpirole more than doubled the density of CH2, from 158 fmol/mg of protein to 383 fmol/mg. On the other hand, both agonists decreased the density of CH3, which has D_2 sequence from the amino-terminal end of TM VI to the carboxy terminus, from 167 to 66 fmol/mg (quinpirole) and from 442 to 120 fmol/mg of protein (NPA). Only NPA decreased the

density of CH4, which has D_2 receptor sequence from the amino-terminal end of TM VII to the carboxy terminus, from 268 to 94 fmol/mg. Because the difference in sequence between CH2 (up-regulated) and CH3 (down-regulated) is in TM V and the third cytoplasmic loop, this region is implicated in the direction of receptor regulation.

DISCUSSION

Although the classical response of receptors to stimulation by an agonist is down-regulation, our results confirm the observation that the density of D_2 dopamine receptors on cells in culture is increased by agonist treatment. NPA was the most potent of the three drugs tested, inducing a proliferation of receptors at a concentration at least 10-fold lower than the lowest effective concentration of dopamine or quinpirole. This is consistent with the greater potency of NPA at D_2 receptors as assessed by inhibition of the binding of radioligands to D_2 receptors (Cox *et al.*, 1992). The effect of 10 μM NPA was maximal after treatment for approximately 10 h. Agonist-induced receptor proliferation has now been described for a cell line that expresses endogenous D_2 receptors (Ivins *et al.*, 1991), as well as recombinant D_2 receptors expressed in HEK-293 cells (Filtz *et al.*, 1993), Chinese hamster ovary (CHO) cells (Zhang *et al.*, 1994), C_6 glioma cells, and Ltk^- cells (present results). As detailed below, however, there are both qualitative and quantitative differences in results that may reflect differences in the cell lines or expression systems.

The proliferation of D_{2L} receptors was induced not only by agonists, but also by three antagonists, epidepride, sulpiride, and domperidone. The magnitude of the increase was less for the antagonists than for the agonists, implying some selectivity for agonists in inducing receptor proliferation. These results are similar to those reported by Filtz et al. (1993).

Comparison of a number of independent clones of C_6-D_{2L} and -D_{2S} indicated that the alternatively spliced forms of D_2 receptors are both up-regulated by treatment with NPA, as reported by Filtz et al. (1993), but in contrast to the results of Zhang et al. (1994), who found that the density of D_{2S} expressed in CHO cells is decreased by treatment with agonists whereas the density of D_{2L} is increased. The present results indicated that in C_6 glioma cells, D_{2S} was up-regulated less than D_{2L} after treatment with 10 μM NPA.

Several manipulations were carried out to assess the mechanism of ligand-induced D_2 receptor proliferation. D_2 dopamine receptors couple to the pertussis toxin-sensitive G proteins,

G_i and G_o, and inactivation of these G proteins by treatment with pertussis toxin prevents most D_2 receptor modulation of second messengers (Vallar and Meldolesi, 1989). Pre-treatment of C_6-D_{2L} cells with 100 ng/ml of pertussis toxin had no effect on NPA-induced D_2 receptor proliferation, indicating that an interaction with pertussis toxin-sensitive G proteins is not necessary for receptor proliferation to occur. The lack of effect of pertussis toxin on receptor proliferation does not exclude the possibility that an interaction with pertussis toxin-insensitive G proteins is necessary for receptor proliferation. Since several responses to activation of D_2 receptors are not inhibited by pertussis toxin (Ganz *et al.*, 1990; Neve *et al.*, 1992; Senogles, 1994), modulation of one of these signaling pathways could be involved in pertussis toxin-insensitive receptor proliferation. Nevertheless, the insensitivity to pertussis toxin is consistent with the finding that antagonists can induce receptor up-regulation, since antagonists would not be expected to induce coupling of receptors to G proteins. Together these data suggest that modulation of second messengers is not necessary for ligand-induced receptor up-regulation. Our results are at variance with those of Zhang et al. (1994), who reported that treatment with 1 μg/ml of pertussis toxin prevents NPA-induced proliferation of D_{2L} receptors. The discrepancy could by due either to the 10-fold higher concentrations of pertussis toxin used by Zhang et al. or to differences between C_6 and CHO cells.

To assess if protein synthesis was necessary for NPA-induced proliferation of D_{2L} receptors, we treated cells with cycloheximide. We observed that cycloheximide treatment by itself decreased the density of receptors. Comparison of the density of receptors in cycloheximide-treated cells with the density in cells treated with both 10 μM NPA and cycloheximide indicated that inhibition of protein synthesis reduced, but did not prevent, NPA-induced receptor proliferation. All experiments summarized above used the substituted benzamide derivative [^{125}I]epidepride to quantify the density of D_2 receptors, but the effect of NPA and cycloheximide was quantified using the butyrophenone radioligand [^{3}H]spiperone. Although there is one report that the effect of agonist treatment may differ according to whether D_2 receptor density is assessed using a butyrophenone or a substituted benzamide radioligand (Barton *et al.*, 1991), in the present experiments the effect of treatment with NPA was the same regardless of the radioligand used.

We recently characterized chimeric D_1/D_2 receptors, determining that CH2, with the first and second cytoplasmic loops from the D_1 receptor and the third cytoplasmic loop and C-terminus

from the D_2 receptor, is not able to stimulate or inhibit adenylyl cyclase (Kozell *et al.*, 1994). In contrast, CH3 and CH4, with all 3 cytoplasmic loops from the D_1 receptor and the C-terminus from the D_2 receptor, stimulate but do not inhibit adenylyl cyclase. Furthermore, quinpirole, which has virtually no ability to stimulate adenylyl cyclase *via* D_1 receptors or CH4, gains the ability to stimulate adenylyl cyclase with the addition of TM VI from the D_2 receptor (CH3), but then loses coupling to adenylyl cyclase with the further addition of the third cytoplasmic loop and TM V from the D_2 receptor (CH2). Analysis of chimeric receptor regulation by agonists should suggest important domains for receptor regulation.

As we reported previously (Machida *et al.*, 1992), treatment with a D_1 receptor agonist decreases the density of recombinant D_1 receptors. Quinpirole had no effect on the density of D_1 receptors, which is consistent with its low affinity and efficacy for this subtype (Kozell *et al.*, 1994). The mixed D_1/D_2 agonist NPA increased the density of CH2, but decreased the density of CH3. Since the sequence difference between these two chimeric receptors is in TM V and the third cytoplasmic loop, this suggests that somewhere within this region are key structural determinants of receptor regulation. These data do not differentiate between two possibilities. First, the D_2 receptor TM V and third cytoplasmic loop could be important for up-regulation. Second, the D_1 receptor TM V and third cytoplasmic loop, and the ability to couple to G_s and to stimulate adenylyl cyclase activity that is conferred by this domain, could be important for agonist-induced down-regulation. In support of the second possibility, agonists that stimulate adenylyl cyclase activity at CH3, CH4, and D_1 receptors decreased the density of each of these receptors. In particular, quinpirole gains the ability to stimulate adenylyl cyclase *via* CH3, and also down-regulated this chimera but not CH4 or D_1 receptors. The first possibility mentioned above could also be correct, since the two are not mutually exclusive, but it is at least equally likely that it is the absence of the D_1 third cytoplasmic loop rather than the presence of the D_2 third cytoplasmic loop that leads to up-regulation of CH2 and D_2 receptors.

Agonist-induced proliferation of receptors is unusual, but not unprecedented, and seems to involve multiple mechanisms that may vary depending on the tissue or receptor. The density of 5-HT_2 receptors on cultured cerebellar neurons and β_3-adrenergic receptors on 3T3 cells is increased by persistent stimulation with agonists, due to an increased abundance of receptor mRNA (Akiyoshi *et al.*, 1993; Thomas *et al.*, 1992). The density of GnRH receptors is increased

by low concentrations of agonist, apparently as a result of increased efficiency of translation of an unchanged pool of mRNA (Tsutsumi *et al.*, 1993). The density of vitamin D_3 receptors is also increased by agonists, and at least two mechanisms appear to be involved. The receptors appear to be more stable when in an agonist-binding conformation; i.e., the rate of degradation of receptors that are occupied by agonist is decreased (Costa and Feldman, 1987; Wiese *et al.*, 1992). In addition to a large decrease in the rate of receptor degradation, agonist binding causes a smaller increase in the rate of synthesis of the receptors (Costa and Feldman, 1987), probably due to increased gene expression (Mangelsdorf *et al.*, 1987; Kamei *et al.*, 1993). Our data do not unequivocally identify a mechanism for ligand-induced proliferation of D_2 receptors. Several lines of evidence, however, including the antagonist-induced proliferation, the lack of effect of pertussis toxin, and the failure of cycloheximide to prevent the NPA-induced increase, suggest that stabilization of the receptors by ligands is a likely mechanism.

III. Structural Determinants Of D_2 Dopamine Receptor Function And Regulation

ABSTRACT:

Molecular modeling studies of the D_{2L} dopamine receptor suggest that amino acid residues phenylalanine 110 (F110) and serine (S167), and the carboxyl terminal region of the third intracellular loop (IC3), are important for ligand binding and receptor activation (Trumpp-Kallmeyer *et al.*, 1992; Burstein *et al.*, 1995; Shapiro *et al.*, 1993). In vitro mutagenesis was used to substitute alanine residues for F110 (F110A) and S167 (S167A), and to create the deletion mutant receptor D_2(del 365-374). HEK 293 cells were stably transfected with D_{2L} or mutant receptor cDNAs and clonal cell lines with receptor densities ranging from 0.7 to 2 pmol/mg protein were selected. In assays to determine the ability to inhibit forskolin-stimulated adenylate cyclase activity, the EC_{50} values for DA, NPA, and quinpirole were similar for D_{2L} and mutant receptors, though these agonists had somewhat decreased efficacy at D_2(del 365-374) receptors. D_{2L}, S167A, and D_2(del 365-374) receptors had similar affinities for antagonists. In contrast, F110A receptors had increased affinity for the antagonist haloperidol, yet had decreased affinity for some other antagonists and agonists. In competition binding studies with agonists, without GTP, there were high and low affinity states for D_{2L} and mutant receptors indicating interactions with G proteins. D_{2L} and S167A receptors were all shifted to the low affinity state with the addition of GTP. D_2(del 365-374) receptors were resistant to the addition of GTP, as agonist competition curves in the presence of GTP were best fit by assuming the presence of high- and low- affinity classes of binding sites. Also D_2(del 365-374) receptors had over 160-fold higher affinity for agonists compared to D_{2L} receptors in the presence of GTP. Some D_2 antagonists have been reported to act as inverse agonists at D_{2L} receptors (Kozell and Neve, 1997). Several antagonists acted as inverse agonists on cells expressing D_{2L}, F110A, S167A, and D_2(del 365-374) receptors. Cells expressing D_2(del 365-374) receptors produced very large increases in forskolin-stimulated cAMP accumulation in the presence of some antagonists suggesting a greater degree of constitutive activity of this mutant. Higher affinity for agonists, resistance to effects of GTP in competition binding assays with agonists, and inverse agonist activity of some antagonists are all characteristics of constitutively active receptors. In other studies, regulation of receptor density after overnight treatment with agonists was assessed. Treatment with agonists alone gave

no change in D_{2L} receptor density, small increases in F110A receptor density, and robust increases for S167A and D_2(del 365-374) receptors. D_{2L} receptor density was significantly increased after overnight treatment with agonists plus forskolin. The addition of forskolin did not enhance the receptor density increases caused by NPA treatment of mutant receptors.

INTRODUCTION:

D_2 dopamine receptors have been implicated in the clinical manifestations of Parkinson's disease and schizophrenia (Seeman and Van Tol, 1994). D_2 receptors are a member of the seven transmembrane domain, GPCR superfamily (Trumpp-Kallmeyer *et al.*, 1992). D_2 receptors inhibit the enzyme adenylate cyclase via coupling to the pertussis toxin-sensitive G proteins $G_{i/o}$ (Creese, 1987). By studying dopamine receptors in cultured cells, receptor subtypes can be isolated such that ligand-receptor interactions and the activation of second messenger systems can be examined (Neve *et al.*, 1989).

Specific amino acid residues or regions of dopamine receptors are crucial for ligand binding and receptor function due to the inherent properties of the residue side chains such as hydrophobicity, charge, size, and spatial orientation. To test if an amino acid residue or region contributes to binding or function, the residue can be replaced with one that has a side chain with different properties (Cavalli *et al.*, 1996; Cho *et al.*, 1996; Cox *et al.*, 1992; Kjelsberg *et al.*, 1992; Trumpp-Kallmeyer *et al.*, 1992) or the region of interest can be replaced with the corresponding region from a homologous receptor (Cotecchia *et al.*, 1990; Kozell and Neve, 1997; Wang *et al.*, 1995), or deleted (Shapiro *et al.*, 1993; Takhar *et al.*, 1996). If the hypothesis that the residue or region of the receptor contributes to binding or function of the receptor is correct, then the mutant receptor may exhibit differences in the affinity of ligand binding, ability to couple to second messenger systems, or agonist-induced changes in receptor density. We chose to replace F110 and S167, which are found in transmembrane domains 3 and 4, respectively, with alanine residues, as well as to delete the 10 carboxyl terminal residues of IC3 D_2(del 365-374) of the D_{2L} receptor to create three mutant receptors. F110 and S167 as well as a number of residues within the deleted region are conserved among catecholamine receptors. The assumption here is that residues that are conserved within specific classes of GPCRs may be important for ligand binding and receptor activation (Trumpp-Kallmeyer *et al.*, 1992). Phenylalanine, has an aromatic side chain that may stabilize the electrostatic interaction between D114 and the amine group of

the ligand, participate in ring-ring interactions with the aromatic ring of ligands (Malmberg *et al.*, 1994), and may mediate changes between different receptor conformations (Trumpp-Kallmeyer *et al.*, 1992). F110 has specifically been suggested to be important for ligand binding from molecular modeling studies of D_2 DA receptors (Moereels and Leysen, 1993). Serine has a hydrophilic side chain that can form hydrogen bonds. S167 has also been specifically suggested by D_2 DA receptor homology modeling studies (Teeter *et al.*, 1994). These residues have been substituted with alanine, which has a hydrophobic non-reactive side chain and should abolish the inherent properties of the wild type side chain. The region of IC3 which was deleted has been implicated in G protein coupling in other GPCRs (Burstein *et al.*, 1995; Shapiro *et al.*, 1993). Studies in which point mutations were introduced in this region resulted in constitutively active receptors (Kjelsberg *et al.*, 1992; Zhou *et al.*, 1997).

MATERIALS AND METHODS

Materials [^{3}H]Spiperone was purchased from Amersham (Arlington Heights, IL). [^{3}H]cAMP was purchased from New England Nuclear (Boston, MA). NPA, forskolin, quinpirole, SCH23390, butaclamol, and sulpiride were purchased from Research Biochemicals (Natick, MA). Spiperone and domperidone (Janssen), epidepride (Dr. T. de Paulis, Vanderbilt University, and a cDNA for the rat long (444-amino-acid) form of the D_2 receptor (Dr. O. Civelli, Oregon Health Sciences University) were generous gifts. Dopamine (3-hydroxytyramine) and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Construction of mutant receptor cDNAs Mutant D_{2L} cDNAs were generated in which either F110 or S167 was changed to alanine (F110A and S167A) or in which the carboxyl terminus of IC3 was deleted using the method of trans-PCR (Neve *et al.*, 1991) and ligated into the pRSV vector. Mutant D_{2L} cDNAs in which S365 or T373 was changed to alanine (S365A and T373A) were generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene), with D_{2L} cDNA in the pcDNA3 vector as template.

Expression Of Recombinant Receptors D_{2l}, F110A, S167A, and D_2(del 365-374) cDNAs were stably expressed in HEK 293 cells using the method of electroporation as previously described (Kozell and Neve, 1997). S365A and S373A cDNAs were stably expressed in HEK 293 cells using the method of calcium phosphate precipitation as previously described (Cox *et al.*, 1992). Transfectants were selected using growth medium containing puromycin (2ug/Ml) as previously

described (Kozell and Neve, 1997). Puromycin resistant colonies were transferred to 6-well clusters and screened for specific binding of [^{3}H]spiperone.

Radioligand binding assay The binding of [^{3}H]spiperone was assessed as described (Cox *et al.*, 1992). Aliquots of the membrane preparation (3-40 μg of protein) were added to duplicate assay tubes containing the following (final concentrations): 50 mM Tris-HCl, pH 7.4 with 155 mM NaCl (Tris-buffered saline), 0.001 % bovine serum albumin, radioligand, and appropriate drugs. (+)-Butaclamol (2 μM) was used to define nonspecific binding of [^{3}H]spiperone. Incubations were carried out at 37° C for 60 min, in a volume of 1.0 ml.

Competition binding assays were carried essentially as previously described (Wiens *et al.*, 1997). The crude membrane fraction was resuspended in HEPES assay buffer (20mM K^+-HEPES, pH 7.4, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.01% BSA, 0.025% ascorbic acid). Membrane proteins were incubated in duplicate in a total reaction volume of 1 ml in the presence of [^{3}H]spiperone (0.05 nM) and the appropriate concentration of the competing drug for competition binding. Drug concentrations were diluted and assay buffer was added using a Biomek 2000 (Beckman). Incubations were terminated by filtration through glass-fiber filters on a 96-well Tomtec cell harvester, dried, and soaked with BetaPlate scintillation fluid. Radioactivity was counted using a Wallac (Gaithersburg, MD) 1205 BetaPlate scintillation counter.

Data analysis Data were analyzed by nonlinear regression using the program GraphPAD Prism. The free concentration of radioligand was calculated as the concentration added minus the concentration bound. Averages for K_D and K_i values are the geometric means [the antilogarithm of mean logarithms (Fleming *et al.*, 1972)].

Adenylate Cyclase Activity Cells were plated in 48 well clusters at a concentration of 100,000 cells/well and grown to confluence. All assays included 10, 30, or 60 μM forskolin plus increasing concentrations of agonist in Earle's balanced salt solution containing 0.02% ascorbic acid and 2% calf bovine serum. Cells were incubated for 10 min. at 37° C, medium was then removed and ice cold 3% TCA was added to lyse cells. Plates were stored at 4° C, then centrifuged at 1000 x g for 10 minutes prior to the cAMP competitive binding assay (Watts and Neve, 1996).

Quantification of cAMP cAMP was quantified using a competitive binding assay with the cAMP binding protein PKA and [^{3}H]cAMP as previously described (Watts and Neve, 1996).

Drug-induced up-regulation of D_2 and mutant receptors Overnight treatment with agonists was carried out essentially as previously described (Starr *et al.*, 1995).

RESULTS:

Binding of [^{3}H]spiperone to wild type and mutant D_2 receptors HEK 293 cells were stably transfected with D_{2L}, F110A, S167A, or D_2(del 365-374) cDNAs. Saturation analysis using [^{3}H]spiperone gave average densities of 1900 $\pm$ 229 fmol/mg of membrane protein for D_{2L}, 1406 $\pm$ 146 fmol/mg for F110A, 741 $\pm$ 96 fmol/mg for S167A, and 862 $\pm$ 124 fmol/mg for D_2 (del 365-374) with average affinity values of 50 (37-69) pM for D_{2L}, 20 (16-25) pM for F110A, 44 (33-60) pM for S167A, and 74 (52-106) pM for D_2(del 365-374) (Fig. 1).

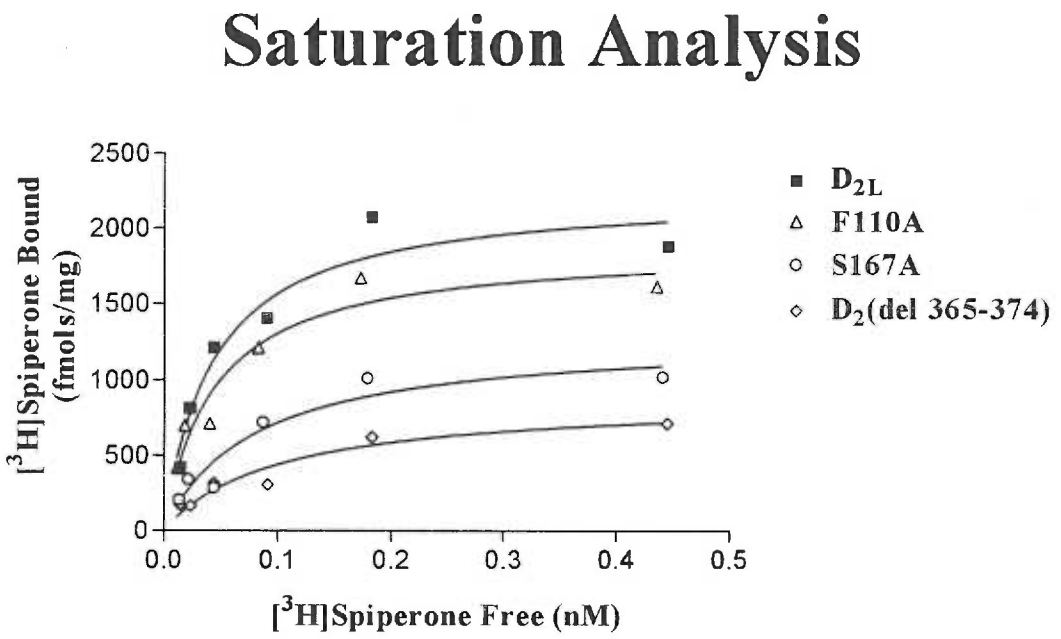


Fig. 1. The density of the receptors was determined by saturation analysis of the binding of [^{3}H]spiperone to membranes prepared from HEK 293 cells expressing D_{2L}, F110A, S167A, or D_2(del 365-374) receptors. The experiment shown is representative of 8-16 independent experiments.

Inhibition of adenylate cyclase Cells expressing D_{2L} or mutant receptors were used in functional assays to assess receptor mediated inhibition of forskolin-stimulated adenylate cyclase activity. The concentration at which 50% of forskolin-stimulated cAMP accumulation was inhibited (EC_{50}) in cells expressing D_{2L}, F110A, S167A, and D_2(del 365-374) receptors were

between 0.8 and 6 nM for DA, and between 1 and 25 nM quinpirole and between 0.2 and 0.3 nM for NPA (Table 1). There was high efficacy for the inhibition of forskolin-stimulated adenylate cyclase activity in cells expressing D_{2L}, F110A, and S167A receptors, resulting in 90% inhibition of forskolin-stimulated activity. All three agonist were somewhat less efficacious at D_2(del 365-374) producing only 70 to 80% inhibition (Fig. 2, Table 1). Cells expressing D_2(del 365-374) receptors had low levels of forskolin-stimulated cAMP accumulation of approximately 20-50 pmol per well compared with 150-200 pmols per well for cells expressing wild type and the other mutant receptors.

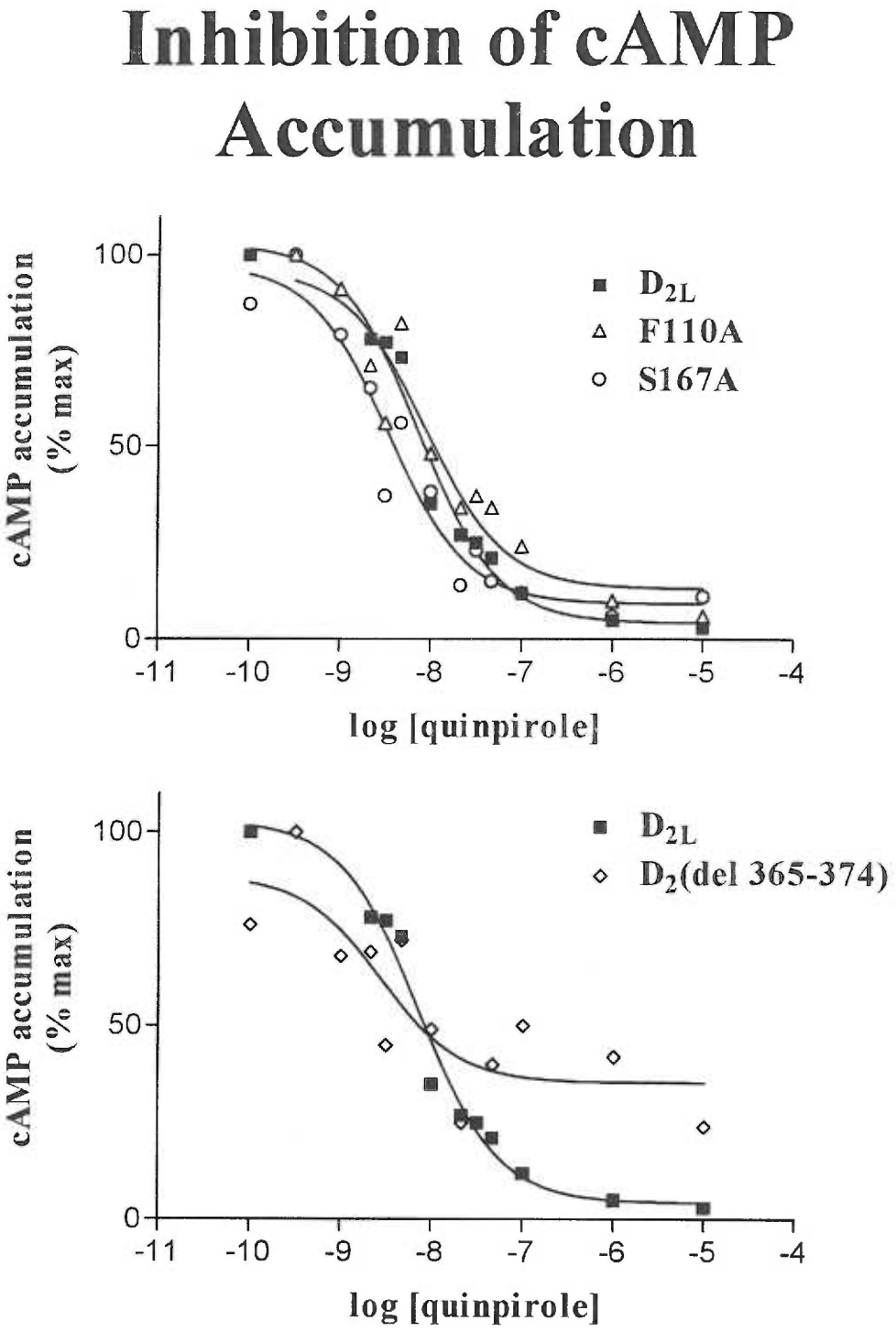


Fig 2. Results are expressed as % of maximum cAMP accumulation stimulated by 30 μM forskolin. The experiment shown is representative of 3-20 independent experiments.

Table 1. Effects of mutations on inhibition of forskolin-stimulated cyclic AMP accumulation by D_2 receptor agonists

	D_{2L}	F110A	S167A	D_2(del 365-374)
Dopamine				
EC_{50}	3 nM (2-5)	6 nM (3-13)	1 nM (0.4-2)	0.8 nM (0.2-3)
Max	91 ± 2%	91 ± 2%	90 ± 2%	69 ± 6%
NPA				
EC_{50}	0.2 nM (0.1-0.4)	0.2 nM (0.009-0.3)	0.2 nM (0.1-0.4)	0.3 nM (0.1-0.5)
Max	90 ± 1%	93 ± 2%	92 ± 1%	71 ± 6%
Quinpirole				
EC_{50}	25 nM (7-95)	6 nM (0.4-71)	5 nM (3-8)	1 nM (0.5-3)
Max	94 ± 4%	87 ± 6%	94 ± 4%	82 ± 11%

Results are shown from 3-20 independent experiments in which inhibition of forskolin-stimulated cyclic AMP accumulation was determined in HEK cells expressing D_{2L} or mutant receptors. The concentration at which 50% maximal inhibition was observed (EC_{50}) was determined by nonlinear regression. Values are the geometric means, followed by the 95% confidence limits of the mean. The maximal inhibition observed (Max) is expressed as a percentage of total forskolin-stimulated cAMP accumulation. The means ± SEM are shown.

Competition binding of antagonists Competition binding assays were carried out to determine the apparent affinity of wild type and mutant receptors for antagonists. The affinity of F110A for haloperidol was increased 25-fold compared to D_{2L}, whereas the affinity of the mutant for several other antagonists was decreased approximately 10-fold (Fig. 3, Table 2). The affinity of S167A receptors was significantly decreased for the substituted benzamide class of antagonists compared to wild type (Table 2). All three mutant receptors had significantly decreased affinity for epidepride (Table 2).

F110A Receptors have Increased Affinity for Haloperidol and Decreased Affinity for Sulpiride

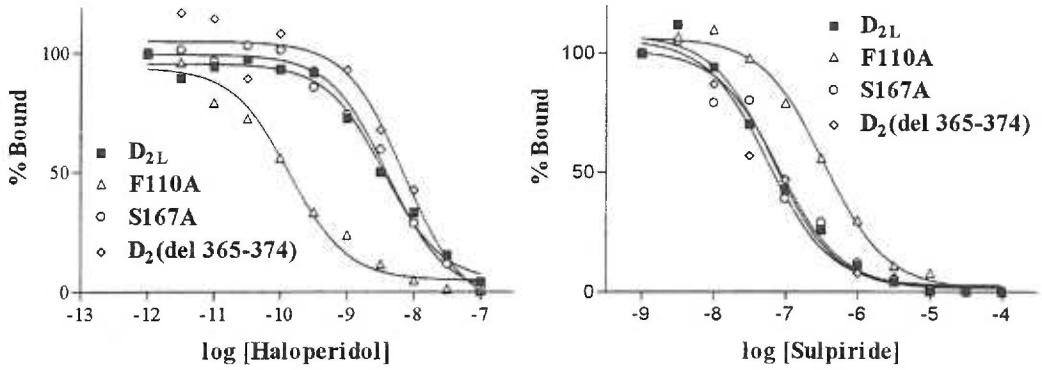


Fig 3. The binding of [^{3}H]spiperone to D_{2L} and mutant receptors was inhibited by increasing concentrations of antagonist. The experiment shown is one of 3-4 independent experiments. Results are plotted as radioligand bound, expressed as the percentage of specific binding in the absence of inhibitor, versus the concentration of inhibitor.

Table 2 Inhibition of radioligand binding by antagonists

	Ki (nM)			
	D_{2L}	F110A	S167A	D2(del 365-374)
Butaclamol	2 (0.1-0.4)	1 (0.4-5)	0.4 (0.2-0.5)	0.4 (0.2-0.8)
Domperidone	1 (0.8-2)	0.7 (0.4-1)	1 (0.4-3)	2 (1-3)
Haloperidol	1 (0.7-1)	0.04** (0.03-0.04)	1 (0.9-2)	1 (0.8-2)
Sulpiride	37 (31-45)	303** (297-301)	86** (64-115)	45 (43-46)
Cis-flupenthixol	0.3 (0.2-0.6)	4** (3-6)	0.9 (0.5-2)	0.6 (0.3-2)
Clozapine	132 (61-285)	1520** (971-2380)	238 (136-417)	188 (89-395)
Epidepride	71 (64-79)	360** (275-470)	220** (175-277)	113* (97-131)
Eticlopride	0.01 (0.007-0.02)	0.5** (0.4-0.8)	0.04* (0.02-0.09)	0.03 (0.02-0.03)

The apparent affinities (Ki) of the antagonists were determined by inhibition of the binding of [^{3}H]spiperone to membranes prepared from HEK 293 cells expressing D_{2L} or mutant receptors. Results are shown from 3-4 independent experiments. Values are the geometric means, followed by the 95% confidence limits of the mean. *$p < 0.05$, **$p < 0.01$ compared to wildtype (Dunnett's *post hoc* analysis of variance).

Competition binding of agonists To assess receptor: G protein interactions, agonist inhibition of the binding of [^{3}H]spiperone was carried out in the presence and absence of GTP. In the absence of GTP, the competition curves for DA, NPA, and quinpirole were best fit by assuming the presence of two classes of binding sites with 41%, 51%, and 42% of the wild type D_{2L} receptors in the high affinity state (Fig. 4). Addition of GTP abolished high affinity binding of DA so that curves were shifted to a single lower affinity state. On the other hand, D_2(del 365-374)

receptors were resistant to GTP and competition curves were best fit by assuming two classes of binding sites. There was a shift in overall affinity of the high and low states for D_2(del 365-374) with the addition of GTP. The Hill slope for D_2(del 365-374) remains less than unity consistent with the presence of more than one binding state. Also, the D_2(del 365-374) receptors had over 160-fold higher affinity for the agonists DA, quinpirole, and NPA compared to D_{2L} receptors. The F110A receptor had greatly reduced affinity for the agonist quinpirole as well as moderately decreased affinity for DA and NPA. S167A receptors had similar affinity values to D_{2L} (Table 3).

High Affinity State of Agonist Binding to D_2(365-374) is Resistant to Effects of GTP

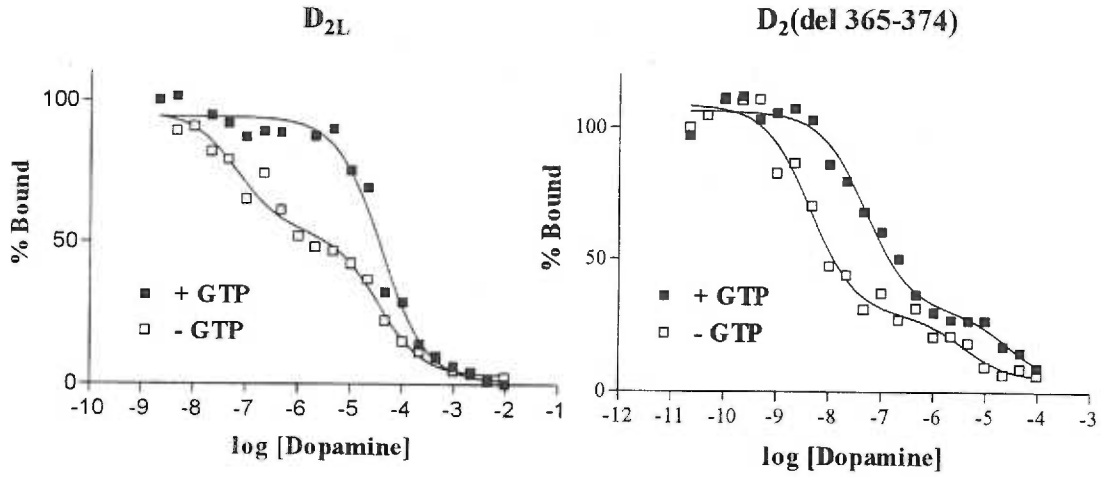


Fig 4. The binding of [^{3}H]spiperone to D_{2L} and mutant receptors was inhibited by increasing concentrations of agonist. The experiment shown is one of 4-6 independent experiments. Results are plotted as radioligand bound, expressed as the percentage of specific binding in the absence of inhibitor, versus the concentration of inhibitor.

Table 3. Inhibition of radioligand binding by agonists

	+ GTP		-GTP				
			one site		two sites		
	IC_{50}	Hill	IC_{50}	Hill	K_H	%hi	K_L
Dopamine (μM)							
D_{2L}	43 (38-50)	0.89	6.9 (2.5-1.9)	0.48	0.2 (0.04-0.4)	41	11 (7.3-17)
F110A	126 (74-215)	0.54	45 (26-77)	0.61	1.6 (1.1-5.5)*	44	55 (44-194)
S167A	61 (31-118)	0.77	3.5 (1-12)	0.51	0.4 (0.2-.08)	69	40 (25-63)
D_2(del 365-374)	0.2 (0.1-0.3)**	0.58	0.04 (0.01-0.1)**	0.40	0.002 (0.001-0.003)*	59	0.5 (0.8-1.6)**
NPA (nM)							
D_{2L}	32 (25-41)	0.92	1.6 (0.4-59)	0.54	0.05 (0.02-0.2)	51	5 (2-12)
F110A	93 (48-183)*	0.68	38 (18-82)	0.52	0.6 (0.3-1)	37	42 (30-60)
S167A	10 (7-14)*	0.65	1.7 (0.8-3.7)	0.52	0.02 (0.001-0.4)	47	4 (3-5)
D_2(del 365-374)	0.3 (0.04-7)**	0.63	0.07 (0.04-0.1)*	0.49	0.001 (0.00005-0.02)*	64	0.8 (0.08-7)*
Quinpirole (μM)							
D_{2L}	54 (36-83)	0.86	7.7 (3-20)	0.52	0.1 (0.05-0.3)	42	11 (6-18)
F110A	11,484 (130-10,145)*	0.63	6,837 (68-68,474)*	0.40	23 (1-370)**	26	92 (63-136)*
S167A	7 (0.5-95)	0.84	2 (0.2-31)	0.57	0.04 (0.003-0.4)	42	3 (0.5-16)
D_2(del 365-374)	0.2 (0.07-0.5)**	1.1	0.6 (0.1-3)**	0.55	0.002 (0.0006-0.004)*	31	0.1 (0.05-0.3)**

The concentration at which 50% of [^{3}H]spiperone binding was inhibited (IC50) and the apparent affinities (Ki) of the agonists were determined by inhibition of the binding of [^{3}H]spiperone to membranes prepared from HEK 293 cells expressing wildtype or mutant receptors. Results are shown from 4 to 6 independent experiments. Values are the geometric means, followed by the 95% confidence limits of the mean. *$p < 0.05$, $p^{**} < 0.01$ compared to wild type (Dunnett's post hoc analysis of variance).

Inverse agonist effect of antagonists In studies to assess the ability of antagonists to act as inverse agonists at D_{2L} or mutant receptors, there was marked potentiation of forskolin-stimulated cAMP accumulation in the presence of D_2-selective antagonists in cell lines expressing D_{2L} and mutant receptors. Studies with cells expressing D_{2L} receptors have shown some D_2-selective antagonists to act as inverse agonists (Kozell and Neve, 1997). The antagonists may act to block the inherent constitutive activity of the receptor, thus relieving tonic inhibition of adenylate cyclase. Increases in cAMP accumulation ranged from about 50 to 100% for D_{2L}, 100 to 150% for F110A, 40 to 140% for S167A, and 250 to 350% for D_2(del 365-374) receptors for all D_2-selective antagonists tested except for clozapine at D_2(del 365-374) receptors (Fig. 5). Similar increases in cAMP accumulation with the addition of inverse agonists have been reported for cells expressing D_{2L} receptors (Kozell and Neve, 1997). With the exception of clozapine, cells expressing D_2(del 365-374) receptors had greatly enhanced increases in cAMP accumulation in the presence of D_2-selective antagonists compared to wild type or the other mutant receptors.

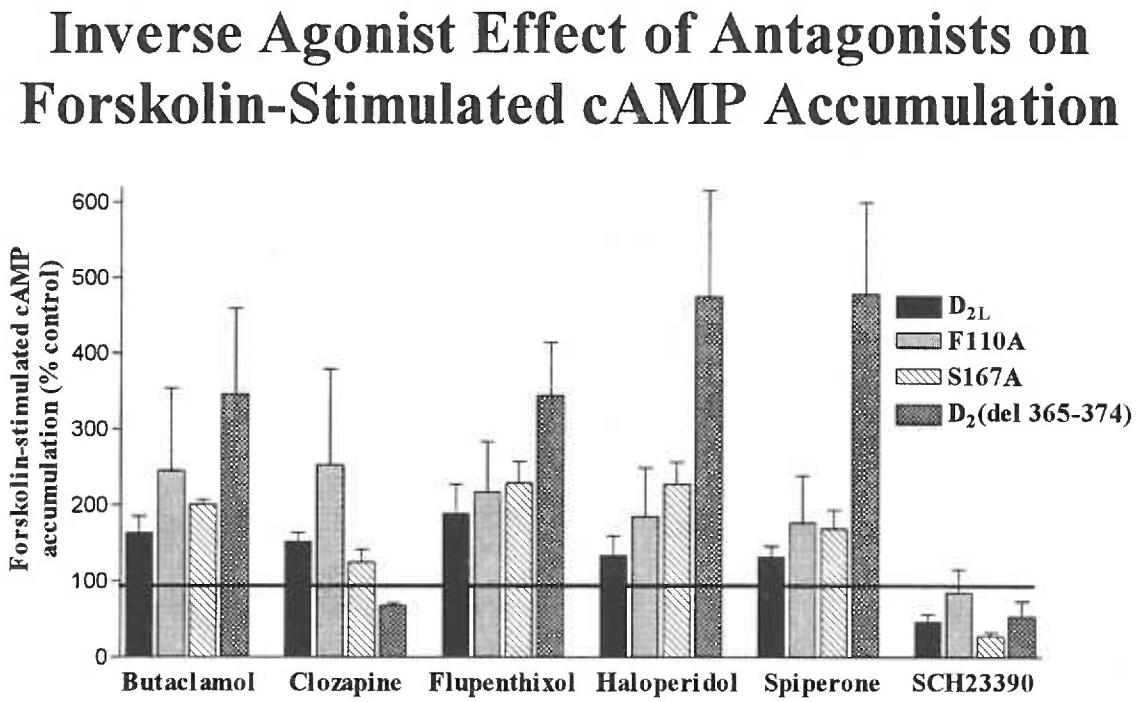


Fig. 5 Results are expressed as a percentage of maximum cAMP accumulation stimulated by 60 μM forskolin in the absence of antagonist.

Agonist-induced up-regulation of D_{2L} and mutant receptors Additional studies were carried out to examine agonist-induced regulation of D_{2L} and mutant receptor density. Cells expressing D_{2L} or mutant receptors were treated for 18 hours with 3 μM NPA, 3 μM NPA plus 10 μM forskolin, or 10 μM forskolin. There was little change in receptor density for D_{2L} receptors after treatment with NPA alone, but F110A, S167A, and D_2(del 365-374) receptors were increased by 40% , 390%, and 860% above control levels, respectively (Fig. 6). Treatment of D_{2L} and mutant receptors with NPA plus forskolin resulted in increases of 88% (D_{2L}), 50% (F110A), 420% (S167A), and 1440% D_2(del 365-374). Forskolin alone had negligible effects on D_{2L} or mutant receptors (Fig. 6).

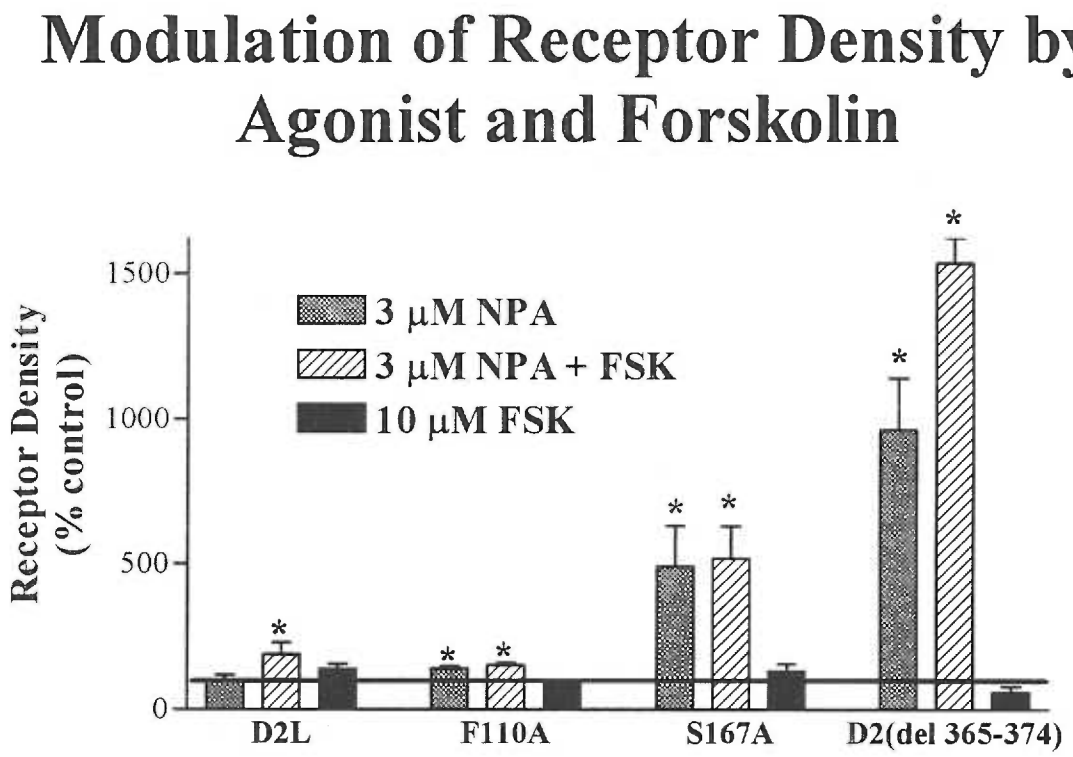


Fig. 6. D_{2L} receptor density was increased by overnight treatment with agonist plus forskolin. F110A receptor density was increased after treatment with agonist alone or agonist plus forskolin. The density of S167A and D_2(del 365-374) receptors was robustly increased with agonist alone or agonist plus forskolin. The density of receptors on membranes prepared from treated cells was determined by saturation analysis of the binding of [^{3}H]spiperone. Data shown are means ± S.E.M. from 3-7 independent experiments, only 2 independent experiments for D_2(del 365-374) treated with 3μM NPA + FSK. *$p < 0.01$ compared with control receptor density, t test.

DISCUSSION It is apparent from this study that the amino acid substitutions, F110A and S167A, suggested to be important for ligand binding and receptor activation through molecular modeling studies (Malmberg, *et al.*, 1994; Moereels and Leysen, 1993; Teeter, *et al.*, 1994; and Trumpp-Kallmeyer, *et al.*, 1992), and deletion D_2(del 365-374), suggested from studies with other GPCRs (Burstein *et al.*, 1995; Kjelsberg *et al.*, 1992; Shapiro et al. 1993; and Zhou *et al.*, 1997) are important for ligand binding, receptor activation, and constitutive activity of the D_{2L} receptor.

In this study the D_{2L} receptor had anticipated affinity values in saturation analyses and competition binding assays with antagonists and agonists, as well as expected values for EC_{50} and efficacy for the inhibition of forskolin-stimulated cAMP accumulation. In competition binding studies with agonists, in the absence of GTP curves were fit best by assuming both high- and low-affinity states, whereas in the presence of GTP curves were best fit by assuming a single affinity state. Addition of antagonists to assays for forskolin-stimulated cAMP accumulation resulted in increases in cAMP accumulation, presumable due to the constitutive activity of the D_{2L} receptor.

One recent theory explaining constitutively active receptors is the allosteric ternary complex model. In this model there is an equilibrium between two states of the receptor, inactive (R) and active (R*). This equilibrium defines the basal level of receptor mediated response and varies for individual receptors. The level of agonist-independent activity induced by constitutively activating mutations would vary as well. In the absence of agonist, structural constraints are put on the wild type receptor R, reducing the likelihood of spontaneously changing to R*. Agonist binding to R may relax the constraints, permitting the conformational change to the active state (R*). It has been hypothesized that constitutively active mutations may mimic this conformational change (Scheer and Cotecchia, 1997).

Negative antagonists, also referred to as inverse agonists, have properties that are reciprocal to agonists. Agonists would preferentially bind to R*, which promotes G protein activation, whereas inverse agonists preferentially bind to R (Samana *et al.*, 1993). In terms of the allosteric ternary complex model, there would be a spectrum of efficacies ranging from positive (agonist) to zero (neutral antagonists) to negative (inverse agonists). As inverse agonists bind preferentially to R the proportion of receptors in the R* state is decreased and G protein coupling would be decreased as well (Scheer and Cotecchia, 1997). For the D_{2L} receptor the

presence of an inverse agonist would preferentially bind to the R form of the receptor which would decrease activation of G_i. Since G_i is inhibitory toward adenylate cyclase, the presence of inverse agonist would result in increases in cAMP accumulation.

Agonist treatment of cultured cells expressing D_{2L} receptors has resulted in increases in receptor density (Ivins *et al.*, 1991; Filtz *et al.*, 1993, 1994; Zhang *et al.*, 1994; Boundy *et al.*, 1995; Starr *et al.*, 1995). This phenomena is interesting because it conflicts with the compensatory model of receptor regulation in which the over-stimulation of the receptors decreases receptor density. The results of the regulation studies are contradictory to agonist-induced up-regulation found after overnight treatment with DA, NPA, or quinpirole observed in C_6 glioma cells expressing D_{2L} receptors (Starr *et al.*, 1995), in HEK293 cells expressing D_{2L} receptors (Filtz *et al.*, 1993, 1994; Boundy et al, 1995), in SUP1 cells which endogenously express D_2 receptors (Ivins *et al.*, 1991), and in CHO cells expressing D_{2L} receptors (Zhang *et al.*, 1994), in which there was a doubling in receptor density. The discrepancy between the results that we observed for D_{2L} receptors expressed in HEK293 cells, in which there were only increases in receptor density with the addition of agonist plus forskolin, and C_6 cells, where agonist alone caused receptor density increases, may be due to different characteristics of the cell lines. Previously, differences between the characteristics of receptor up-regulation in CHO cells and other cell lines have been noted (Zhang et al, 1994; Filtz *et al.*, 1993; 1994; Starr *et al.*, 1995). The differences in results found within the same cell line may be due to species differences in DNA (rat D_{2L} in our work vs. human D_{2L} in work by Filtz et al.).

Saturation analysis of [^{3}H]spiperone binding to F110A receptors resulted in a significant increase in affinity compared to D_{2L} receptors. Spiperone is a member of the butyrophenone class of ligands. In competition binding assays with antagonists the F110A mutant receptor had an unexpected increased affinity of 25-fold for the butyrophenone haloperidol and 10-fold decreased affinity for some other D_2 antagonists including all substituted benzamides tested. Modeling studies have suggested that F110 is located one residue away from the extracellular surface of TM3 and one α-helical turn away from aspartate 114 (D114) which resides in TM3. D114 has been implicated in electrostatic interactions with agonists and antagonists in site-directed mutagenesis studies in which the inherent negative charge of the residue is altered (Mansour *et al.*, 1992; Neve *et al.*, 1994). It is possible that by removing the large hydrophobic side chain,

phenylalanine, and replacing it with an alanine, that the conformation of the receptor is changed such that the binding pocket facilitates interaction of D114 with ligands of the butyrophenone class.

The ability of agonists to inhibit forskolin-stimulated cAMP accumulation via F110A was similar to the D_{2L} receptors in both potency and efficacy. Mutation of this residue does not seem to interfere with the ability of the receptor to regulate adenylate cyclase activity.

In competition binding studies with agonists, the affinity of the F110A receptor for agonists was somewhat decreased compared to D_{2L}, with the affinity for quinpirole being particularly low. DA, NPA, and quinpirole were 3-fold, 3-fold, and 213-fold less potent, respectively, at F110A receptors compared to D_{2L} in competition binding assays in the presence of GTP and Hill slopes were 0.54, 0.68, and 0.63, consistent with the presence of more than one affinity state of the receptor. In the absence of GTP, K_H values for F110A receptors were 37-fold, 30-fold, and 230-fold less potent for DA, NPA, and quinpirole, respectively, compared to D_{2L} and 44%, 37%, and 26% of the receptors were in the high affinity state. The lower potency for agonists at F110A may indicate that the phenylalanine ring of the side chain is necessary for stabilization of the receptor ligand complex. D_2-selective antagonists act as inverse agonists in cells expressing the F110A receptor, potentiating cAMP accumulation as is found in cells expressing the native D_{2L} receptor. Assessment of receptor turnover resulted in small increases in F110A receptor density after overnight treatment with NPA alone. This is contrary to results of receptor regulation studies with D_{2L} receptors in which there was no increase in receptor density after overnight treatment with agonist alone.

DA, NPA, and quinpirole had similar potency and efficacy for inhibition of forskolin-stimulated adenylate cyclase activity in cells expressing S167A receptors compared to D_{2L} receptors. In competition binding studies with agonists and antagonists K_i values were comparable to wild type. D_2-selective antagonists acted as inverse agonists at S167A receptors as well. These results suggest that the inherent properties of the native side chain may not be crucial for ligand binding or for agonist -induced and constitutive activation of the wild type receptor. Surprisingly, there were robust increases in receptor density of over 400% after overnight treatment with the agonist NPA, in contrast to wild type receptors which required addition of forskolin to see significant receptor proliferation.

D_2(del 365-374) receptors show several characteristics of constitutively active receptors. D_2(del 365-374) receptors had higher affinity for agonists in competition binding assays, and the high-affinity agonist binding was resistant to the effects GTP. Furthermore, some antagonists acted as inverse agonists at D_2(del 365-374) receptors, producing greater increases in cAMP accumulation than in cells expressing the wild type receptor.

Although the EC_{50} values for agonists acting to inhibit forskolin-stimulated cAMP accumulation in cells expressing the D_2(del 365-374) receptor were not decreased, as would be expected for a constitutively active receptor, there were extremely low levels of forskolin-stimulated cAMP accumulation in the absence of agonist. If the receptor is already in the active conformation R*, there would conceivably be some tonic inhibition of adenylate cyclase activity which for a G_i-coupled receptor would lead to low levels of forskolin-stimulated cAMP accumulation. When D_2 antagonists were added to assays for adenylate cyclase activity most acted as inverse agonists resulting in large increases in cAMP accumulation compared to modest increases with wild type receptors. The levels of forskolin-stimulated cAMP accumulation in the presence of inverse agonists for cells expressing D_2(365-374) were similar to wild type receptors in the absence of antagonist.

In competition binding studies with antagonists there were no differences in affinity between D_2(del 365-374) and D_{2L} receptors. In competition binding studies with agonists, D_2(del 365-374) receptors had over 160-fold higher affinity for DA, NPA, and quinpirole compared to D_{2L} receptors. The addition of GTP did not shift the mutant receptors to the low affinity state for DA and NPA, as the Hill slopes remained at 0.58 and 0.63, respectively. One finding which differed was that there was a Hill slope of 1.1 in competition assays with quinpirole in the presence of GTP which is indicative of a single class of binding sites. These interactions with agonists give further evidence of the constitutive nature of D_2(del 365-374) receptors.

The carboxyl terminal region of IC3 has been reported to be important for G protein coupling as both substitutions and deletions in this region have lead to diminished activation of G proteins. On the other hand, point mutations and reciprocal mutations with homologous receptors in this region have resulted in constitutively active receptors in other GPCRs. In contrast to our results, when the same region was deleted in other GPCRs, phosphoinositide turnover was abolished in the mouse m1 muscarinic acetylcholine receptor (Shapiro *et al.*, 1993),

and there was no coupling to Gα14, in the α1-adrenergic receptor (Wu *et al.*, 1995). When point mutations were made in this region of other GPCRs there were marked increases in EC_{50} values for the human m5 muscarinic receptor (Burstein *et al.*, 1995), a loss in phosphoinositide but not cAMP signaling in the thyrotropin receptor (Kosugi *et al.*, 1992), and defective mediation of phosphatidylinositol turnover in the muscarinic Hm1 receptor (Moro *et al.*, 1993).

On the other hand, point mutations in this region in the α1-adrenergic receptor (Cotecchia *et al.*, 1990), the thyrotropin receptor (Parma *et al.*, 1993), the α1b-adrenergic receptor (Kjelsberg *et al.*, 1992), the α2-adrenergic receptor (Ren *et al.*, 1993), the D_1 receptor (Charpentier *et al.*, 1996), the D_2 receptor (Zhou *et al.*, 1997) and the muscarinic acetylcholine Hm1 receptor (Hogger *et al.*, 1995), resulted in constitutively active receptors. Other constitutively active receptors have resulted from reciprocal mutations in which IC3 has been replaced with the IC3 of a homologous receptor. For example the IC3 of the β2-adrenergic receptor has been replace with the IC3 of the α1b receptor (Samana *et al.*, 1993), TM5 and 6, and IC3 of the D_2 dopamine receptor with have been substituted with D_1 sequence in this region (Kozell and Neve, 1997), and IC3 of the angiotensin II type 2 receptor (AT2) has been replaced with IC3 of angiotensin II type 1 (AT1) (Wang *et al.*, 1995).

Current studies in our laboratory involve a point mutation at threonine 373, located within the region deleted in D_2(del 365-374). This substitution is analogous to point mutations described above for other GPCRs which have resulted in constitutive activation of the receptors. This threonine residue has been replaced with alanine (T373A) and preliminary results suggest constitutive activity, as some antagonists acted as inverse agonists producing large increases in cAMP accumulation in cells expressing the T373A receptor.

In regulation studies with mutant receptors there were small increases in receptor density after overnight treatment with agonist for F110A, but both S167A and D_2(del 365-374) gave robust increases in receptor density. In a study with a constitutively active β2-adrenergic (CAM) receptor, density was more than doubled after 48 hour treatment with agonist or inverse agonists (Gether *et al.*, 1997). Gether suggests that the presence of ligand has a stabilizing effect and that the protein is inherently unstable. This explanation seems plausible since increases in receptor density were found with both agonists and antagonists. It is possible that occupancy of the receptor by a ligand decreases the rate of degradation of the receptor.

IV. DISCUSSION AND CONCLUSIONS :

Agonist-dependent increases in receptor density in cells lines is a perplexing phenomenon which is contrary to the compensatory model of receptor regulation in which prolonged agonist treatment decreases receptor density. Presumably, the compensatory model would result in maintenance of a relatively constant level of stimulation downstream. In these studies, agonist treatment of C_6 cells expressing D_{2S} and D_{2L} receptors resulted in significant increases in receptor density, though treatment of HEK 293 cell expressing D_{2L} receptors did not. Only treatments with agonist plus FSK increased D_{2L} receptors expressed in HEK 293 cells. Overnight treatment with NPA resulted in modest increases in receptor density for HEK 293 cells expressing F110A receptors, and large receptor density increases for HEK 293 cells expressing S167A or D_2(del 365-374) receptors.

Overnight treatment of HEK 293 cells expressing D_{2L} receptors with NPA plus FSK, resulted in receptor proliferation when treatment with NPA alone did not. Similar increases in receptor density resulted from the addition of the cAMP analog dbcAMP to agonist treatments. These studies suggest a possible role for the second messenger system cascade that begins with the activation of adenylate cyclase. It is known that the prolonged occupation of the receptor by agonist leads to receptor desensitization, thus removing the inhibitory properties toward adenylate cyclase. In addition, prolonged stimulation of G_i-coupled receptors leads to sensitization of adenylate cyclase resulting in increases in basal or forskolin-stimulated cAMP levels (Watts and Neve, 1996). Another hypothesis is that receptor occupancy by agonist produces a conformational change which allows a post-translational modification in response to exposure to cAMP analogs thus providing an initial pathway for the up-regulation (Filtz *et al.,* 1994).

The large increases in receptor density after overnight treatment with agonist found in HEK 293 cells expressing S167A and D_2(del 365-374) were surprising. By changing the inherent properties of the side chain of the mutant receptors the entire conformation of the receptor may be different from the native protein and may lead to decreased degradation in the presence of ligands. One explanation for the large increases in receptor density after overnight treatment with agonists found for the constitutively active mutant, D_2(365-374), is that constitutively active receptors are inherently more flexible than wild type receptors and ligands may act to stabilize the

mutant receptors (Gether *et al.*, 1997). S167A receptors may be more flexible as well and the presence of ligands may act similarly to stabilize the receptor.

The advent of molecular biology has given us the ability to test specific amino acid residues or regions of receptors which may be critical for ligand binding and receptor activation. This approach is validated by previous work in our laboratory. Aspartate-80 of the D_2 dopamine receptor was replaced with an alanine or a glutamate residue. In cells expressing alanine-80 D_2 receptors (D80A), the mutant receptors are unable to mediate the inhibition of adenylate cyclase. Glutamate-80 D_2 receptors (D80A) display decreased ability to inhibit adenylate cyclase activity. Sodium and pH sensitivity are greatly decreased for D80A receptors and modestly decreased for D80E receptors, as determined by effects on the apparent affinity of epidepride, sulpiride, NPA, and dopamine (Neve *et al.*, 1991). In another in vitro mutagenesis study in our laboratory, serines 193, 194, and 197 were substituted with alanine residues (S193A, S194A, and S197A). S193A receptors have decreased affinity for a number of agonists including DA and NPA. In cells expressing S193A and S197A receptors, the potency of DA for inhibition of adenylate cyclase activity was decreased. In cells expressing S194A receptors, there was no inhibition of adenylate cyclase activity by DA or *p*-tyramine suggesting hydrogen bonding between the *p*-hydroxyl of these ligands with the side chain of the native residue (Cox *et al.*, 1992). Recent studies of these serine mutants have revealed evidence that supports the existence of multiple active receptor conformations that are differentially sensitive to each of the three mutations (Wiens *et al.*, 1997). Further studies in our laboratory involved chimeric D_1/D_2 receptors which have D_1 sequence at the amino-terminal end and D_2 sequence at the carboxyl-terminus, and in which specific regions of each receptor subtype were exchanged with each other. Results of these studies suggest that transmembrane region (TM) VII contributes disproportionately to ligand selectivity, and that the third cytoplasmic loop of D_1 is crucial for activation of adenylate cyclase activity, whereas both the second and third cytoplasmic loops of D_2 are required for inhibition of adenylate cyclase (Kozell *et al.*, 1994). In other D_1/ D_2 chimeric studies, the third cytoplasmic loop and (TM) V of D_2 was implicated in the agonist-induced receptor up-regulation (Starr *et al.*, 1995). A recent study of D_1/D_2 chimeric receptors in which TM5 and 6, and IC3 of the D_1 receptor were substituted with D_2 sequence, resulted in constitutive activation of the chimeric receptors (Kozell and Neve, 1997).

In this study of D_2 DA receptors, five mutant receptors were created. Residues F110, S167, S365A, and T373 were replaced with alanine residues and the 10 carboxy terminal residues of IC3 were deleted. F110A receptors had increased affinity for the antagonist haloperidol and decreased affinity for other D_2-selective antagonists and agonists in competition binding studies. There was some inverse agonist effect of antagonists on cells expressing F110A receptors. There were small but significant increases in receptor density in cells expressing F110A receptors, after overnight treatment with agonists. S167A receptors had affinities comparable to wild type receptors for inhibition of forskolin-stimulated adenylate cyclase activity. D_2(del 365-374) receptors had some characteristics of constitutively active receptors including increased affinity for agonists in competition binding studies, resistance to GTP in competition binding studies with agonists, and some antagonists acted as inverse agonists on cells expressing D_2(del 365-374) receptors. In preliminary studies with cells expressing T373A receptors, antagonists-induced increases in cAMP accumulation were observed, suggesting constitutive activity.

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