

CHARACTERIZATION OF EPITOPE-TAGGED DOPAMINE D₄ RECEPTORS

by

James N. Oak

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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"Our ignorance is not so vast as our failure to use what we know."

M. King Hubbert

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Master of Science, 1999

James N. Oak

Department of Pharmacology, University of Toronto

ABSTRACT

The dopamine D₄ receptor is a polymorphic G protein-coupled receptor with a high affinity for antipsychotics. This thesis describes the characterization of several wildtype and mutant forms of the D₄ receptor. A tagged D₄ containing an amino-terminal FLAG or HA epitope sequence had unaltered ligand binding and functional coupling compared with untagged receptors. HA-tagged D₄ was also shown to couple to the MAPK cascade, which represents a previously undescribed signalling pathway for this receptor. Western blotting detected the presence of glycosylated and unglycosylated forms of the tagged receptors. Three receptors with mutations in the third cytoplasmic loop were also studied. Two receptors with deletions in the third intracellular loop had normal ligand binding, but both were functionally impaired. Deletion of residues 221-337 (Δ 221-337) from D_{4,4} completely abolished the inhibition of forskolin-stimulated cAMP by the receptor, while Δ 221-315 retained coupling but was less effective than full-length D₄. A third mutant containing a point mutation (M345A) near the sixth trans-membrane domain of D_{4,4} had defective ligand binding.

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TABLE OF CONTENTS

I. INTRODUCTION	1
A. The Dopamine Receptors	1
B. The D ₂ -like Dopamine Receptors	2
1. Structure	2
a. Overview	2
b. Transmembrane Domains	6
c. Extracellular Domains	7
d. Intracellular Domains	9
i. Alternate Splicing and Polymorphisms	9
ii. Post-translational Modifications	10
iii. G protein-Coupling and Activation	10
e. Dimerization	12
2. Pharmacology	13
a. D ₂ -like Receptor Antagonists	13
b. Selective Antagonists	14
c. Inverse Agonists	16
3. Functional Coupling of D ₂ -like Dopamine Receptors	16
a. Native Tissue	16
b. Adenylyl Cyclase	17
c. Arachidonic Acid and Phosphatidylinositol Hydrolysis	17
d. Transporters and Ion Channels	20
e. Effects on Morphology and Growth	20
f. G Protein Specificity	21
4. Insights from Dopamine Receptor Knockout Animals	22
C. Activation of MAPK by GPCRs	23
1. The MAPK Pathway	23
2. Activation of MAPK by G Protein-Coupled Receptors	25
a. GPCRs as Oncogenes	25
b. MAPK Activation by G $\beta\gamma$	25
c. Phosphoinositide 3-Kinase and Protein Tyrosine Kinases	26

d. Activation of MAPK by G _q	29
e. Receptor Transactivation	30
f. Endocytosis in MAPK Activation	30
g. G _s and G _o Activation of MAPK	31
h. MAPK in Native Tissue	31
i. D ₂ Receptors and MAPK	33
D. Desensitization	34
1. Heterologous Desensitization	34
2. Homologous Desensitization	35
3. Receptor Sequestration	37
4. Desensitization of D ₂ -like Receptors	38
E. RESEARCH OBJECTIVES	40
1. General Objective	40
2. FLAG-tagged Dopamine Receptors	41
3. Desensitization of the D ₄ Receptor	41
4. HA-tagged Dopamine Receptors	41
5. Mutant Dopamine Receptors	41
6. Coupling of Dopamine D ₄ Receptors to MAPK	42
 II. MATERIALS	43
A. GENERAL CHEMICALS	43
B. BACTERIAL AND TISSUE CULTURE	43
C. MOLECULAR BIOLOGY	44
D. VECTORS AND STRAINS	44
E. DRUGS, INHIBITORS AND RADIOCHEMICALS	45
F. PROTEIN ANALYSIS AND IMMUNOCHEMISTRY	45
G. OTHER LAB SUPPLIES	46
 III. METHODS	47
A. MOLECULAR BIOLOGY	47
1. Preparation of Competent <i>E. coli</i> Bacteria	47

2. Transformation of Competent <i>E. coli</i>	47
3. Preparation of Plasmid DNA	48
a. Rapid Minipreparation of DNA	48
b. QIAGEN Minipreparation of DNA	48
c. Large-scale DNA Preparation	49
4. Restriction Enzyme Digests	49
5. Subcloning Techniques	50
6. Sequencing	51
7. Cloning of Epitope-tagged Dopamine Receptors	52
a. FLAG Epitope-tagged D _{4,4} Receptor (FD4.4)	52
b. FLAG Epitope-tagged D _{4,4} Receptor Mutant (FD4.4(M345A))	52
c. HA Epitope-tagged Dopamine D ₄ and D _{2L} Receptors	53
B. TISSUE CULTURE	53
1. Growth and Maintenance of Mammalian Cells	53
2. Stable cell lines	54
C. RECEPTOR PHARMACOLOGY	54
1. Radioligand Binding	54
2. Saturation Binding	55
3. Drug Competition	55
4. Dopamine Binding	56
5. [³⁵ S]GTPγS Binding	56
D. FUNCTIONAL ASSAYS	57
1. Drug Incubations	57
a. Intracellular cAMP Assay	57
b. MAPK Assays	57
2. cAMP Radioimmunoassay (RIA)	58
3. MAPK Assays	59
a. MAPK Activity Assay	59
b. Phospho-MAPK Western Blotting	60
E. RECEPTOR IMMUNOPRECIPITATION AND WESTERN BLOTTING	60
1. FLAG-Tagged Receptors	60

a. Immunoprecipitation	60
b. Western Blotting	61
2. HA-Tagged Receptors	61
a. Immunoprecipitation	61
b. Western Blotting	62
3. BCA Protein Assay	62
 IV. RESULTS	64
A. SELECTION OF STABLE CELL LINES	64
B. PHARMACOLOGY OF EPITOPE-TAGGED RECEPTORS	64
1. Saturation Binding	64
2. Antagonist Binding	68
3. Dopamine Pharmacology	68
C. WESTERN BLOTTING OF EPITOPE-TAGGED RECEPTORS	75
1. Detection of FLAG-tagged Wildtype and Mutant D _{4,4} Receptors	75
2. Detection of HA-tagged Wildtype and Mutant D ₄ and D _{2L} Receptors	75
D. FUNCTIONAL COUPLING OF EPITOPE-TAGGED RECEPTORS	78
1. Inhibition of Adenylyl Cyclase	78
2. Stimulation of [³⁵ S]GTPγS Binding	80
3. Sensitization of Adenylyl Cyclase after Quinpirole Preincubation	84
4. Activation of MAPK by Dopamine D ₄ and D _{2L} Receptors	84
 V. DISCUSSION	92
A. THE POLYMORPHIC D ₄ RECEPTOR	92
B. EPITOPE-TAGGED D ₄ RECEPTORS	93
C. RECEPTOR PHARMACOLOGY	95
1. Wildtype Receptors	95
2. Mutant Receptors	96
D. IMMUNOBLOTTING OF D ₄ AND D _{2L} RECEPTORS	98
E. FUNCTIONAL COUPLING	100
1. Epitope-tagged D ₄ and D _{2L} Receptors	100

2. Desensitization of the Dopamine D ₄ Receptor	102
3. Mutant D ₄ Receptors	104
a. D _{4,4} Deletion Mutants Δ221-315 and Δ221-337	104
b. D _{4,4} Point Mutant M345A	108
F. Localization of Epitope-tagged D ₄ Receptors	109
VI. SUMMARY AND CONCLUSIONS	111
VII. FUTURE RESEARCH	113
VIII. REFERENCES	115

LIST OF TABLES

Table		Page
1.	Biochemical and anatomical properties of the D ₂ -like dopamine receptors	3
2.	Ligand binding constants for selected D ₂ -like agonists and antagonists	15
3.	Functional coupling of the D2, D3, and D4 dopamine receptors.	18-19
4.	[³ H]Spiperone dissociation constant (Kd) and receptor density (Bmax) for HA-tagged wildtype and mutant D4 and D2L receptors	67
5.	Inhibition constants of antagonists for FLAG- and HA-tagged wildtype and mutant D ₄ and D _{2L} receptors determined with [³ H]spiperone.	70
6.	Inhibition constants of antagonists for FLAG-tagged wildtype and mutant D4 receptors determined with [³ H]nemonapride (YM09151-2)	71
7.	Dopamine binding properties of FLAG- and HA-tagged receptors	74

LIST OF FIGURES

Figure	Page
1. Amino acid sequence of the dopamine D ₄ receptor	4
2. The G protein-coupled receptor activation cycle	5
3. The proposed arrangement of transmembrane (TM) α -helices in the D4 receptor	8
4. Activation of the MAPK pathway by G $\beta\gamma$ and G αq	27
5. Third intracellular loop amino acid sequence alignment for wildtype and mutant D4 and D2L receptors	65
6. Saturation binding of [³ H]spiperone to membranes from Chinese hamster ovary cells expressing HA-tagged D4.4.	66
7. Antagonist competition of radioligand binding to FLAG-tagged wildtype and mutant D ₄ receptors	69
8. Antagonist competition of [³ H]spiperone binding to HA-tagged wildtype and mutant D ₄ and D _{2L} receptors	72
9. Dopamine competition curves for FLAG-tagged wildtype and mutant D ₄ receptors	73
10. Dopamine competition curves for HA-tagged wildtype and mutant D ₄ receptors	76
11. Western blot of FLAG epitope-tagged wildtype and mutant D ₄ receptors.	77
12. Western blot of HA epitope-tagged wildtype and mutant D ₄ and D _{2L} receptors	79

13.	Inhibition of forskolin-stimulated cAMP by FLAG-tagged wildtype and mutant D ₄ receptors	81
14.	Inhibition of forskolin-stimulated cAMP by HA-tagged wildtype and mutant D ₄ receptors	82
15.	Dopamine stimulation of [³⁵ S]GTPγS binding to membranes from CHO cells expressing FLAG- and HA-tagged D ₄ receptors	83
16.	Sensitization of adenylyl cyclase activity in CHO cells expressing FD4.4	85
17.	Stimulation of MAPK activity and phosphorylation by dopamine	86
18.	Time course of MAPK activation by dopamine	88
19.	Dose-response of MAPK activation by dopamine	89
20.	Impairment of MAPK activation in dopamine D ₄ deletion mutants	89
21.	Haloperidol and pertussis-toxin sensitivity of MAPK activation by dopamine	90

ABBREVIATIONS

AA	arachidonic acid
ACII	adenylyl cyclase II
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
ApCAM	<i>Aplysia</i> cell adhesion molecule
APS	ammonium persulfate
AR	adrenergic receptor
AT ₁ , AT ₂	angiotensin 1 and 2
ATP	adenosine triphosphate
β ARK	β -adrenergic receptor kinase
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
bFGF	brain fibroblast growth factor
BSA	bovine serum albumin
ATP	adenosine triphosphate
CaM	calmodulin
CaMK II	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CAM	constitutively active mutant
CD	circular dichroism
CNS	central nervous system
cPLA ₂	cytosolic phospholipase A ₂
COMT	catecholamine-O-methyl transferase
CTP	cytosine triphosphate
CTX	cholera toxin
DA	dopamine (3-hydroxytyramine)
DAG	1,2-diacylglycerol
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxynucleotide triphosphate
dsDNA	double-stranded DNA
EC ₅₀	median effective concentration
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPS	extrapyramidal side effects
EPSP	excitatory post-synaptic potential
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FD4.4	FLAG epitope-tagged dopamine D _{4.4} receptor
G418	Geneticin
Gab1	Grb2 activating protein-1
GAD	glutamic acid decarboxylase
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GIRK	G protein-coupled inwardly-rectifying potassium channel

GPCR	G protein-coupled receptor
Gpp[NH]p	5'-guanylyl-imidodiphosphate
Grb2	growth-factor-receptor binding protein 2
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
HAD4	HA epitope-tagged dopamine D ₄ receptor
5-HT	5-hydroxytryptamine (serotonin)
HRP	horseradish peroxidase
HS	horse serum
i2	intracellular loop 2
i3	intracellular loop 3
IBMX	3-isobutyl-1-methylxanthine
IC ₅₀	median inhibitory concentration
IEG	immediate early gene
IGF-IIR	insulin-like growth factor II receptor
Ins(1,4,5)P ₃	inositol (1,4,5)trisphosphate
IP3	inositol (1,4,5)trisphosphate
IRK	inwardly rectifying potassium channel
JIP-1	JNK interacting protein-1
JNK	Jun N-terminal kinase
K _d	equilibrium dissociation constant (saturation binding)
K _i	equilibrium inhibition constant (competitive binding)
Kir	inwardly rectifying potassium channel
LPA	lysophosphatidic acid
LTF	long-term facilitation
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MAO	monoamine oxidase
MAP-1	microtubule-associated protein-1
MAPK	mitogen-activated/microtubule-associated protein kinase (eg. ERK, JNK)
MAPKK	MAPK kinase (eg. MEK, SEK)
MAPKKK	MAPK kinase kinase (eg. Raf, MEKK)
MBP	myelin basic protein
MEK	MAPK/ERK kinase (a MAPKK)
MEKK	MEK kinase (a MAPKKK)
mGluR	metabotropic glutamate receptor
MP1	MEK partner 1
Nck	novel cytoplasmic protein
NGF	nerve growth factor
NMDA	N-methyl D-aspartate
NMR	nuclear magnetic resonance
NPA	N-propylmorphine
NT	neurotrophin
NTP	nucleoside triphosphate
PAF	platelet activating factor
PAK	p21-activated kinase

PDE	phosphodiesterase
PH	pleckstrin homology
PHF	paired helical filament
PLA ₂	phospholipase A ₂
PI3-K, PI3-kinase	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristate 13-acetate
PtdIns(3)P	phosphatidylinositol 3-phosphate
PtdIns(3,4)P ₂	phosphatidylinositol (3,4)bisphosphate
PtdIns(4,5)P ₂	phosphatidylinositol (4,5)bisphosphate
PtdIns(3,4,5)P ₃	phosphatidylinositol (3,4,5)trisphosphate
PIP2	phosphatidylinositol (4,5)bisphosphate
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTX	pertussis toxin
PVDF	polyvinylidene difluorine
Pyk2	proline-rich tyrosine kinase 2
RAFTK	related focal adhesion tyrosine kinase
Ras	rat sarcoma virus p21 GTPase
Ras-GRF	Ras guanine nucleotide exchange factor
RIA	radioimmunoassay
RTK	receptor tyrosine kinase
SAP-1	serum activated-protein 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	SH2 domain-containing, α 2-collagen-related protein
SH-PTP-2	phosphotyrosine phosphatase-2
SHPS	SHP (SH2 domain-containing phosphatase) substrate
Sos	Son of sevenless guanine nucleotide exchange factor
TEMED	N,N,N,N-tetramethyl-ethylenediamine
TH	tyrosine hydroxylase
TM	transmembrane
TPP	thymidine triphosphate
TUN	tunicamycin

I. INTRODUCTION

A. The Dopamine Receptors

Dopamine is the predominant catecholamine neurotransmitter in the brain and is also active in peripheral tissue such as the heart and kidney. Within the CNS, there exist three main dopaminergic pathways: the nigrostriatal, mesolimbic/mesocortical, and tuberoinfundibular, which are involved in movement, mood/cognition, and pituitary hormone secretion, respectively. Clinically, dopamine has been implicated in several neurological disorders. Degeneration of dopamine-containing neurons in the substantia nigra is responsible for Parkinson's disease, and can be treated by administration of L-DOPA, which is decarboxylated to form dopamine in the brain (Haavik & Toska, 1998). The usefulness of dopamine antagonists in the treatment of schizophrenia has led to the suggestion that dopamine overactivity in the limbic system may contribute to this disease (Seeman, 1987; Hietala & Syvalahti, 1996).

Historically, two populations of dopamine receptors were identified in the brain by their pharmacological and functional characteristics. Kebabian and Calne reviewed this evidence and designated the receptors D-1 and D-2 (Kebabian & Calne, 1979). D-1 receptors stimulated adenylyl cyclase, while D-2 receptors had a higher affinity for dopamine and butyrophenone antipsychotics. Concurrently, a unique functional activity of D-2 receptors was identified in extracts of pituitary tumours, where dopamine reduced intracellular cAMP levels (De Camilli *et al.*, 1979).

The D₂ receptor was first cloned from rat based on its homology to the β₂-adrenergic receptor (Bunzow *et al.*, 1988), which was followed by the cloning of the human D₂ receptor (Grandy *et al.*, 1989). Subsequently, rapid progress was made with the cloning of the D₁ receptor (Zhou *et al.*, 1990b), as well as the additional receptor variants D₃ (Sokoloff *et al.*, 1990), D₄ (Van Tol *et al.*, 1991), and D₅ (Sunahara *et al.*, 1991) (see reviews by Civelli *et al.*, 1991; Seeman & Van Tol, 1993; Gingrich & Caron, 1993; Lachowicz & Sibley, 1997b; Missale *et al.*, 1998). All five members of the dopamine receptor family have a primary sequence containing seven hydrophobic domains typical of the family of G protein-coupled receptors (GPCRs), which includes over 2000 proteins. The dopamine receptors are considered to have properties similar to those of receptors for other bioamines (epinephrine, serotonin, histamine, acetylcholine), nucleotides, eicosanoids, and lipids (Ji *et al.*, 1998).

Despite the number of dopamine receptors cloned, the five receptors can be classified as

D₁-like or D₂-like, based on their pharmacological and biochemical characteristics. Thus the D₁-like (D₁ and D₅) receptors have almost identical affinities for many ligands and stimulate adenylyl cyclase by coupling to G_s. Similarly, D₂, D₃, and D₄ receptors have a high affinity for butyrophenones and have been shown to inhibit adenylyl cyclase in response to agonists. Recent evidence from D₁ transgenic mice suggests that there exists at least one additional D₁-like receptor coupled to the stimulation of phosphoinositide hydrolysis (Friedman *et al.*, 1997).

B. The D₂-like Dopamine Receptors

1. Structure

a. Overview

The members of the D₂-like receptor family share a high sequence identity suggesting they emerged from a common progenitor by gene duplication (Table 1) (Vernier *et al.*, 1995). Dopamine receptors share the characteristic extracellular amino terminus, seven hydrophobic transmembrane domains, and intracellular C-terminus found in G protein-coupled receptors such as rhodopsin and the adrenergic receptors (reviewed by Strader *et al.*, 1994; Wess, 1997; Ji *et al.*, 1998; Gether & Kobilka, 1998). D₂, D₃, and D₄ all share a large third cytoplasmic loop typical of aminergic GPCRs coupled to G_i, as shown with D₄ in Figure 1.

One model for GPCR activation, the extended ternary complex model, proposes that an equilibrium of inactive (R), active (R*), and intermediate (R', R") receptors exist, with the R* state capable of strong coupling to G proteins (Figure 2). For example, at least five thermal intermediate states exist between the inactive and active conformations of rhodopsin (Helmreich & Hofmann, 1996). Evidence suggests that the opsin apoprotein, as well as metarhodopsin(II) (R*), can couple to G proteins with low affinity (Surya *et al.*, 1998). This coupling is believed to have physiological importance in light/dark adaptation. The existence of inverse agonists (ie. compounds which reduce basal receptor activity) indicates that drugs can promote a state with less spontaneous activity (R°), while partial agonists are proposed to favour the R' or R" intermediates (Gether & Kobilka, 1998). Neurotransmitters activate intracellular signalling pathways by binding to a GPCR and stabilizing the conformation of the R* state. This results in the formation of an agonist-receptor-heterotrimeric G protein complex at the membrane, which catalyzes the exchange of GTP for GDP on the G protein α subunit. Nucleotide exchange results in dissociation of the G protein into free G α and G $\beta\gamma$, which activate intracellular effectors.

Table 1. Biochemical and anatomical properties of the D₂-like dopamine receptors.

	D ₂	D ₃	D ₄
Chromosomal location	11q22-23	3q13.3	11p15.5
Introns	5/6	5	4
Amino acids	414/443	400	387-515 (D4.2-D4.10)
N-terminal amino acids	33	29	18
3 rd cytoplasmic loop amino acids	134/163	120	101-261
C-terminal amino acids	16	12	14
Sequence identity with D ₂		79%	41%
Transmembrane sequence identity with D ₂		97%	56%
Conserved residues proposed to be involved in dopamine binding or activation (* experimentally demonstrated by site-directed mutagenesis)	D80*, D114*, S193*, S194*, S197*, F389	D75 D110 S192 S193 S196 F345	D80 D115 S196 S197 S200 F362 (D4.4)
Potential N-linked glycosylation sites	3	4	1
Distribution (mRNA or receptor)	Cerebral cortex, basal ganglia, olfactory tubercle, amygdala, mesencephalon, pituitary, retina blood vessels, adrenal gland, kidney, sympathetic ganglia	Parietal cortex, nucleus accumbens, Islands of Calleja, olfactory bulb, cerebellum	Frontal cortex, olfactory bulb, amygdala, hippocampus, hypothalamus, mesencephalon, pituitary, retina heart
Expression level	High (brain) (~300 fmol/mg in rat striatum)	Low (brain)	Low (brain) High (heart) (~20 fmol/mg in rat atria)

References: Missale *et al.*, 1998; Jackson & Westlind-Danielsson, 1994; Seeman, 1987; Picetti *et al.*, 1997; Ricci *et al.*, 1998.

1 MGNRSTADADGLLAGRGPAAGASAGASAGLAGQQGAAA TM 1
e1 60

61 TERALQTPTN TM 2
i1 e2 TM 3
60 120

121 VDRFVAVAVPLRYNRQGGSRR TM 4
i2 DVRGR 180

181 DPAVCRLIEDRD TM 5
e3 i3 240

241 PGPPSPTP TM 6
PAPRLPQDP CGPDCAP PAPGLPRGPCGPDCAP AAPSLPQDP CGPDCAP PAPG 300
α β θ ζ

301 LPPDPCGSNCAP TM 7
PDAVRAAALPPQT PPQT RRRRRAKI TGRERKAMR 360
i3

361 QALCPACSVP TM 8
e4 i4 419

FNAEFRNVFRKALRACC

Figure 1. Amino acid sequence of the dopamine D₄ receptor.

The amino acid sequence of the dopamine D_{4,4} receptor (ie. the D₄ receptor variant containing 4 repeats) is shown. The proposed topology of the transmembrane domains (TM 1 - 7), extracellular domains (e1 - 4), and intracellular domains (i1 - 4) is shown. Boxed sequences labelled by Greek letter delineate the position of the polymorphic repeat region. Underlined residues indicate proposed sites of post-translational modification (glycosylation and palmitoylation) and regions putatively involved in ligand binding and G protein activation.

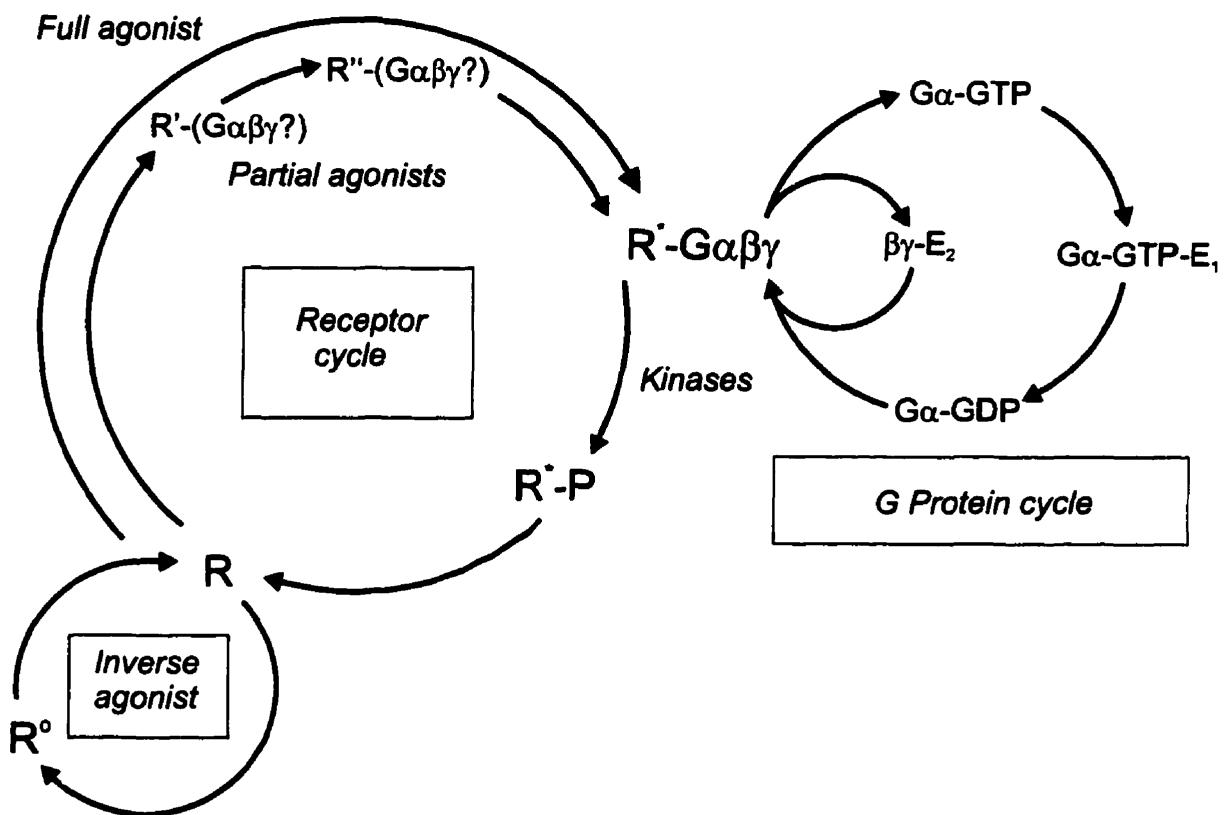


Figure 2. The G protein-coupled receptor activation cycle.

Abbreviations: R° , inverse agonist-stabilized receptor; R , inactive receptor; R' , R'' , intermediate receptor conformations; R^* , activated receptor, R^*-P , phosphorylated receptor; E , G protein effectors; GDP, guanosine diphosphate; GTP, guanosine triphosphate; $G\alpha\beta\gamma$, G protein α , β , and γ subunits (Adapted with modifications from Helmreich & Hofmann, 1996; Gether & Kobilka, 1998).

Removal of the agonist or receptor phosphorylation may cease activation. Signalling by G proteins is terminated by the hydrolysis of GTP to GDP by the GTPase activity of the α subunit, followed by the reformation of the inactive $G\alpha\beta\gamma$ complex at the membrane.

This model, while based on indirect evidence, accounts for many of the observations regarding GPCR activation. However, others have pointed out the failings of this G protein “shuttling” mechanism. It has been speculated that receptor-G protein-effector complexes may form, which would allow greater control of signalling to specific pathways (Chidiac, 1998). In addition, the ternary complex model fails to adequately explain the effect of guanine nucleotides on the affinity of agonist binding. Recently, a model which includes multiple states of affinity has been proposed based on the binding of an agonist to cardiac muscarinic receptors (Chidiac *et al.*, 1997). This more complex model is better at describing the cooperativity in binding and the dependence of agonist affinity on guanine nucleotides that is observed in the experimental data. Therefore caution must be exercised in data analysis with the knowledge that the ternary complex model is only a model, and firm biochemical characterization of how receptors activate G proteins is still needed.

b. Transmembrane Domains

A recently reported three-dimensional structure of the of the seven transmembrane proton pump bacteriorhodopsin (Pebay-Peyroula *et al.*, 1997) confirmed previous low-resolution projection-density mapping of rhodopsin, which established the arrangement of seven membrane-spanning α -helices in a circular bundle (Baldwin *et al.*, 1997). These α -helices are arranged such that hydrophobic residues form a boundary with the plasma membrane and the less hydrophobic residues face the interior, where 11-cis retinal is located in rhodopsin. Activation of GPCRs by bioamines is believed to occur upon agonist binding at conserved residues within the transmembrane domains.

Our understanding of the structure and function of bioamine GPCRs has been led by studies of the adrenergic receptors (AR). In the β -ARs, an aspartate in transmembrane domain (TM) 3 acts as a counterion to the amine group of the agonist, while conserved serines in helix 5 can act as hydrogen bond acceptor sites for the hydroxyl groups of the catechol ring (Strader *et al.*, 1988; Strader *et al.*, 1989). A phenylalanine in helix 6 appears to interact with the aromatic catechol ring of the agonist to mediate activation (Strader *et al.*, 1994). Another aspartate in

helix 2 is highly conserved in GPCRs and is important in producing the conformation change required for activation (Strader *et al.*, 1988). It is believed that in the inactive state, GPCRs are held in a constrained configuration which, upon disruption by agonist binding but not antagonist binding, results in a conformational change that allows an interaction with G proteins. In the α_{1b} -AR, site-directed mutations indicate that the constraint is introduced by a salt bridge between the conserved aspartate in TM 3 and a lysine in TM 7, with mutations at either residue leading to enhanced constitutive signalling (Porter *et al.*, 1996). The response to mutations at this conserved residue varies, however, as mutation of aspartate 113 in the β_2 -AR results in a 10,000-fold reduction in agonist and antagonist affinity (Strader *et al.*, 1994).

Site-directed mutagenesis of the D_2 receptor has confirmed the importance of serine 193, 194, and 197 in dopamine binding and activation, although the relative importance of each differed from those of the β -AR (Cox *et al.*, 1992; Woodward *et al.*, 1996). Mutagenesis of an aspartate at position 80 of D_2 abolished functional coupling of the receptor but preserved dopamine binding (Neve *et al.*, 1991), while mutating aspartate 114 resulted in a total loss of agonist and antagonist binding (Mansour *et al.*, 1992). The combination of biophysical data from rhodopsin, sequence conservation within GPCRs, and mutagenesis studies all support a model for the transmembrane arrangement of the D_2 receptor consisting of seven α -helices of approximately 25 amino acids transversing the membrane. These helices are arranged around a central ligand-binding pocket in a counterclockwise orientation (TM 1 to TM 7) when viewed from the extracellular surface (Baldwin *et al.*, 1997). Using a template based on the β_2 -AR, a model for arrangement of α -helices in the D_2 receptor is shown in Figure 3.

Recently, progress has been made in mapping the binding site crevice of the D_2 receptor. Using a substituted-cysteine accessibility method, Javitch *et al.* (Javitch *et al.*, 1998) have mapped water-accessible residues on TM 3, TM 5, TM 6 and TM 7. Using competition by agonists to define the hydrophilic binding site, their work confirmed the importance of D114, S203, S204, and S207, as well as a cluster of aromatic residues on TM 6.

c. Extracellular Domains

In contrast to the transmembrane domains, no defined secondary structure has been identified for the amino terminus or the extracellular loops of the bioamine GPCRs. Two highly conserved cysteine residues in extracellular domains 2 and 3 are believed to form a disulfide

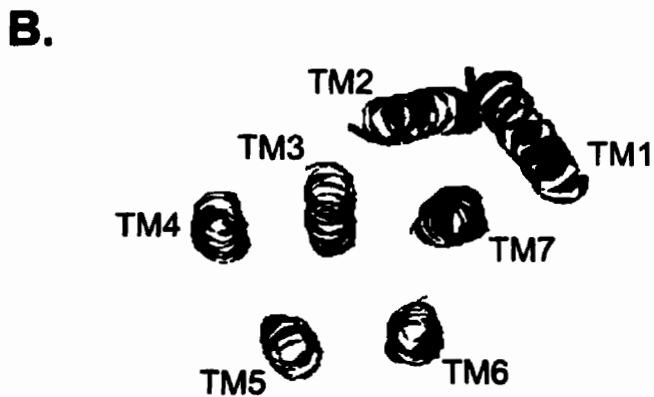
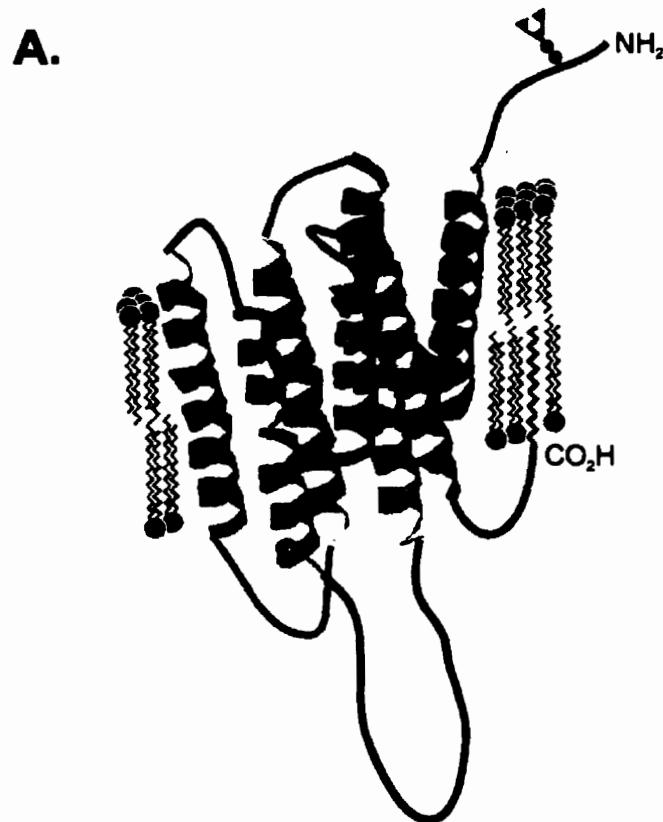


Figure 3. The proposed arrangement of transmembrane (TM) α -helices in the D₄ receptor.
A. The model of the D₄ receptor transmembrane α -helices was produced with RasMol v2.5 (Glaxo Research and Development, Greenford, Middlesex, U.K.) using co-ordinates produced by SWISS-MODEL in GPCR mode. The theoretical D₄ TM domains were aligned to conserved residues in the β_2 -adrenergic receptor and modelled using the β_2 -AR group-specific template (Peitsch, 1995; Peitsch, 1996; Guex & Peitsch, 1997). The intervening protein sequences are indicated by grey lines and are structurally undefined. Potential sites of N-linked glycosylation at the amino (NH₂) terminus (●, ▼) and palmitoylation at the carboxyl (CO₂H) terminus are also shown.
B. Circular arrangement of D₄ membrane-spanning α -helices as viewed from outside the cell.

bond (Fraser, 1989; Hwa *et al.*, 1997). In addition, the extracellular region of D₂-like receptors contain up to four putative N-linked glycosylation sites (NXS/T). Expression studies of D₂ and D₄ receptors has confirmed that these receptors are glycosylated when expressed in cultured cells (Grünewald *et al.*, 1996a; Lanau *et al.*, 1997). Glycosylation does not appear to be required for ligand binding or activation in bioamine GPCRs (George *et al.*, 1986; Fukushima *et al.*, 1995). Some diversity in primary structure is known to occur in the amino-terminal extracellular domain of D₄. A polymorphism has been identified in exon 1 of the D₄ receptor which exists as either one or two 12 bp repeats, leading to a receptor variant with four additional residues before the first TM domain (Catalano *et al.*, 1993).

d. Intracellular Domains

i. Alternate Splicing and Polymorphisms

After the cloning of the rat D₂ receptor, a human D₂ receptor was cloned which contained an additional 29 amino acids within the third cytoplasmic loop (Dal Toso *et al.*, 1989). Both subtypes were found to exist in rat and human and have been designated D_{2S} (or D_{2B}, D₂₍₄₁₅₎) and D_{2L} (or D_{2A}, D₂₍₄₄₄₎) (Giros *et al.*, 1989). The long isoform is generated by alternative RNA splicing which results in the inclusion of an additional exon. Similarly, the mouse D₃ receptor can undergo alternate RNA splicing by using an internal acceptor site, which results in an additional 21 amino acids in the third cytoplasmic loop (Fishburn *et al.*, 1993). Fishburn *et al.* (1995) have also shown that the D_{2L} and D_{2S} isoforms differ in their protein processing, with D_{2L} glycosylation occurring more slowly and in a different intracellular compartment as compared to D_{2S}. A recent study using D_{2S}- and D_{2L}-specific antibodies indicated that the D_{2S} isoform is situated primarily on dopamine-producing cells, while D_{2L} is located as a postsynaptic dopamine receptor (Khan *et al.*, 1998). This suggests that D_{2S} may represent the dopamine autoreceptor.

The length of the D₄ receptor third cytoplasmic loop also varies. A polymorphism consisting of a variable number of imperfect 48 bp repeats has been identified in humans and other primates (Van Tol *et al.*, 1992; Livak *et al.*, 1995). Analysis of this region has identified individuals having between 2 and 10 repeats, with the first (α) and last (ζ) repeat highly conserved (Lichter *et al.*, 1993). This study determined the haplotype frequencies of the various D₄ receptor isoforms in 178 individuals of diverse ethnicity and found the most abundant D₄ subtypes were D_{4,2} (4.5%), D_{4,4} (66%), and D_{4,7} (23.6%). Therefore, the D₄ receptor with two,

four, and seven 16-amino acid repeats are the most abundant variants, although the precise amino acid sequence may vary due to the irregular order of the repeats. However, the three most common sequences of the two, four, and seven-repeat variants alone accounted for 83.2% of all human D₄ receptors.

ii. Post-translational Modifications

Carboxyl-terminal cysteines, which are present in the three D₂-like receptors, are another feature conserved in the GPCR family. Palmitoylation of the C-terminus by a thioester linkage has been shown in numerous GPCRs (Morello & Bouvier, 1996). [³H]-palmitate labelling demonstrated that this modification occurs on baculovirus-expressed D_{2L} receptors (Ng *et al.*, 1994b). The addition of this 16-carbon fatty acid is proposed to anchor the C-terminus to the plasma membrane, thereby forming a fourth intracellular loop. In the β₂-AR and the D₁ receptor, palmitoylation state appears to be regulated in response to agonist activation (Mouillac *et al.*, 1992; Ng *et al.*, 1994a). While cysteine mutants of the β₂-AR were unable to couple to adenylyl cyclase, the functional activity of other GPCRs such as the D₁, α₂-AR, and muscarinic M2 receptor were unaffected by these mutations (Jin *et al.*, 1997).

Phosphorylation of intracellular serine and threonine residues by PKA, PKC, Casein Kinase 1α, and GRKs is an important mechanism for attenuating GPCR signalling (Böhm *et al.*, 1997a; Tobin *et al.*, 1997). In addition, phosphorylation by PKA is known to switch the coupling of the β₂-AR from G_i to G_s, resulting in the activation of the MAPK pathway (Daaka *et al.*, 1997). Analysis of the amino acid sequence of D₂ identifies the presence of consensus sequences for 3 PKA sites, 6 PKC sites, and 4 Casein kinase II sites in the intracellular domains. D₃ and D₄ have fewer potential phosphorylation sites, with D₃ possessing 2 PKA and 3 PKC consensus sequences, while D₄ has one site each for PKA, PKC, and CaMK II. Agonist-independent phosphorylation has been demonstrated to occur on D₂ *in vivo* (Ng *et al.*, 1994b). However, the phosphorylation state of D₃ and D₄ is presently unknown.

iii. G Protein-Coupling and Activation

The intracellular loops of GPCRs act to transmit conformational changes into G protein activation. At the N-terminal region of the second intracellular loop, the conserved (D/E)RY (or DRF in the D₄ receptor) has been shown to be a key determinant in activation. Replacement of this aspartate with a neutral amino acid abolishes G protein activation in the β-AR, and experiments with rhodopsin suggest that the protonation of this negatively charged residue may be

involved in activation (Helmreich & Hofmann, 1996).

The third intracellular loop has been established as the primary determinant for G protein selectivity in 7 TM receptors, while the importance of intracellular loop 2 (i2) and the C-terminus varies among receptors. The importance of the third loop has been clearly demonstrated using peptides derived from the C-terminal portion of i3. Expression of a peptide based on the third cytoplasmic loop of the α_{1B} -adrenergic receptor blocked activation of PLC by the cotransfected full-length receptor, but did not inhibit the activation of adenylyl cyclase by cotransfected dopamine D₁ receptors (Luttrell *et al.*, 1993). A 12-amino acid peptide derived from the C-terminal portion of the β -adrenergic receptor i3 is capable of coupling to the stimulation of adenylyl cyclase, with structural studies indicating that the peptide contains an α -helical C-terminal domain and a flexible N-terminal region when bound to micelles (Jung *et al.*, 1995). In the G_i-coupled α_{2A} -adrenergic receptor, 12 and 14 amino acid peptides from i2 and i3 were found to affect G protein coupling and activation (Dalman & Neubig, 1991). Specifically, the i2 peptide appears to interfere with high affinity binding of agonists but does not affect GTPase activity. In contrast, the peptide consisting of the C-terminal portion of i3 appears to act by mimicking the receptor and binding to the G protein, since this fragment can inhibit α_2 -receptor-mediated GTPase activity. Another study using peptides derived from the C-terminus and N-terminus of the α_2 -AR third intracellular loop indicated a dimer of the two regions was most effective in activating G_o (Wade *et al.*, 1996). These studies have supported the importance of a basic 15-20 amino acid region preceding TM 6, which is absolutely required for activation of G proteins, and a region adjacent to TM 6 which is important in the affinity for particular G proteins.

Point mutations in these regions have further defined the importance of specific amino acids conserved among GPCRs. O'Dowd *et al.* (1988) studied 19 mutants of the intracellular domains of the β -adrenergic receptor. They determined that while the N-terminal portion of i3 was not implicated in G protein coupling specificity, the C terminal portion of i3 as well as the N-terminal portion of the cytoplasmic tail were important in distinguishing between G_s and G_i. In the α_2 C10-AR, point mutation of a threonine near the i3/TM 6 boundary confers constitutive activity in this G_i-coupled receptor (Ren *et al.*, 1993).

The importance of these regions in i3 are supported by data from chimeric receptors of the α_2 - and β_2 -adrenergic receptors. Replacement of the region from the second extracellular

loop to TM 6 of the α_2 -AR with the corresponding region of the β_2 -AR resulted in a receptor with the pharmacology of the α_2 -AR but which coupled to adenylyl cyclase stimulation in response to epinephrine, a characteristic of the β_2 -AR (Kobilka *et al.*, 1988). Interestingly, this study showed that “split receptors”, with one consisting of the N-terminus to the mid-point of i3 in β_2 and another extending from the same position in i3 to the C-terminus of β_2 , had normal pharmacology and could stimulate adenylyl cyclase when co-expressed. However, despite the importance of individual residues in G protein coupling, alignment of the amino acid sequences of many GPCRs which activate the same effectors failed to reveal individual sequences that would predict which signalling pathways are activated (Hedin *et al.*, 1993).

Dopamine receptor chimeras between D₁ and D₂ have also been constructed (Kozell *et al.*, 1994; MacKenzie *et al.*, 1993). In a D₁/D₂ chimera with the junction preceding TM 3, D₂ agonists were able to couple to G_i, although the efficacy was reduced. Another D₁/D₂ chimera with the junction after TM 4 could not inhibit cAMP levels, suggesting the i2 loop is important for functional coupling of D₂. Although D₃ can couple to G_i in some cell lines, it is much less effective than D₂ (Lajiness *et al.*, 1995). However, D₃, i2 or i3, when switched to D₂, are capable of robust inhibition of adenylyl cyclase, indicating that factors outside the loops are responsible for the poor coupling of D₃ (Robinson & Caron, 1996). Lachowicz and Sibley (1997a) created chimeric D₂/D₃ and D₃/D₂ receptors with the fusion in the middle of i3 and found both were functionally coupled, while wildtype D₃ was not, suggesting that the N- and C-terminal regions of i3 can couple to G_i independently. Chimeras of D_{2L} and D_{4,2} which can bind agonists and antagonists have also been constructed, but functional coupling data was not reported (Shih *et al.*, 1997).

e. Dimerization

Evidence that GPCRs may associate as dimers has arisen from studies of several receptors (reviewed by Hebert & Bouvier, 1998). Hebert *et al.* (1996) studied dimerization of the β_2 -AR using differential epitope-tagging and found that SDS-resistant homodimers were present. In addition, a peptide of TM 6 was able to block dimerization and receptor activation, indicating a functional role may be associated with receptor interactions. Using two reciprocal chimeras of the muscarinic M3 receptor and the α_2 -AR, heterodimer formation was found to restore ligand binding, which was dependent on the presence of a large i3 domain (Maggio *et al.*,

1996). Metabotropic glutamate (mGluR5) receptors also undergo dimerization by the formation of disulfide bonds in the large extracellular domain (Romano *et al.*, 1996), and the δ -opioid receptor has been shown to dimerize in co-immunoprecipitation and cross-linking experiments, with deletion of 15 residues at the C-terminus preventing dimer formation (Cvejic & Devi, 1997). In the β_2 -AR, dimerization is stabilized by agonists, while in the δ -opioid receptor agonist stimulation decreased the level of dimers. Thus, the regions important for dimer formation and the role of dimerization in GPCR function appears to vary among receptors.

In the D_2 receptor, several lines of evidence have pointed to the existence of dimers. Seeman and Van Tol (1993) have reported that the benzamides [3H]nemagonpride and [3H]raclopride label 1.7 - 2-fold more D_2 binding sites than the butyrophenone [3H]spiperone in both cultured cells and striatal tissue. Similar results with D_4 indicate that [3H]nemagonpride binds 1.25-fold more receptor sites in COS-1 cells (Hidaka *et al.*, 1995). These findings have led to the proposal that different molecular forms of the receptor may be recognized by these antagonists, with benzamides binding monomers while butyrophenones bind dimers. However, these findings have been disputed on the basis that they were experimental artefacts resulting from ligand depletion or nonattainment of equilibrium (Malmberg *et al.*, 1996).

Biochemical evidence of D_2 and D_3 dimers also exists. Ng *et al.* (1996) reported that D_2 receptors in brain membranes exist as spiperone-sensitive monomers and nemagonpride-labelled monomers and dimers. Co-incubation of peptides from TM 6 and TM 7 of D_2 specifically resulted in the elimination of the dimeric form of D_2 without affecting D_1 or serotonin 5-HT_{1B} homodimers. A report of D_3 dimers and tetramers in brain and GH3 cells has also emerged recently and suggests that this may be a common feature of dopamine receptors (Nimchinsky *et al.*, 1997). However, two recent studies using mouse and human D_4 -specific antibodies failed to report the presence of oligomers (Lanau *et al.*, 1997; Mauger *et al.*, 1998).

2. Pharmacology

a. D_2 -like Receptor Antagonists

Dopamine receptor pharmacology has received attention due the efficacy of D_2 -like receptor antagonists in reducing the positive symptoms of schizophrenia (psychosis, paranoia, hallucinations) (reviewed in Seeman & Van Tol, 1993; Wilson *et al.*, 1998). The discovery that neuroleptic drugs such as (+)-butaclamol stereo-selectively block [3H]dopamine binding in brain

tissue at nanomolar concentrations led to the hypothesis that overactive dopaminergic transmission may underlie this disorder (Seeman, 1987). Later, the site of action was further isolated to the D₂-subpopulation of dopamine receptors. Thus it has been proposed that the beneficial aspects of antipsychotic drugs are due to the blockade of limbic D₂-like receptors, while interference with striatal dopamine signalling results in unwanted Parkinson's-like extra-pyramidal side effects (EPS) which impair motor control.

All D₂-like receptors share a common pharmacology, with the variants having minor differences in their affinity for the phenothiazine, butyrophenone, and substituted benzamide classes of antipsychotics (Table 2). A strong correlation exists between the clinical potency of these drugs and their affinity for D₂ (Seeman & Van Tol, 1993). More recently, a group of atypical antipsychotics have been identified, including clozapine and risperidone. These drugs are effective at treating the negative symptoms of schizophrenia (lack of motivation, emotional blunting) with a lower propensity for EPS.

The cloning of novel D₂-like receptors has spurned the search for selective ligands for D₃ and D₄. Interestingly, clozapine was found to have a higher affinity for the D₄ receptor compared to D₂ and D₃, leading to speculation that clozapine's unique profile may be attributed to preferential binding to this receptor (Van Tol *et al.*, 1991). It was postulated that the existence of clozapine-refractory patients may be due to the D₄ polymorphism. However, binding analysis of D₄ variants indicated that any difference in the K_i for clozapine due to the size of the i3 loop were minor (Asghari *et al.*, 1994). Interestingly, the D₄ receptor was recently found to bind the agonists norepinephrine and epinephrine with a similar affinity as the α- and β-adrenergic receptor, compared to a low affinity of these drugs for D₂ (Newman-Tancredi *et al.*, 1997b). This may have functional relevance given that D₄ expression is high in the heart (Ricci *et al.*, 1998).

b. Selective Antagonists

In the last two years, several antagonists which discriminate between D₄ and D₂/D₃ have been identified, some of which are mentioned in Table 2. Despite the presence of D₄ in the limbic area of the brain, D₄-selective L-745,870 failed to attenuate amphetamine-induced hyperactivity or conditioned avoidance in rodents, two tests used to predict antipsychotic potential (Bristow *et al.*, 1997). In a small clinical trial, this compound was also ineffective as a neuroleptic (Kramer *et al.*, 1997). In contrast, another recent study using the pre-pulse inhibition

Table 2. Ligand binding constants for selected D₂-like agonists and antagonists.

	Affinity constant (K_d or K_i) (nM)		
	D ₂	D ₃	D ₄
<i>Antagonists</i>			
Phenothiazines			
chlorpromazine	1 - 8	1.2 - 6.1	8 - 37
Butyrophenones			
haloperidol	0.3 - 3	1 - 10	1 - 5
spiperone	0.02 - 0.14	0.03 - 0.61	0.08 - 0.4
Substituted benzamides			
nemonapride (YM09151-2)	0.02 - 0.09	0.04 - 0.06	0.04 - 0.6
raclopride	1 - 10	2	240 - 2000
Atypical			
clozapine	17 - 158	74 - 620	9 - 32
<i>Agonists</i>			
dopamine (high)	4 - 50	0.8 - 4	0.6 ^a - 28
dopamine (low)	650 - 17,000	22 - 99	17 ^a - 19,000
quinpirole (high)	6	0.4 - 40	2 - 46
<i>Selective antagonists for D₄</i>			K_i (nM)
L-745,870 (Patel <i>et al.</i> , 1996)	960	2200	0.43
RBI-257 (Kula <i>et al.</i> , 1997)	568	145	0.33
U101,387 (Merchant <i>et al.</i> , 1996)	>5000	>2780	3.6

^a(Asghari *et al.*, 1994)

Values are from Neve & Neve, 1997, unless otherwise indicated.

model to predict antipsychotic potential of L-745,870 and two other selective D₄ antagonists (CP-293,019, U-101,387) concluded that these compounds did have antipsychotic potential (Mansbach *et al.*, 1998). The conflicting results from these and other studies demonstrates that further evidence is required before we have a clear understanding of the role of D₄ in schizophrenia, although results with L-745,870 appear to indicate that D₄ alone is not responsible for the antipsychotic action of clozapine (Bristow *et al.*, 1997).

c. Inverse Agonists

Since the cloning of numerous GPCRs, the concept of agonists and antagonists has required modification. Specifically, many antagonists have been reclassified as inverse agonists due to their ability to reduce basal (spontaneous) activation of receptors (Milligan & Bond, 1997). Evidence has emerged that the D₂ antagonists haloperidol and flupenthixol can act as inverse agonists by inducing prolactin release in GH₄C₁ pituitary cells expressing D_{2S}, while other antagonists block this effect (Nilsson *et al.*, 1996). Hall and Strange (1997), using CHO K1 cells expressing D_{2S}, found that a broad range of antagonists acted as inverse agonists by increasing basal cAMP levels. Similarly with the D₃, the antipsychotics haloperidol and fluphenazine inhibited the baseline level of [³H]thymidine incorporation in these cells (Griffon *et al.*, 1996). [³⁵S]GTPγS-binding experiments, which measure receptor activation of G proteins, also showed that basal D₃ receptor coupling was inhibited by haloperidol and raclopride, while clozapine was a partial inverse agonist (Malmberg *et al.*, 1998). In contrast, a recent study of [³⁵S]GTPγS binding in CHO cells expressing the D_{4.4} receptor did not detect any effects of these antagonists on basal G protein coupling (Newman-Tancredi *et al.*, 1997a), perhaps due to the relatively low expression level of this receptor as compared to D₂ and D₃.

3. Functional Coupling of D₂-like Dopamine Receptors

a. Native Tissue

The D₂-like family of receptors couple to multiple intracellular mechanisms (reviewed by Huff, 1996). D₂ receptor inhibition of adenylyl cyclase was first identified in the pituitary prior to cloning (De Camilli *et al.*, 1979; Onalli *et al.*, 1981). In the mouse retina, the D₄ receptor has also been shown to reduce dark-adapted cAMP levels indicating that this subtype is active *in vivo* (Cohen *et al.*, 1992). In addition, D₂-like receptors also couple to the inhibition of inositol

phosphate hydrolysis, inhibition of arachidonic acid release, opening of potassium channel, and inhibition of calcium channel in native tissue (Huff, 1996). Due to a historical lack of selective agonists and antagonists, the contribution of individual receptor subtypes has not been determined by *in vivo* studies, although it is likely that the more abundant D₂ receptor is responsible for most of these actions.

b. Adenylyl Cyclase

Since the cloning of D₂, D₃, and D₄, all have been shown to couple to cAMP inhibition when expressed in cultured cells, although results differ between cell lines (Table 3). Although initial reports found that D₃ was unable to inhibit cAMP (Sokoloff *et al.*, 1990; Tang *et al.*, 1994), further study has found that the receptor can couple to this pathway in selected systems, albeit less effectively than D₂ or D₄ (Chio *et al.*, 1993; Robinson & Caron, 1997). Inhibition of adenylyl cyclase and other signalling events mediated by D₂-like receptors are pertussis-sensitive. Pertussis toxin (PTX) blocks coupling through G_i/G_o by catalyzing the ADP-ribosylation of the α-subunit, indicating that the D₂-like receptors activate these G proteins (Neer, 1995).

c. Arachidonic Acid and Phosphatidylinositol Hydrolysis

Several authors have measured a potentiation of ATP- or Ca²⁺ ionophore-stimulated release of arachidonic acid (AA) in CHO cells mediated by D₂ and D₄ (Table 3). This pathway appears to require a pertussis toxin-sensitive G protein but is not dependent on cAMP inhibition (Kanterman *et al.*, 1991), with some reports indicating that PKC is required for the potentiation of AA release. For D_{2S} expressed in CHO cells, PKC has been proposed to “switch” coupling from cAMP inhibition to facilitation of arachidonic acid release by cPLA₂ (Di Marzo *et al.*, 1993). A recent report by Nilsson *et al.* (Nilsson *et al.*, 1998) has indicated that D_{2L} can cause AA release without prior stimulation with calcium mobilization inhibitors, with PTX and downregulation of PKC abolishing the effect.

Literature reporting the effect of D₂ on the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PtdIns(4,5)P₂) has been contradictory, as shown in Table 3. It was reported that D₂ in pituitary cells can inhibit inositol monophosphate, bisphosphate, and trisphosphate formation via a PTX-sensitive inhibition of calcium channels (Enjalbert *et al.*, 1990). In contrast to this report, others have found that D₂ can stimulate the release of inositol phosphates in Ltk⁻ fibroblasts and

Table 3. Functional coupling of the D₂, D₃, and D₄ dopamine receptors.

Functional Response	Receptor	Tissue / Cell Line	Coupling	Reference
Adenylyl cyclase inhibition	D ₂ -like (rat)	anterior pituitary		(Onalli <i>et al.</i> , 1981)
	D _{2L} (rat)	GH ₄ C ₁	PTX-sensitive	(Albert <i>et al.</i> , 1990)
	D _{2L} (rat)	CCL1.3 (Ltk ⁻) MN9D	Gα _{i2} , Gα _{i3} Gα _{i2}	(O'Hara <i>et al.</i> , 1996)
	D _{2S} D _{2L}	GH ₄ C ₁	PTX-sensitive Gα _{i2}	(Liu <i>et al.</i> , 1994)
	D _{2S} D _{2L}	GH ₄ C ₁	Gα _{i2} Gα _{i3}	(Senogles, 1994a)
	D _{2S} D _{2L}	JEG3, NCB20 JEG3, NCB20	Gα _{i1} or Gα _{i3} Gα _{i2} > Gα _{i1/3}	(Montmayer <i>et al.</i> , 1993)
	D ₃	NG108-15		(Griffon <i>et al.</i> , 1997)
	D ₄ (mouse)	retina		(Cohen <i>et al.</i> , 1992)
	D ₄	CHO lacI	PTX-sensitive	(Chio <i>et al.</i> , 1994)
	D _{2L} (rat) D ₄ (rat)	MN9D, CCL1.3 MN9D	Gα _{i2} PTX-sensitive	(Tang <i>et al.</i> , 1994)
	D _{4.2} , D _{4.4} , D _{4.7}	GH ₄ C ₁		(Sanyal & Van Tol, 1997)
	D ₄ (rat)	MN9D	Gα _{i2}	(Yamaguchi <i>et al.</i> , 1997)
	D _{4.2} , D _{4.4} , D _{4.7}	CHO K1		(Asghari <i>et al.</i> , 1995)
	D ₂ , D ₃ , D ₄	CHO 10001	PTX-sensitive	(Lajiness <i>et al.</i> , 1995)
	D _{2S} , D ₃ , D ₄	HEK 293		(McAllister <i>et al.</i> , 1995)
Potentiation of ACII stimulation	D _{2L} , D ₄	HEK 293	Gβγ	(Watts & Neve, 1997)
Arachidonic acid release (direct)	D ₂ (rat)	CHO 10001	PKC	(Nilsson <i>et al.</i> , 1998)
Arachidonic acid release (potentiation)	D _{2L} (rat)	CHO	PTX-sensitive	(Piomelli <i>et al.</i> , 1991)
	D _{2L}	CHO	PTX/PKC insensitive	(Kanterman <i>et al.</i> , 1991)
	D ₄	CHO lacI	PTX-sensitive	(Chio <i>et al.</i> , 1994)
	D ₂ , D ₄	CHO 10001	PTX-sensitive/PKC	(Lajiness <i>et al.</i> , 1995)
Phosphoinositide hydrolysis (1)	D _{2L} (rat)	CCL1.3	PTX-sensitive	(Tang <i>et al.</i> , 1994)
	D ₂ (rat)	Ltk ⁻ fibroblasts	PTX-sensitive	(Valler <i>et al.</i> , 1990)
Phosphoinositide hydrolysis (1)	D ₂ -like	Lactotroph primary culture	Ca ²⁺ -influx PLC?	(Enjalbert <i>et al.</i> , 1990)

Table 3. Functional coupling of the D₂, D₃, and D₄ dopamine receptors (cont'd)

Functional Response	Receptor	Tissue / Cell Line	Coupling	Reference
Na ⁺ /H ⁺ antiporter stimulation	D _{2S} and D _{2L}	C ₆ glioma L fibroblasts	Insensitive to PTX	(Neve <i>et al.</i> , 1992)
	D ₂ and D ₃	CHO 1000I	PTX-sensitive	(Chio <i>et al.</i> , 1993)
	D ₂ , D ₃ , D ₄	CHO 1000I	PTX-sensitive	(Lajiness <i>et al.</i> , 1995)
K ⁺ -channel activation (voltage-dependent))	D ₂ -like	rat melanotrophs	PTX-sensitive	(Williams <i>et al.</i> , 1989)
(voltage-dependent)	D ₂ (rat)	Lactotroph primary culture	Gα _i	(Lledo <i>et al.</i> , 1992)
(voltage-dependent)	D _{2S} D _{2L} , D ₃	NG108-15 NG108-15	Gα/Gα _{o/r} Gα _o	(Liu <i>et al.</i> , 1996)
(voltage-independent)	D ₂ -like	rat neuron primary culture	PTX-sensitive	(Belousov & Van Den Pol, 1997)
(Kir3)	D ₂ , D ₃ , D ₄	Xenopus oocytes	PTX-sensitive	(Werner <i>et al.</i> , 1996)
(85 pS IRK)	D ₂ -like	striatal neurons		(Waszczak <i>et al.</i> , 1998)
K ⁺ -channel inhibition (voltage-dependent)	D ₄	hypophysial nerve terminals		(Wilke <i>et al.</i> , 1998)
Ca ²⁺ -channel current inhibition (T and L-type)	D ₂ (rat)	Lactotroph primary culture	Gα _o	(Lledo <i>et al.</i> , 1992)
	D ₂ and D ₄	GH ₄ C ₁		(Seabrook <i>et al.</i> , 1994)
	D _{2S} (rat) D _{2L} (hum.)	GH ₄ C ₁	Gα _o Gα _o	(Liu <i>et al.</i> , 1994)
Cl ⁻ influx	D ₂ , D ₃ , D ₄	Xenopus oocytes	PTX-sensitive	(Jensen <i>et al.</i> , 1997)
Changes to neural morphology	D ₂ , D ₃ , D ₄	Fetal cortical neurons		(Swarzenski <i>et al.</i> , 1994)
Apoptosis and Differentiation	D ₂ (rat)	Olfactory epithelium		(Coronas <i>et al.</i> , 1997)
Antiproliferation (DNA synthesis !)	D ₂ (rat)	GH ₄ C ₁	PTX-sensitive PTP-associated	(Florio <i>et al.</i> , 1992)
	D ₂ (rat)	GH ₄ C ₁	Insensitive to PTX PKCε-dependent	(Senogles, 1994b)
Mitogenesis (DNA synthesis !)	D ₂ , D ₃ , D ₄	CHO 1000I	PTX-sensitive	(Lajiness <i>et al.</i> , 1995)
	D _{2L} (rat)	C ₆ glioma	PTX-sensitive/Ras	(Luo <i>et al.</i> , 1998b)
	D ₃	NG108-15	TK-dependent	(Griffon <i>et al.</i> , 1997)
MAPK activation (ERK1/2)	D ₂	COS-7	Gβγ	(Faure <i>et al.</i> , 1994)
	D _{2S} and D _{2L}	CHO	PTX-sensitive	(Welsh <i>et al.</i> , 1998)
	D _{2L} (rat)	C ₆ glioma	PTX-sensitive/Ras	(Luo <i>et al.</i> , 1998b)

CCL1.3 cells, probably as a result of increased $[Ca^{2+}]_i$ (Valler *et al.*, 1990; Tang *et al.*, 1994). The latter study found that D_3 and D_4 had no effect. As with other functional responses, it is likely that these events are cell-line dependent.

d. Transporters and Ion Channels

The D_2 -like receptors stimulate extracellular acidification by activation of the amiloride-sensitive Na^+/H^+ antiporter, although reports on PTX sensitivity conflict (Neve *et al.*, 1992; Lajiness *et al.*, 1995). An inhibitory effect on Ca^{2+} channels by D_2 and D_4 is also well documented, again by way of PTX-sensitive G_i/G_o (Table 3). The inhibition of K^+ current by D_2 in PC12 cells (Zhu *et al.*, 1997) and by a D_4 -specific agonist has recently been reported (Wilke *et al.*, 1998). However the predominant effect of dopamine D_2 receptors on K^+ channels has been PTX-sensitive activation leading to hyperpolarization. Inhibition of prolactin release occurs via D_2 receptors in pituitary lactotrophs. In these cells, D_2 couples to the inhibition of two calcium currents and the activation of two potassium currents, producing a hyperpolarizing postsynaptic potential (Lledo *et al.*, 1992; Williams *et al.*, 1989). The inhibition of Ca^{2+} channels was blocked by anti- $G\alpha_o$ antibodies, while the K^+ channel activation could be blocked by anti- $G\alpha_{i3}$. Inhibition of dopamine release in the striatum by D_2 (autoreceptor activity) is invoked through a K^+ channel-dependent mechanism, whereby hyperpolarization reduces presynaptic excitability (Cass & Zahniser, 1991). Both voltage-dependent (I_A and I_K) and voltage-independent K^+ channels (IRKs) appear to be activated by dopamine D_2 receptors (Lledo *et al.*, 1992; Belousov & Van Den Pol, 1997). More specifically, the Kir3 family of potassium channels has been shown to be stimulated by all D_2 -like receptors (Werner *et al.*, 1996).

e. Effects on Morphology and Growth

When D_2 , D_3 , and D_4 are transfected into the mesencephalic cell line MN9D, these receptors can increase the number, branching, and extension of neurites with sub-type selective differences (Swarzenski *et al.*, 1994). Opposite effects on cell growth (as measured by $[^3H]$ thymidine incorporation) have been reported when D_2 -like receptors are expressed in different cell lines, with dopamine inhibiting growth in pituitary-derived cells (Florio *et al.*, 1992; Senogles, 1994b) and stimulating growth in CHO or NG108-15 cells (Lajiness *et al.*, 1995; Pilon *et al.*, 1994). D_3 -mediated mitogenesis was shown to be potentiated by PKA, leading to the suggestion

that there may be cross-talk with D₁ (Griffon *et al.*, 1997). However, in CHO cells, the D₂ effect on mitogenesis was independent of cAMP but blocked by genistein, a tyrosine kinase inhibitor (Lajiness *et al.*, 1993). Recently, it was shown by Luo *et al.* (1998b) that the mitogenic effect of D₂ occurs through a pathway involving G_i/G_o and Ras/MEK. Other reports have indicated that D₂ can activate the MAPK pathway by a G protein-dependent mechanism (Faure *et al.*, 1994; Welsh *et al.*, 1998) (see Section I.C.2.e), adding another chapter to the long story of D₂-like receptor signalling.

f. G Protein Specificity

Considerable effort has gone into elucidating which G proteins couple to the different D₂ receptor family members. Despite conflicting results, most likely arising from the variety of cell lines employed, it seems clear that selectivity in coupling occurs. Almost all reports have shown that functional coupling is dependent on a PTX-sensitive G protein (ie. G_i/G_o). Effects on Ca²⁺ channels appear to involve G_o for both D_{2S} and D_{2L} (Lledo *et al.*, 1992). In contrast, coupling to other G proteins seems to differ between the alternatively spliced variants. Coupling to a voltage-dependent K⁺ current by D_{2S} appears to be inhibited by CTX, implicating G_s, whereas D_{2L} and D₃ were PTX-sensitive (Liu *et al.*, 1996). Senogles (1994a) used Gα_i mutants to determine that D_{2S} coupled to inhibit cAMP by Gα_{i2}, while D_{2L} coupled by Gα_{i3}. In another cell line, however, D_{2S} was more efficient at coupling to G_{i1/3} than D_{2L}, with the latter requiring Gα_{i2} for maximum activity (Montmayer *et al.*, 1993). Reports indicating that D_{2L} couples to Gα_{i2} seems to form a consensus (O'Hara *et al.*, 1996; Liu *et al.*, 1994; Guiramand *et al.*, 1995; Tang *et al.*, 1994). In Sf9 cells, D_{2S} preferentially couples to Gα_{i1} (Grünewald *et al.*, 1996b). D₃ and D₄ do not appear to use this Gα subunit (Tang *et al.*, 1994). In MN9D cells, the D₄ receptor fails to couple to Gα_{oA}, Gα_{oB}, and Gα_{i1/2/3} (O'Hara *et al.*, 1996; Tang *et al.*, 1994). However, use of a PTX-resistant mutant of Gα_{i2} (transducin) has revealed that D₄ can activate this G protein in MN9D mesencephalic cells (Yamaguchi *et al.*, 1997). G_{i2} was shown to express outside the retina and to inhibit adenylyl cyclase upon activation by D₄. Despite identifying D₄ as the first non-opsin GPCR to couple to G_{i2}, Yamaguchi *et al.* (1997) did not identify any effects on cGMP phosphodiesterase activity, the effector of rhodopsin-stimulated Gα_r. Therefore the ultimate target of D₄ signalling in MN9D cells remains to be determined. Huff (1996) has reported that the potentiation of AA release by D₂ does not involve G_{i2/3}, but does involve G_{i/o},

further demonstrating that multiple G proteins can be activated by these receptors.

As well as mediating effects through the α -subunit of G proteins, it has become increasingly evident that receptor signals can be transduced by $G\beta\gamma$. The cloning of G protein-coupled inward rectifying potassium channels such as Kir3.1 (GIRK1) has revealed that these proteins are regulated by $G\beta\gamma$ subunits released by muscarinic receptor activation (Wickman *et al.*, 1994; Reuveny *et al.*, 1994). The activation of Kir3 in *Xenopus* oocytes by all D₂-like receptors suggests that they can open K⁺ channels via $G\beta\gamma$ as well (Werner *et al.*, 1996). A recent report showed that potentiation of PLA₂ or G_s-stimulated adenylyl cyclase (ACII) by D_{2L} and D₄ (but not D₃) was most likely due to dopamine-activated $G\beta\gamma$ subunits (Watts & Neve, 1997).

In conclusion, while the simple description of D₂-like receptors as "G_i/G_o" coupled is still valid, considerably more complexity has been elucidated in the last decade. In particular, the coupling of D_{2S}, D_{2L}, D₃, and D₄ through specific G α (and now G $\beta\gamma$) subunits has demonstrated that the multiplicity of D₂-like dopamine receptor subtypes is not redundant at the level of basic biochemical function. Further complicating our picture of receptor signalling pathways are recent advances in research with the adrenergic receptors. Phosphorylation of the β_2 -AR by PKA results in the switching of coupling from G_s to G_i, allowing the receptor to activate MAPK by G $\beta\gamma$ (Daaka *et al.*, 1997). Switching also seems to occur in the α_2 -AR, where nanomolar concentrations of the agonist norepinephrine inhibit cAMP, while micromolar amounts stimulate cAMP, producing biphasic activation curves (Jasper *et al.*, 1998). Alternate splicing of G_s can also have prominent effects, with a 15 amino acid insert in the Ras-like domain of G α_s producing a β_2 -AR with properties of a constitutively active receptor (Seifert *et al.*, 1998). G α_{i2} is also known to undergo alternate splicing (Montmayer & Borrelli, 1994). The additional complication of more than 20 α , 5 β , and 12 γ subunits, which can form hundreds of valid heterotrimer combinations (Hamm, 1998), as well as the apparent compartmentation of receptors and their effectors (Huang *et al.*, 1997) makes understanding these systems a daunting task. Hopefully, characterization of this interesting family of neurotransmitter receptors, as well as knowledge gained from other GPCRs, will clarify the ambiguities that have arisen from studying them in various cell lines.

4. Insights from Dopamine Receptor Knockout Animals

Transgenic animals have emphasized the importance of D₂ receptors *in vivo*. Mice

lacking the D₂ receptor display the abnormal posture, akinesia, and bradykinesia that is typical of Parkinson's disease, as well as increased enkephalin and GAD mRNA and a reduction in the size of the reproductive organs (Baik *et al.*, 1995). These D2R-/- mice also show altered neuronal plasticity and a lack of morphine's rewarding effect (Calebrese *et al.*, 1997; Maldonado *et al.*, 1997). D₂ receptors of the mesolimbic system are believed to participate in opiate dependence and withdrawal (Nestler & Aghajanian, 1997).

As may be expected from their much lower expression in the CNS, D₃ and D₄ knockout mice did not display as strong a phenotype. D₃ receptor mutants did demonstrate hyperactivity and reduced anxiety-associated behavior (Drago *et al.*, 1998). A recently described dopamine D₄ knockout mouse was supersensitive to the motor effects of ethanol, cocaine, and methamphetamine, and also had higher rates of dopamine synthesis and degradation (Rubinstein *et al.*, 1997). This study also supported the hypothesis that the high affinity of the antipsychotic clozapine for D₄ receptors may have physiological relevance, as this drug was less capable of reversing apomorphine-induced locomotor activity in the knockout animals compared to wildtype mice. These knockout studies appear to establish that the heterogeneity of D₂-like dopamine receptors is physiologically significant, with D₂, D₃, and D₄ all having roles in the brain.

C. Activation of MAPK by GPCRs

1. The MAPK Pathway

MAP kinases, which include ERKs (extracellular signal-regulated kinase) 1 and 2, JNK/SAPK (Jun N-terminal kinase/stress-activated protein kinase), and p38 MAP kinase, are a family of serine/threonine kinases involved in the transduction of extracellular signals to induce responses such as cell differentiation and proliferation. Upon phosphorylation at threonine and tyrosine residues by a MAP kinase kinase, MAP kinases become activated and may phosphorylate cytoplasmic effectors and structural proteins, as well as translocating to the nucleus and activating transcription factors (Davis, 1993; Hill & Treisman, 1995).

The best characterized MAPK pathway is the activation of ERK1 and 2 (p44 and p42) by growth factor receptors (reviewed by Malarkey *et al.*, 1995; Seger & Krebs, 1995). Activation of transmembrane receptors (eg. PDGF and EGF receptors) results in their dimerization and the autophosphorylation of intracellular tyrosine residues (Heldin, 1995). Phosphorylated tyrosines and surrounding residues are specifically recognized by the SH2 (Src homology 2) domains of

adapter proteins such as Grb2 and Shc. Many other proteins can dock with activated RTKs, including Src-family protein tyrosine kinases (PTK), phospholipase C γ (PLC γ), the p85 subunit of phosphoinositol 3-kinase (PI3-kinase), GTPase activating protein (GAP), and the phosphotyrosine phosphatase SH-PTP-2 (Pawson, 1995). Src tyrosine kinases in particular have a long list of substrates and diverse functions in the cell, including phosphorylation of cytoskeletal proteins and many proteins involved in mitogenic signalling (Brown & Cooper, 1996). Some receptors (eg. cytokine receptors) lack catalytic activity and are therefore dependent on protein tyrosine kinases for phosphorylation and activation (Heldin, 1995).

The association of Grb2 or Shc/Grb2 with the activated RTK translocates Sos, which is associated with Grb2 by two SH3 interactions, to the plasma membrane (Egan *et al.*, 1993). Sos, a GEF (guanine-nucleotide exchange factor) for p21 Ras, is activated by membrane targeting and catalyzes the exchange of Ras-bound GDP for GTP, thereby activating Ras (Aronheim *et al.*, 1994). Activated GTP-Ras translocates the cytoplasmic Raf-1 (a MAPKKK) to the membrane, resulting in autophosphorylation and activation. Raf proceeds to catalyze the phosphorylation of MEK (a MAPKK). Finally, MEK activates ERK (MAPK) by phosphorylation, leading to the phosphorylation of cytoplasmic targets such as MBP and cPLA₂, as well as the translocation of ERK to the nucleus, allowing activation of transcription factors such as Elk-1 and SAP-1 (Seger & Krebs, 1995). Ras appears to act as a key “molecular switch” in the MAPK pathway, with Ras GAPs (GTPase activating proteins) stimulating the GTPase activity of Ras, thereby returning it to the inactive GDP-bound state (Quilliam *et al.*, 1995). The duration of MAPK signalling can also be controlled by the activity of several phosphatases (Hunter, 1995).

Other MAPKs, such as JNK and p38, also act through a MAPKKK – MAPKK – MAPK pathway. For example, Rac1 (another p21 GTPase) activates a sequential phosphorylation cascade which includes MEKK, SEK, and JNK (Coso *et al.*, 1995), whereas p38 MAPK appears to be activated by MEK in PC12 cells to induce neurite outgrowth (Morooka & Nishida, 1998). The latter finding demonstrates that activation of a common signalling molecule, MEK, can lead to either differentiation or proliferation depending on the cell type. In PC12 cells, it appears that transient vs. sustained activation of MEK may determine whether mitogenesis (via ERK) or neuronal differentiation (via p38) is stimulated (Morooka & Nishida, 1998).

2. Activation of MAPK by G Protein-Coupled Receptors

a. GPCRs as Oncogenes

It has become clear that GPCRs can also activate MAPK signalling pathways (reviewed in van Biesen *et al.*, 1996b; Gutkind, 1998; Lopez-Illasaca *et al.*, 1998). Early evidence for GPCR involvement in mitogenesis came from the discovery of GTPase deficient, oncogenic activating mutations of $\text{G}\alpha_s$ (*gsp* oncogene) in pituitary adenomas and $\text{G}\alpha_{12}$ (*gip* 2 oncogene) in adrenal cortical tumours (Lyons *et al.*, 1990). The tumorigenicity of *gsp* and *gip* is cell line specific, however, and increased levels of cAMP results in growth arrest in fibroblasts (Cook & McCormick, 1993).

The oncogenic potential of GPCRs themselves has also been established. NIH 3T3 fibroblasts transformed with the serotonin 5-HT_{1c} receptor form foci dependent on activation by serotonin, with tumour formation resulting after transfer to nude mice (Julius *et al.*, 1989) Allen and coworkers (1991) found that a constitutively active mutant (CAM) α_{1c} -AR was also capable of enhancing mitogenesis in cultured fibroblasts without addition of agonists. More recently, the Kaposi's sarcoma-associated herpesvirus was found to express an oncogenic GPCR with constitutive signalling (Bais *et al.*, 1998). This receptor activates JNK/SAPK and p38 MAPK, resulting in transformation and tumorigenicity. Elucidation of the pathways involved in MAPK activation by GPCRs has progressed rapidly as of late. However, these studies have also demonstrated the complexity and cell-type specificity of many of these signalling pathways.

b. MAPK Activation by $\text{G}\beta\gamma$

Elucidation of the role of $\text{G}\beta\gamma$ in cell cycle control was led by studies of the yeast pheromone response pathway. The G protein-coupled STE2 and STE3 receptors are activated in response to mating factors α and α . This results in the activation of a MAPK cascade through STE4 and STE18, which are the yeast homologs of $\text{G}\beta$ and $\text{G}\gamma$ (Herskowitz, 1995; Clapham & Neer, 1997). $\text{G}\beta\gamma$ acts by a pathway involving SET20, and STE5 to activate STE11 (MEKK), STE7 (MEK), and finally the MAPKs FUS3 and KSS1. The leads to the activation of transcription factors which control the expression of genes necessary for cell fusion. $\text{G}\beta\gamma$ appears to initiate the cascade by binding STE5 and targetting it to the membrane (Pryciak & Huntress, 1998). This allows STE5 to act as a scaffold for elements of the MAPK cascade: STE11, STE7, and FUS3. The kinase STE20 (a member of the PAK kinase family of MAPKKKs) interacts with $\text{G}\beta\gamma$ and phosphorylates STE11, leading to the activation of the multienzyme complex

bound to STE5 (Leeuw *et al.*, 1998). STE5 appears to act to colocalize elements of the MAPK pathway, specifically activating the pheromone response and preventing cross-talk, since STE11 is active in three of the four MAPK cascade modules in *S. cerevisiae* (Elion, 1998). Very recently, yeast two-hybrid screening identified two proteins that are structurally unrelated to STE5 but which act in a similar manner in mammalian cells: JIP-1 and MP1 (Schaeffer *et al.*, 1998; Whitmarsh *et al.*, 1998). For example, MP1 binds MEK1 and ERK1, promoting their activation by B-Raf.

Many other GPCRs (eg. M2 mAChR, α_2 -AR, LPA receptor, opioid receptors) activate the Ras/MAPK pathway in a PTX-sensitive manner (Koch *et al.*, 1994; Luttrell *et al.*, 1996; Belcheva *et al.*, 1998). Several groups concurrently demonstrated the role of G $\beta\gamma$ in this process in mammalian cells (Figure 4). Crespo *et al.* (1994) found that expression of G α_q , G α_{12} , and G α_{13} in COS-7 cells failed to enhance ERK phosphorylation. In contrast, overexpression of G α_i , which sequesters G $\beta\gamma$, blocked M1 and M2 mAChR activation of ERK. The overexpression of G $\beta 1\gamma 2$ alone was found to potently stimulate MAPK phosphorylation in COS-7 cells (Faure *et al.*, 1994). Using another G $\beta\gamma$ sequestrant, β ARKct (containing the C-terminal PH domain of β ARK), activation of Ras and MAPK by G i -coupled LPA, α_2 -AR, and M2 mAChR was inhibited, while α_1 -AR (G q -coupled) and EGF receptor stimulation of ERK was not altered (Koch *et al.*, 1994). Thus, Koch *et al.* (1994) established that G $\beta\gamma$ was acting upstream of Ras and that an alternate MAPK pathway must exist for G q -coupled receptors. G $\beta\gamma$ has also been found to stimulate the tyrosine phosphorylation of the adapter protein Shc, indicating that G i -coupled receptors recruit the same Shc-Grb2-Sos complex formation involved in RTK activation of Ras/MAPK (van Biesen *et al.*, 1995; Luttrell *et al.*, 1995). Interestingly, it has also been found that the insulin-like growth factor 1 (IGF1) receptor, a RTK, is sensitive to PTX and G $\beta\gamma$ sequestration, indicating that some of the mechanisms responsible for GPCR activation of MAPK are shared by a receptor with intrinsic tyrosine kinase activity (Luttrell *et al.*, 1995).

c. Phosphoinositide 3-Kinase and Protein Tyrosine Kinases

The pathway connecting G $\beta\gamma$ to Shc has received much attention recently, but the results are far from clear and significant diversity may exist among different systems. The specific PI3-kinase inhibitors wortmannin and LY294002, and a dominant negative mutant of the p85 subunit of PI3-kinase, demonstrated that this kinase is an essential component in the activation

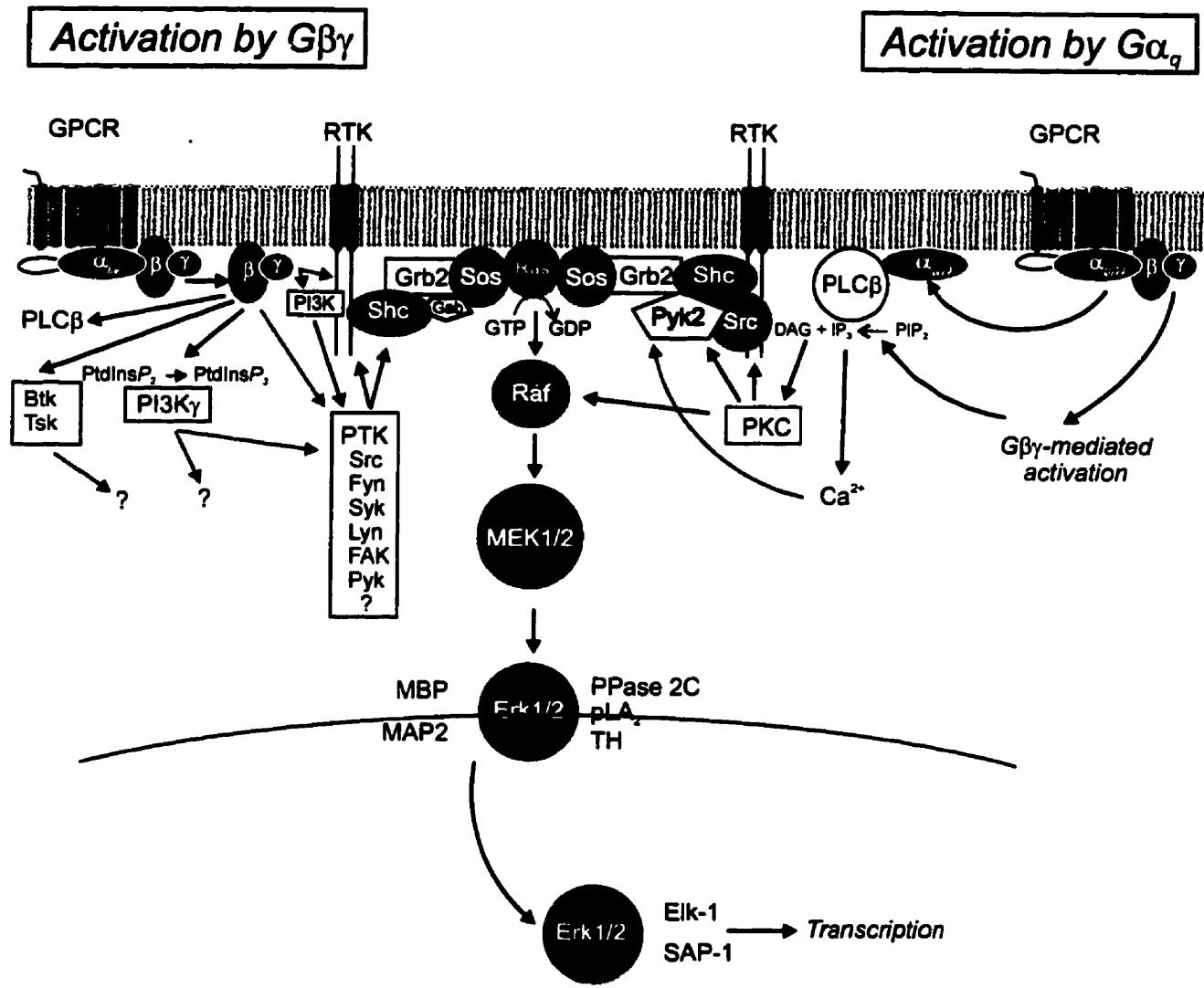


Figure 4. Activation of the MAPK pathway by G $\beta\gamma$ and G α_q .
Refer to ABBREVIATIONS for the full names of the pathway components.

of MAPK by LPA and α_{2A} -AR receptors in CHO and COS-7 cells (Hawes *et al.*, 1996). The finding that the PI3-kinase product PtdIns(3,4,5)P₃ can interact with SH2 domains has also implicated this phospholipid in adapter protein recruitment (Rameh *et al.*, 1995). A PI3-kinase isotype that is activated in response to G α and G $\beta\gamma$, p110 γ , has recently been identified, but this subtype does not associate with p85 (Stoyanov *et al.*, 1995). ERK activation by the M2 mAChR was shown to be potentiated by expression of the novel PI3-kinase γ (Lopez-Illasaca *et al.*, 1997). The ability of a Src inhibitor, PP1, to block this pathway suggests that one mechanism of GPCR-mediated ERK activation may involve G $\beta\gamma$ - PI3 kinase γ - Src-family PTK - Shc - Grb2/Sos - Ras - - - MAPK (Figure 4).

The nonreceptor PTK c-Src is also phosphorylated and associates with Shc in LPA-stimulated COS-7 cells (Luttrell *et al.*, 1996). A direct role for this PTK was implicated since expression of Csk, which inactivates c-Src, was found to block LPA stimulation of MAPK. Another Src-related tyrosine kinase, Lyn, was activated by G protein-coupled NFP receptors, producing Lyn-Shc-p85 PI3-kinase complexes. This led to the activation of Rac, a GTPase in the JNK pathway (Ptaszniak *et al.*, 1995). Lyn and the PTK Syk are also involved in the stimulation of MAPK by the M1 mAChR (G q -coupled) in DT40 cells, while the M2 mAChR (G i -coupled) only required Syk (Wan *et al.*, 1996). In this study, transfection of the related tyrosine kinases Fyn, Lck, and Src could compensate for a Lyn deficiency, indicating that the pattern of expression in different cells may be a prime determinant in the mechanism of MAPK activation. It is also unclear, in the case of the M1 mAChR, whether MAPK activation was mediated by G $\beta\gamma$ or G α_q (Section I.C.2.d). Another PTK which is highly expressed in the brain, Pyk2 (also called related focal adhesion tyrosine kinase, RAFTK), has recently been identified. Pyk2 can recruit Shc/Grb2/Sos complexes in PC12 cells in response to LPA (G i -coupled) and bradykinin (G q -coupled), with Src playing a role in the activation of Pyk2 (Lev *et al.*, 1995; Dikic *et al.*, 1996). An emerging picture appears to be that G i -coupled receptors (via G $\beta\gamma$) and G q -coupled receptors (by G α_q and/or G $\beta\gamma$) may share downstream components, such as the tyrosine kinases Src and Pyk, leading to the activation of MAPK (Figure 4). Additional PH domain-containing tyrosine kinaes, Tsk and Btk, are also known to be activated by G $\beta\gamma$ upon cotransfection, although their role *in vivo* has not been established (Langhans-Rajasekaran *et al.*, 1995). LPA stimulation in Dami cells induces phosphorylation of the protein-tyrosine phosphatase SH-PTP1 in a G $\beta\gamma$ and PKC-dependent manner, which may provide a mechanism for Src-family PTK activation by

dephosphorylating autoinhibitory phosphotyrosine-containing SH2 domains, or may indicate negative feedback (Gaits *et al.*, 1996). Another pathway mediated by G $\beta\gamma$ and dependent on Ras-GRF (CDC25), a GEF independent of Sos, has been proposed to mediate Ras activation (Mattingly & Macara, 1996). In Neutrophils, the activation of Ras by G i_2 -coupled C5a and FMLP receptors was independent of Grb2/Sos, but involved the inhibition of Ras-GAP, suggesting yet another possible mechanism of activation (Zheng *et al.*, 1997).

d. Activation of MAPK by G α_q

While MAPK activation by G i -coupled receptors is clearly dependent on G $\beta\gamma$, it appears that both the α and $\beta\gamma$ subunits of G q can activate this pathway. While some researchers (Crespo *et al.*, 1994; Igishi & Gutkind, 1998) have attributed M1 mAChR-mediated MAPK activation to G $\beta\gamma$, another group showed that constitutively-active G α_q could increase MAPK activity (Faure *et al.*, 1994). In addition, Faure and others found that sequestration of G $\beta\gamma$ did not affect G q -coupled activation of MAPK by M1 mAChR, α_{1B} -AR, and bombesin receptors (Hawes *et al.*, 1995). The expression of β ARKct and dominant negative RasN17 also failed to inhibit signalling to MAPK by M1 mAChR and α_{2A} -AR in COS-7 and CHO cells, although dominant negative Raf and PKC depletion were effective, indicating the influence of G α_q on ERK occurs through a separate process (Hawes *et al.*, 1995). PKC α has been shown to phosphorylate Rafl, providing one mechanism for G α_q -activation of MAPK (Kolch *et al.*, 1993).

Other studies point to a common pathway in G α_q and G $\beta\gamma$ activation, involving PTK-mediated formation of the Shc/Grb2/Sos complex. Activation of G q -coupled α_{1B} -AR and bradykinin receptors stimulated MAPK in PC12 and HEK 293 cells (Dikic *et al.*, 1996; Della Rocca *et al.*, 1997). In the latter study, both the G $i/\beta\gamma$ - and G q -mediated pathways activated Ras and required PLC, Ca $^{2+}$, calmodulin, and Pyk2. This is in contrast to α_{1C} -AR activation in COS-7 cells, which is not Ras-dependent (Hawes *et al.*, 1995). PLC β is known to be activated by both G α_q and G $\beta\gamma$, and it appears this mechanism is functioning in 293 cells. The PTK Pyk2, which is regulated by Ca $^{2+}$ via an unknown mechanism, was found to be required in this case. Therefore the proposed MAPK activation sequence in this instance was G q /G $\beta\gamma$ → PLC β → Ca $^{2+}$ → CaM → Pyk2 → Src → Shc/Grb2/Sos → Ras → MAPK (Figure 4). In HEK 293 cells, a similar pathway for M1 mAChR activation of Pyk2, followed by association with Src and Grb2, was found to be dependent on both Ca $^{2+}$ and PKC (Felsch *et al.*, 1998). Angiotensin 2 (AT $_2$) receptors in kidney

cells are also G_q-coupled and were recently shown to activate MAPK by a novel mechanism involving the stimulation of PLA₂ (Jiao *et al.*, 1998). AT₂ receptors also produced a PTX-insensitive activation of SH-PTP1 in a neuroblastoma cell line (Bedecs *et al.*, 1997).

e. Receptor Transactivation

Luttrell *et al.* (1995) have proposed that the EGF receptor may act as a scaffold that is phosphorylated by Src in response to LPA activation, leading to Shc/Grb2/Sos binding and activation of Ras. He found that LPA stimulation produced an EGFR/Shc/Grb2 complex that was Src kinase-dependent. Others have found that G_i- and G_q-coupled receptors stimulate EGFR tyrosine phosphorylation, and a dominant negative EGFR reduced the activation of MAPK by GPCRs in several cell lines (Daub *et al.*, 1996; Daub *et al.*, 1997). The latter report found that inhibition of PI3-kinase and Src blocked MAPK activation but not EGFR phosphorylation in response to GPCR activation, implying that the sequence of activation was different than that proposed by Luttrell *et al.* (1995). Another crucial adapter protein in the pathway proposed by Daub *et al.* (Daub *et al.*, 1997) is Gab1, which bound to Grb2.

A study found that LPA stimulation of EGFR in HeLa cells was required for maximum activation MEK1/2, supporting the hypothesis that RTK transactivation precedes downstream events, although an EGFR-independent pathway was also detected (Cunnick *et al.*, 1998). ROS (reactive oxygen species) appeared to mediate this LPA-stimulated EGFR phosphorylation, perhaps by oxidative inhibition of a tyrosine phosphatase. In other cell lines, LPA-mediated effects were dependent on activation PDGF receptors (Herrlich *et al.*, 1998) and SHPS-1 receptors (Takeda *et al.*, 1998). P_{2Y2} purine receptor activation of MAPK appears to involve PKC-dependent Pyk2 and EGFR activation, leading to Shc/Grb2/Sos activation (Soltoff *et al.*, 1998). A fascinating example of “inside-out” signalling was recently described in the M3 mAChR transactivation of integrins (Slack, 1998). In this case, a GPCR activated the integrin receptor intracellularly, but was dependent on the presence of the integrin ligand fibronectin. The integrin ligand itself was unable to activate the receptor.

f. Endocytosis in MAPK Activation

Another interesting aspect of GPCR-mediated MAPK signalling is the interdependence of receptor endocytosis and MEK activation. Using inhibitors of clathrin-mediated endocytosis,

Luttrell and coworkers (1997) demonstrated that LPA- and thrombin-induced activation of ERK1/2 in Rat-1a fibroblasts was dependent on receptor internalization. This finding was furthered with the β_2 -AR in HEK 293 cells, where it was shown that β -arrestin1 and dynamin were important for Raf-mediated MEK activation by this receptor, but not for conventional second-messenger signalling or Raf activation (Daaka *et al.*, 1998). β -arrestins are essential for targeting many GPCRs for internalization after they are phosphorylated by GRKs. With the μ -opioid receptor, MAPK inhibitors were found to block receptor desensitization in response to agonists (Polakiewicz *et al.*, 1998). Therefore a relationship between GPCR internalization and MAPK activation is presently emerging, although further research is necessary to determine whether this mechanism is absolutely essential in the $G\beta\gamma$ activation pathway. However, a similar dependence between signalling and receptor endocytosis has been found with EGF receptors, where dominant-negative dynamin was found to suppress MAPK and PI3-kinase activation (Vieira *et al.*, 1996).

g. G_s and G_o Activation of MAPK

In CHO cells, the G_o -coupled platelet-activating factor (PAF) receptor can activate MAPK independent of $G\beta\gamma$ and Ras but dependent of PKC, although little is known about this mechanism (van Biesen *et al.*, 1996a). Cell line-specific responses to cAMP have also been reported. In fibroblasts, increased cAMP inhibits MAPK (Cook & McCormick, 1993), while in PC12 cells cAMP potentiated MAPK activation by NGF (Calleja *et al.*, 1997). PKA is known to mediate the inhibition of Raf1 by phosphorylation, reducing its affinity for Ras (Wu *et al.*, 1993). Recently, it was reported that cAMP can activate B-Raf and inhibit Raf1 in PC12 cells by activation of Rap1, which may be a source of variability among cell types, which may or may not express B-Raf and Rap1 (Vossler *et al.*, 1997). Another recent study found that the β_2 -AR, generally regarded as G_s -coupled, can activate the MAPK pathway via the $\beta\gamma$ subunits of PTX-sensitive G-proteins (Daaka *et al.*, 1997). This was apparently mediated by PKA phosphorylation of the receptor, resulting in the “switching” of G-protein coupling.

h. MAPK in Native Tissue

Since much of the research on GPCR-mediated MAPK activation has utilized cultured, immortalized cells, which are most often fibroblastic, there remains a question as to role this may

play *in vivo*. The α_1 -AR (G_q -coupled) agonist phenylephrine, which is known to cause hypertrophic growth of ventricular myocytes, stimulated the activation of p38 MAPK, JNK, and ERK1/2 in perfused rat heart by a PKC-dependent mechanism (Lazou *et al.*, 1998). Rat hepatocytes in primary culture responded to the GPCR agonists norepinephrine, prostaglandin F_{2 α} , vasopressin, and angiotensin II; all activated MAPK to an extent comparable to that of EGF (Koch *et al.*, 1994). This response lasted several hours and was blocked by pertussis toxin. Thus MAPK in untransformed cells can also be stimulated by G proteins.

MAPK has also been shown to have a role in the brain (reviewed by Fukunaga & Miyamoto, 1998). In rats, ERK1 and ERK2 are expressed at high levels in the CNS, with each having a distinct regional distribution (Thomas & Hunt, 1993). Nerve growth factor (NGF) and other neurotrophic factors (BDNF, NT-3/4/5) as well as bFGF, EGF, and Ca²⁺-influx are coupled to MAPK and involved in transcription, differentiation, plasticity, and survival (Fukunaga & Miyamoto, 1998). MAPK has also been implicated in the formation of PHFs (paired helical filaments) in Alzheimer's disease by phosphorylating tau, a microtubule-associated protein (Drewes *et al.*, 1992). In addition, JNK and p38 MAPK are active in cultured neurons and appear to be involved in apoptosis (Fukunaga & Miyamoto, 1998). Although Shc is barely detectable in neural tissue, two homologs, N-Shc and Sck, have been identified in the brain (Nakamura *et al.*, 1998). While these forms share some functional properties, they appear to have differing responses to Src kinase and a unique expression pattern.

Recently, a role for G protein-coupled activation of MAPK in neuronal tissue has emerged. In primary culture of rat cortical glia, activation of mGluR5 resulted in the transient phosphorylation of ERK1/2, with maximum activation after 5 minutes. (Peavy & Conn, 1998). Stimulation of the G protein-coupled PAF receptor in cultured neurons activated MAPK, phosphorylated synapsin I, and led to spontaneous AMPA receptor-ion channel activity similar to that seen in response to BDNF (Fukunaga & Miyamoto, 1998). Activation of the LPA receptor has been shown to result in the closing of connexin43 Gap junctions by a Src-dependent mechanism involving connexin43, although MAPK itself was not involved (Postma *et al.*, 1998).

In neurons, the AT₁ receptor was recently shown to stimulate the transcription of important metabolic enzymes such as tyrosine hydroxylase (TH) and dopamine β -hydroxylase (D β H) (Yang *et al.*, 1997). This fascinating study also implicated MAPK in the translocation of the AT₁ GPCR to the nucleus. AT₁ receptors were found to coimmunoprecipitate with MAPK

and were also substrates for this kinase. Yang's report suggests that MAPK-dependent GPCR endocytosis (Section I.C.2.f) may have important functional consequences in the brain, in this case resulting in enhanced catecholamine synthesis due to the AT₁ receptor.

The biological basis of learning is believed to be mediated by long-term synaptic plasticity. Long-term facilitation (LTF) in *Aplysia* was studied using reconstituted sensory-motor synapses in culture (Martin *et al.*, 1997). In this report, it was found that serotonin induced the long-term facilitation (as measured by an increase in EPSP size) at the presynaptic neuron. LTF was dependent on nuclear translocation of MAPK, while short-term facilitation was not. In addition, cAMP was found to induce MAPK translocation, and PKA and MAPK are both believed to mediate the activation of transcription factors such as CREB1 and 2. Martin *et al.* (1997) showed that the latter mechanism also occurs in the hippocampus of mice. They suggest that the coactivation of PKA and MAPK may provide a combinatorial threshold for memory.

The mechanism of memory formation was addressed in a report that the expression of mutant apCAM lacking MAPK consensus sites or administration of specific MAPK inhibitors can block the 5-HT-stimulated endocytosis of apCAM adhesion molecules in *Aplysia* (Martin *et al.*, 1997). This process is required for synaptic remodelling. Also supporting a role for MAPK in memory is the finding that knockout mice lacking the neural GEF Ras-GRF were severely impaired in amygdala-mediated memory consolidation and also had deficits in plasticity in this brain region, as measured by long term potentiation (LTP) (Brambilla *et al.*, 1997). The muscarinic M1 and M2 mAChRs are known to activate Ras-GRF (Mattingly & Macara, 1996). While the loss of Ras-GRF did not affect hippocampal plasticity in the study by Brambilla *et al.* (1997), a selective MEK inhibitor blocked NMDA receptor-mediated LTP in rat (English & Sweatt, 1997).

i. D₂ Receptors and MAPK

As discussed in Section I.B.3.e, the D₂-like dopamine receptors modulate growth and differentiation in numerous cell lines. Direct evidence of MAPK activation by D₂ was first shown in COS-7 cells, with inhibition by Ga_i implying Gβγ-dependence (Faure *et al.*, 1994). In C6 glioma cells, rat D_{2L} was recently shown to produce a 9-fold increase in MAPK activity and a 3-fold increase in JNK activity (Luo *et al.*, 1998b). Both pathways were PTX-sensitive and dependent on Ras. JNK activation could be blocked specifically by a SEK1 (JNK kinase 1)

inhibitor, while ERK activation was dependent on MEK. Luo's work also found that ERK and JNK were both required for the mitogenic effect of dopamine. In CHO cells, human D_{2S} and D_{2L} were found to transiently activate ERK and p70 S6 kinase in a PTX-dependent manner (Welsh *et al.*, 1998). Activation was blocked 62% by the PI3-kinase inhibitor wortmannin. Although no direct evidence of D₃ and D₄ activating the MAPK pathway has been published, the fact that all D₂-like receptors are capable of altering the morphology of cultured neurons (Swarzenski *et al.*, 1994) and stimulating mitogenesis in CHO 10001 cells (Lajiness *et al.*, 1995) suggests that all three subtypes may couple to MAPK pathways. The specific effects on cell morphology were found to differ among dopamine receptor subtypes, however. D₂ receptor stimulation of mitogenesis was found to be more robust (9-fold increase in [³H]thymidine incorporation) than that of D₃ and D₄ (2.5-fold and 6-fold, respectively) (Lajiness *et al.*, 1995). Subtype-specific differences in G_i-coupled MAPK activation by α₂-AR in CHO cells have also been observed, suggesting that all D₂-like subtypes may not activate the MAPK pathway equally (Flordellis *et al.*, 1995).

D₂-like receptors also modulate postsynaptic gene expression in the CNS by various mechanisms (reviewed by Rogue & Malviya, 1994). Activation of immediate early genes (IEGs), such as c-fos, through dopaminergic mechanisms has also been reported. In the case of neuroleptics (ie. D₂ receptor antagonists), these drugs appear to increase Fos expression in striatum and nucleus accumbens (Robertson & Fibiger, 1992). This was recently shown to occur due to increased cAMP resulting from D₂-like receptor blockade (Adams *et al.*, 1997). In knockout mice lacking the RIIβ-subunit of PKA, haloperidol was unable to induce c-Fos and neurotensin expression. The mechanisms of IEG activation are probably complex, however, and, in 6-hydroxydopamine-lesioned rats, coadministration of D₁ and D₂ agonists stimulates c-fos mRNA in the striatum, indicating synergistic actions (Paul *et al.*, 1992). As demonstrated in *Aplysia*, PKA and MAPK may act in concert to mediate specific effects (Martin *et al.*, 1997).

D. Desensitization

1. Heterologous Desensitization

The response of G protein-coupled receptors to agonists usually diminishes rapidly. The mechanisms by which this occurs are important since they regulate intracellular signalling by GPCRs (for reviews, see Grady *et al.*, 1997; Böhm *et al.*, 1997a). After activation, agonists may

diffuse from the site of the receptor (eg. synapse), get degraded enzymatically, or be taken back up into the cell by specific transporters (Böhm *et al.*, 1997a). For example, dopamine can be inactivated by the enzymes COMT and MAO, or transported back into the presynaptic neuron by the dopamine transporter (DAT) (Kopin, 1994; Gainetdinov *et al.*, 1998). The importance of these mechanisms is underscored by the fact that cocaine acts to block reuptake by DAT, thereby increasing synaptic dopamine levels and prolonging its effect. In the presence of continuous agonist stimulation, however, receptor signalling is usually attenuated by desensitization processes within minutes, whereas long-term exposure (hours to days) is required for down-regulation (Böhm *et al.*, 1997a).

Heterologous desensitization of a receptor may occur due the activation of separate receptors by way of kinases such as PKA and PKC, which are activated by soluble second messengers. For example, PKA phosphorylation of the β_2 -AR is thought to mediate the rapid decrease in sensitivity of the adenylyl cyclase in response to low agonist concentration (Hausdorff *et al.*, 1989). Phosphorylation of two serine residues in the C-terminal tail of the α_{1B} -AR can be catalyzed by PKC and may be important in receptor desensitization (Diviani *et al.*, 1997). These covalent modifications possibly serve to reduce receptor-G protein coupling. Some evidence points to a role for dynamic palmitoylation at C-terminal cysteines in receptor desensitization as well. β_2 -AR and D₁ depalmitoylation occurs after agonist exposure and appears to affect G protein coupling, although no effect was observed in α_{2A} -AR mutants lacking the C-terminal cysteine (Morello & Bouvier, 1996). One theory is that palmitoylation may affect the accessibility of C-terminal cysteines to PKA in these receptors.

2. Homologous Desensitization

Homologous desensitization refers to mechanisms that act specifically on agonist-occupied receptors to reduce signalling. A group of serine/threonine kinases, the G protein receptor kinases (GRKs), have been identified which phosphorylate activated receptors (for reviews, see Ferguson *et al.*, 1996a; Pitcher *et al.*, 1998a). The GRKs include GRK1 (rhodopsin kinase), GRK2/3 (also called β ARK 1/2), GRK4, GRK5, and GRK6, with additional splice variants occurring (Böhm *et al.*, 1997a). Some GRKs (such as GRK2/3) are targeted to the membrane via a N-terminal PH domain-mediated interaction with free G $\beta\gamma$ and PtdIns(3,4)P₂, while others are farnesylated or palmitoylated (Lefkowitz, 1998). GRK phosphorylation has

been implicated in the desensitization of numerous receptors, including rhodopsin, β_1 -AR, α_{1B} -AR, and the M2 mAChRs, to name a few (Helmreich & Hofmann, 1996; Freedman *et al.*, 1995; Diviani *et al.*, 1997; Richardson *et al.*, 1993). The fact that these receptors couple to G_i , G_s , G_q , and $G_{i/o}$, respectively, while they can be phosphorylated by the same GRK (eg. β ARK1), highlights the fact that GRK phosphorylation is not mediated by second messengers. This is even more remarkable considering the structural differences between G_s - and $G_{i/o}$ -coupled receptors, with the latter often having a much larger 3rd cytoplasmic loop and a shorter C-terminal tail. In fact, with the β_2 -AR, serines and threonine residues targeted by GRKs are on the large carboxyl-terminal tail, while $G_{i/o}$ -coupled receptors, such as the M2 mAChR and the α_2 -AR, are phosphorylated on the i3 loop (Ferguson *et al.*, 1997; Pals-Rylaarsdam & Hosey, 1997). Unfortunately, well-defined consensus sequences have not been identified for GRKs (Grady *et al.*, 1997). Since GRKs prefer an agonist-occupied receptor substrate, these kinases tend to be active when high concentrations of agonist are present (Hausdorff *et al.*, 1989).

GRK phosphorylation is believed to facilitate the binding of another group of cytoplasmic proteins, called arrestins, to the intracellular domain of GPCRs. Analogous to the GRKs, several forms exist, including visual arrestin and β -arrestin-1/2 (Ferguson *et al.*, 1996a). Arrestins appear to bind to activated receptors and uncouple them from G proteins, as was shown with the β_1 -AR coupled to G_s (Freedman *et al.*, 1995). In this case, overexpression of either β ARK1/2, GRK5, rhodopsin kinase, or β -arrestin-1 or -2 reduced receptor-stimulated cAMP accumulation, indicating enhanced desensitization. While the arrestins apparently favour binding to activated, phosphorylated receptors (R^*P in Figure 2), experiments have demonstrated that β -arrestins can bind to unphosphorylated, inactive receptors, but with a lower affinity (Ferguson *et al.*, 1996a). Based on work with truncated and chimeric arrestins, Gurevich *et al.* (1995) proposed a model for arrestin binding in which a phosphoprotein recognition domain and an activation recognition domain in the arrestin both contribute to enhanced binding to GPCRs. When receptors are in the R^*P state, they can induce a conformational change in arrestin, possibly by exposing a hydrophobic domain in this protein, providing additional binding sites and increasing the binding affinity. This group recently extended their work by demonstrating that with the β_2 -AR and M2 mAChR, the receptor-arrestin complex has a higher affinity for agonists (10- to 100-fold), analogous to observations with receptor-heterotrimeric G proteins (Gurevich *et al.*, 1997). The similarity to receptor-G protein interactions has led to the hypothesis that the competition

between these two alternate ternary complexes, agonist-receptor-G α β γ and agonist-receptor-arrestin, may be the basis of homologous desensitization. While GTP promotes the dissociation of G α from the receptor and G β γ , GRK phosphorylation promotes the binding of arrestin and receptor uncoupling. Interestingly, recent evidence has shown that activated GPCRs can direct GRK2 phosphorylation of tubulin, the first non-receptor substrate identified for this enzyme (Pitcher *et al.*, 1998b). Thus, GRKs may have a role in cellular signalling as well as desensitization.

3. Receptor Sequestration

Receptor endocytosis is not believed to significantly contribute to desensitization. This is supported by the discovery that mutating a highly conserved tyrosine at the junction of the C-terminal tail and TM 7 of the β_2 -AR did not affect receptor function or rapid desensitization, but did inhibit sequestration (Barak *et al.*, 1994). However, the inability of this mutant to resensitize indicates that endocytosis may be required to restore coupling to G proteins.

Recently, important progress has been made in understanding the mechanism and function of GPCR endocytosis. Overexpression of β -arrestin was able to rescue β_2 -AR sequestration in receptor mutants that were unable to internalize (Ferguson *et al.*, 1996b). In addition, this communication indicated that mutant β -arrestins were able to block wildtype β_2 -AR sequestration. For some GPCRs, such as the β_2 -AR, sequestration was found to be dependent on dynamin, since dominant negative dynamin K44A blocked internalization (Zhang *et al.*, 1996a). However, this construct did not block internalization of the AT_{1A} receptor, indicating other pathways must exist. Yet these results suggest that β -arrestins (but not visual arrestins) can act as adapter proteins to mediate receptor endocytosis by clathrin-coated vesicles. A mechanism by which β -arrestins may target receptors to clathrin coated pits was provided by Goodman *et al.* (1996), who found that β -arrestin-1 and -2 bound with high affinity to clathrin itself, and mutants with reduced clathrin binding had a reduced ability to internalize receptors. In addition, this endocytic pathway involves a discrete group of clathrin coated pits, indicating specialization in the endocytic pathway (Cao *et al.*, 1998). GRK2 apparently internalizes with the β_2 -AR in this pathway, which may provide a mechanism to return the membrane-targeted kinase to the cytoplasm (Ruiz-Gómez & Mayor Jr., 1997). The presence of a GPCR-specific phosphatase in these acidic endosomal vesicles indicates that one function of internalization may be the dephos-

phorylation of desensitized receptors (Pitcher *et al.*, 1995).

A complex process of homologous desensitization has therefore emerged in the last ten years. GRKs catalyze the phosphorylation of activated GPCRs, promoting arrestin binding and uncoupling. β -arrestins also target GPCRs for sequestration by acting as adapter proteins, localizing receptors to clathrin-coated pits. Sequestration of desensitized GPCRs leads to dephosphorylation, making these resensitized receptors available for recycling back to the plasma membrane (Lefkowitz, 1998). The high expression of GRK2/3 and β -arrestins in the brain, as well as pre- and post-synaptic localization of the kinase, has led to the supposition that this system is important in desensitizing signalling by neurotransmitters such as the bioamines and peptide hormones (Böhm *et al.*, 1997a).

As mentioned in Section I.C.2.f, receptor internalization mediated by β -arrestins appears to be involved in MAPK signalling. This has raised the possibility that it may represent a signalling pathway as well as a desensitization/resensitization pathway (Lefkowitz, 1998). In this case, the agonist-occupied GPCR may act as part of a multiprotein complex which activates MEK after endocytosis.

4. Desensitization of D₂-like Receptors

Conflicting results have emerged from the study of D₂ receptor desensitization in various cell lines. *In vivo*, D₂ appears to control the growth of pituitary tumours without becoming desensitized (Ng & George, 1992). In SUP1 cells, which were derived from a rat pituitary tumour and endogenously express D₂ receptors, exposure to 100 μ M dopamine led to a significant increase in receptor density after 7 hours (Ivins *et al.*, 1991). However, in Y-79 retinoblastoma cells expressing an equivalent density of receptors (~ 50 fmol/mg protein), desensitization and decreased binding was observed due to pretreatment with dopamine and other agonists (Barton *et al.*, 1991). In this case, a 70% reduction in the ability of dopamine to inhibit forskolin-stimulated cAMP and a 30-fold increase in the IC₅₀ of dopamine were measured after preincubation for 24 hrs. with 500 μ M dopamine. The desensitization and reduction in binding were temporally distinct, as the t_{1/2} of these processes were 1 hr. and 4 hr., respectively.

Several groups have studied the response of recombinant D₂ receptors to pretreatment with drugs in various cell lines and have found that the receptor B_{max} is increased. In HEK 293 cells, NPA and quinpirole (both D₂ agonists) as well various antagonists led to a dose-dependent

increase in density, while dopamine had a lesser effect (Filtz *et al.*, 1993). In this same report, β_2 -AR receptor density was reduced by the adrenergic agonist isoproterenol, indicating that the response varies among GPCRs. Upregulation of transfected receptors in response to D₂ agonists and antagonists has been confirmed by others in HEK 293, C₆, and Sf9 cells (Boundy *et al.*, 1995; Starr *et al.*, 1995; Ng *et al.*, 1997). Starr *et al.* (1995) found a significantly greater increase with D_{2L} compared to D_{2S}, while D₁/D₂ chimeras with the D₂ i3 loop were also upregulated whereas wildtype D₁ was not. Another study in CHO cells found that exposure to 100 μ M dopamine for 24 hr. decreased D_{2S} binding 25% but increased D_{2L} binding two-fold (Zhang *et al.*, 1994).

Boundy and coworkers (1995) were able to observe desensitization of D₂ due to 5 μ M quinpirole, as measured by the loss of high-affinity agonist binding sites, with a 50% reduction after 15 min. In addition, Starr *et al.* (1995) found that exposure to 10 μ M NPA for 14 hr. significantly reduced the ability of D_{2L} to inhibit cAMP accumulation. While Zhang and coworkers (1994) showed that desensitization in CHO cells resulted in a 5-fold reduction in the IC₅₀ and a 25% drop in D₂-mediated cAMP inhibition, another group reported a much more dramatic 50-fold reduction in potency (ie. IC₅₀) but no change in efficacy for the same response using an Sf9 expression system (Ng *et al.*, 1997). Another study with CHO cells expressing D_{2S} found that the potency of dopamine for reducing forskolin-stimulated cAMP was reduced 4-fold, while the efficacy was unchanged, after preincubation with 1 μ M quinpirole for 1 hr. (Bates *et al.*, 1990). In addition, longer incubation times resulted in the sensitization of the adenylyl cyclase response to stimulation by forskolin, an observation that has been noted by other researchers (Ivins *et al.*, 1991; Boundy *et al.*, 1995).

With respect to sequestration of D₂, two recent studies have come to opposite conclusions. A comparison of whole-cell binding of a charged ligand ([³H]sulpiride) and an uncharged, hydrophobic ligand ([³H]spiperone) on D₂-expressing CHO cells preincubated with 10 μ M dopamine indicated that receptor sequestration takes place (Itokawa *et al.*, 1996). This was manifest in a 44% reduction in D_{2S} [³H]sulpiride binding ($t_{1/2}=19$ min.) and a 22% drop in D_{2L} [³H]sulpiride binding, with no change in the amount of bound [³H]spiperone. Since receptor binding was rapidly restored after agonist removal, it was concluded that D₂ was reversibly sequestered. However, these findings were contradicted by a study that observed increased receptor density in Sf9 cells after exposure to 10 μ M dopamine (Ng *et al.*, 1997). Using ligand

binding in heavy vs light membrane fractions as well as confocal fluorescence microscopy, it was demonstrated that D_{2L} receptors undergo a slow redistribution from intracellular vesicles to the plasma membrane when exposed to dopamine. The reason for these contradictory results are not obvious, although the use of different cell lines may be a factor. Another recent report with D_{2S} found that the high-affinity agonist pergolide induced rapid ($t_{1/2}=6.5$ min.) internalization of 61% of receptors in COS-7 cells (Barbier *et al.*, 1997).

While the results of numerous studies are not always in agreement, several themes can be identified. It is clear that dopamine D₂ receptor desensitization has important differences with that of the better-characterized β₂-AR. Many studies indicate that desensitization is weak or absent, while several show that receptor binding actually increases in response to agonist.

E. RESEARCH OBJECTIVES

1. General Objective

The collective goal of this thesis was to further our understanding of the biological chemistry of the D₄ receptor. In particular, the role of the third cytoplasmic loop in the function of the receptor was investigated. Previous studies in our lab failed to find a significant change in the pharmacology or functional coupling between D₄ receptors with two, four, and seven repeats, although minor differences were observed (Van Tol *et al.*, 1992; Asghari *et al.*, 1994). In fact, deletion of almost the entire repeat region (D4(Δ254-315)) did not impair the binding of ligands or block coupling to G_i (Asghari *et al.*, 1994; Asghari *et al.*, 1995). Thus, the significance of this polymorphism, if any, has remained unknown.

Recently, we demonstrated that the proline-rich third cytoplasmic loop of D₄, which includes the repeat region, can bind to the SH3 domain of the adapter proteins Grb2 and Nck *in vitro* (Oldenhoef *et al.*, 1998). Although the physiological relevance of this interaction has not been determined, the polymorphic repeat region may modulate the interaction. The D₄-Grb2 interaction is apparently mediated by the cooperative binding of two PXXP motifs. These are located amino-terminal and carboxyl-terminal to the polymorphic, SH3 binding domain-containing region of D₄, and interact with the two SH3 domains of Grb2. These results led us to speculate that other proteins may also interact with this region of D₄, and this question was explored using the yeast two-hybrid system.

2. FLAG-tagged Dopamine Receptors

Epitope-tagging has proven to be a useful technique that allows the immunoprecipitation, immunoblotting, and immunohistochemical analysis of G protein-coupled receptors (von Zastrow & Kobilka, 1992). To be useful, such epitope tags should be inert with respect to the structure and function of the receptor. This study first sought to characterize the pharmacology and functional coupling of a dopamine D₄ receptor with an amino-terminal signal sequence and a FLAG epitope-tag (FD4.4). The integrity of this receptor variant was characterized by radio-ligand binding and coupling to the inhibition of adenylyl cyclase in stably transfected CHO K1 cells.

3. Desensitization of the D₄ Receptor

In certain paradigms, the dopamine D₂ receptor appears to undergo desensitization in response to continuous agonist exposure (Section I.D.4). Both heterologous and homologous desensitization is mediated, at least in part, by receptor phosphorylation. Based on the lower number of candidate phosphorylation sites in the D₄ receptor, it was speculated that this receptor may respond differently than D₂. Using a characterized stable cell line expressing the FLAG-tagged D_{4.4} receptor, the ability to desensitize in response to agonist was measured.

4. HA-tagged Dopamine Receptors

In order to expand our ability to detect various dopamine receptors, D₂ and D₄ were produced with a signal sequence followed by an HA epitope. HA epitope-tagged D₄ receptor variants with two, four, and seven repeats (HAD4.2, HAD4.4, HAD4.7) as well as D_{2L} (HAD2L) were characterized pharmacologically and functionally.

5. Mutant Dopamine Receptors

To understand the role if the repeat region, epitope-tagged D_{4.4} mutants with 116, 94, and 61 amino acids deleted from the third cytoplasmic loop were characterized (HAD4(Δ221-337), HAD4(Δ221-315), HA(Δ254-315)). Also, a FLAG-tagged D_{4.4} with a point mutation near the i3/TM 6 junction, converting a methionine to an alanine, was described (FD4.4(M345A)). Mutations in this region of the α_{1B}-, α_{2C10}-, and β₂-adrenergic receptors result in constitutive signalling (Lefkowitz *et al.*, 1993; Ren *et al.*, 1993). In addition, it has been speculated that this

residue may undergo possible post-translational modification by adenosylation via methionine adenosyltransferase (Kramer *et al.*, 1996).

6. Coupling of Dopamine D₄ Receptors to MAPK

Recent research has indicated that many G protein-coupled receptors, including D₂, can activate MAPK pathways (Section I.C). Our observation that the third cytoplasmic loop of D₄ can bind Grb2, an important SH3/SW2 adapter protein involved in mitogenic signalling, raised the possibility that these interactions may contribute to the a receptor/adapter/Ras/Raf complex that has been hypothesized to undergo endocytosis and activate MEK (Lefkowitz, 1998). Therefore we determined whether the D₄ receptor could activate the ERK1/2 in CHO K1 cells stably expressing HA-tagged dopamine receptors. Deletion mutants of D₄ lacking the putative SH3 binding domains in the third cytoplasmic loop were tested to indicate whether interactions with Grb2, Nck, or other adapter proteins are important in the activation of the MAPK pathway.

II. MATERIALS

A. GENERAL CHEMICALS

Ammonium acetate, ammonium chloride, ascorbic acid, boric acid, magnesium sulfate, methanol, potassium acetate, potassium chloride, sodium chloride, sodium hydroxide, and monobasic sodium phosphate were purchased from B.D.H. Inc. (Toronto, ON). Bovine serum albumin, DL-dithiothreitol (DTT), disodium ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), IGEPAL CA-630 (NP-40), lithium acetate, magnesium chloride, magnesium sulfate, 3-[N-morpholino]propanesulfonic acid (MOPS), potassium 2-[N-morpholino]ethanesulfonic acid (KMES), polyethylene glycol (PEG) 3,350, polyoxyethylenesorbitan monolaurate (Tween-20), potassium ferrocyanide, rubidium chloride, sodium azide, succinic anhydride, N,N,N,N-tetramethyl-ethylenediamine (TEMED), triethylamine, Triton X-100, and zinc chloride were purchased from Sigma Chemical Co. (St. Louis, MO).

Acrylamide was obtained from Sangon Ltd. (Toronto, ON). Ammonium persulfate (APS), dimethylsulfoxide (DMSO), and hydrochloric acid were purchased from Caledon Laboratories (Georgetown, ON). bis-Acrylamide and urea were acquired from Biobasic Inc. (Toronto, ON). Calcium chloride, chloroform, glycerol, D-glucose, isopropyl alcohol, dibasic potassium phosphate, and glacial acetic acid were purchased from ACP Chemicals Inc. (Montreal, PQ). Commercial alcohols (Brampton, ON) supplied 95% and anhydrous ethanol. Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were purchased from Schwarz/Mann Biotechnology (ICN) (Aurora, IL). Acetone, isoamyl alcohol (3-methyl-1-butanol), and molecular sieves (3 Å) were obtained from Aldrich Chemical Co. (Milwaukee, WI). N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Gibco BRL (Grand Island, NY). Dibasic sodium phosphate was obtained from J.T. Baker (Phillipsburg, NJ) and monobasic potassium phosphate was purchased from Mallinckrodt (Mississauga, ON).

B. BACTERIAL AND TISSUE CULTURE

Bacto agar, bacto peptone, yeast extract, and yeast nitrogen base (YNB) were obtained from Difco Laboratories (Detroit, MI). All amino acids, adenine hemisulfate, uracil, ampicillin, chloramphenicol, tetracycline, and thiamine were purchased from Sigma Chemical Co. (St.

Louis, MO).

α -Minimal essential media (α -MEM) was purchased from Central Media Preparation Service (U of T, Toronto, ON). Fetal bovine serum (FBS), horse serum (HS), trypsin (2.5%), and Geneticin (G418 sulfate) were bought from Gibco BRL (Grand Island, NY).

Bacterial plates were supplied by Fisher Scientific (Fair Lawn, NJ) and tissue culture plates were purchased from Corning Glass Works (Corning, NY) or Sarstedt Inc. (Newton, NC).

C. MOLECULAR BIOLOGY

Sea Kem (high-melt) agarose and SeaPlaque (low-melt) agarose were purchased from FMC Bioproducts (Rockland, ME). Bromophenol blue, ficoll, xylene cyanol FF, and sodium deoxyribonucleic acid (DNA) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethidium bromide was acquired from Molecular Probes (Eugene, OR). Disodium adenosine-5'-triphosphate (ATP) and RNase A were the product of Boehringer Mannheim (Laval, PQ).

All restriction endonucleases and calf intestinal alkaline phosphatase (CIP) were obtained from New England BioLabs (NEB) (Beverly, MA). T4 DNA Ligase, T4 polynucleotide kinase, 1 kb molecular weight markers, and the Sp6 oligonucleotide (5'-dATTTAGGTGACACTATAG-3') were from Gibco BRL (Grand Island, NY). T7 Sequenase v2.0 was purchased from Amersham Life Science (Oakville, ON). 7-Deaza-dGTP sequencing kits were obtained from United States Biochemical (Cleveland, OH). Pfu polymerase and sequencing oligonucleotides T3 (5'-dATTAACCCTCACTAAAG-3') and T7 (5'-dAATACGACTCACTATAG-3') were from Stratagene (La Jolla, CA). Taq polymerase was obtained from Perkin Elmer (Norwalk, CN). Plasmid maxiprep and spin miniprep kits were purchased from QIAGEN Inc. (Santa Clara, CA).

D. VECTORS AND STRAINS

The eukaryotic expression vector pRC/RSV was purchased from Invitrogen (San Diego, CA). The plasmid pBluescript SK is from Stratagene (La Jolla, CA). The plasmid pBSSF β_2 was obtained as a gift from Dr. B. Kobilka (Stanford University, Stanford, CA). The eukaryotic expression vector pcDNA3 (Invitrogen) expressing an epitope-tagged D4 receptor was the gift of Dr. M. von Zastrow (University of California at San Francisco).

E. coli strain XL-1 was acquired from Stratagene (La Jolla, CA). Chinese hamster ovary cell line K1 (CHO K1) was purchased from the American Type Tissue Culture Collection

(Rockville, MD).

E. DRUGS, INHIBITORS AND RADIOCHEMICALS

Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), 3-amino-1,2,4-triazole (3-AT), dopamine, forskolin, tunicamycin, and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Isobutyl-1-methyl xanthine (IBMX) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Phorbol-12-myristate 13-acetate (PMA), clozapine, tetralithium 5'-guanylyl-imidodiphosphate (Gpp[NH]p), (+)-butaclamol, haloperidol, S(-)-raclopride L-tartrate, and (-)-quinpirole HCl were purchased from RBI (Natick, MA). Nemon-apride (YM09151-2) was purchased from Yamanouchi Pharmaceutical Company (Tokyo, Japan).

[N-methyl-³H]YM09151-2 (85.5 Ci/mmol), [α^{35} S]dATP (1500 Ci/mmol), and adenosine 3',5'-cyclic phosphoric acid, 2'-O-succinyl [¹²⁵I]iodotyrosine methyl ester ([¹²⁵I] cAMP) (3300 Ci/mmol), and [³⁵S]GTP γ S were bought from Dupont NEN Life Science Products (Mississauga, ON). [³H]Spiperone (98 Ci/mmol) was the product of Amersham Life Science (Oakville, ON).

F. PROTEIN ANALYSIS AND IMMUNOCHEMISTRY

Bicotinic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, IL). Prestained molecular weight standards were purchased from BioRad (Hercules, CA) and Helixx Technologies Inc. (Scarborough, ON). Anti-FLAG M1 monoclonal antibody was produced by Eastman Kodak (New Haven, CN). Anti-HA monoclonal antibody (mouse IgG_{2b}, clone 12CA5), anti-HA monoclonal antibody (rat IgG₁, clone 3F10), anti-rat IgG-biotin (sheep IgG F(ab')₂ fragments), and streptavidin-horseradish peroxidase (HRP) were purchased from Boehringer Mannheim (Laval, PQ). Anti-HA hybridoma supernatant was the generous gift of Dr. M. Anafi (University of Toronto). Rabbit anti-mouse IgG-HRP, goat anti-mouse-agarose, and anti-adenosine 3':5'-cyclic monophosphate (anti-cAMP, rabbit whole serum) were bought from Sigma Chemical Co. (St. Louis, MO). Protein A-sepharose CL-4B was from Pharmacia Biotech AB (Uppsala, Sweden). Protein G-agarose was obtained from Gibco-BRL (Grand Island, NY). Tris-glycine gels and polyvinylidene difluorine (PVDF) membranes were bought from Novex Experimental Technologies (San Diego, CA). The ECL and ECL+plus western blot detection kit were from Amersham Life Science (Oakville, ON). New England BioLabs (Beverly, MA) were the suppliers of rabbit polyclonal anti-p44/p42 MAPK antibody, rabbit polyclonal anti-phospho-

MAPK antibody, monoclonal mouse anti-phosphoMAPK antibody (clone E10), and anti-rabbit IgG-HRP. cAMP standard was purchased from Dupont (Wilmington, NC).

G. OTHER LAB SUPPLIES

The water used in all solutions was purified by passage through a Milli-Q UFplus filter cartridge (Millipore Corp., Bedford, MA) and had a resistivity > 18.2 M Ω·cm. Disposable plasticware (Falcon tubes, syringes) was purchased from Becton Dickinson Labware (Lincoln Park, NJ). Milli-GP Filters (0.2 µm) were from Millipore Corp. (Bedford, MA). Flask filters (115 mL, 0.2 µm) were obtained from Nalge Co. (Rochester, NY). All other plasticware (pipette tips, tubes) were purchased from DiaMed Lab Supplies Inc. (Mississauga, ON). The LS6000SC scintillation counter, Gamma 5500B gamma counter and Ready Safe liquid scintillation cocktail was the product of Beckman Instruments Inc. (Fullerton, CA). Glass-fibre filters and the combicell harvester were purchased from Skatron Instruments Inc. (Sterling, VA). BioMax MR film was bought from Eastman Kodak (Rochester, NY) and Polaroid 667 film was from Polaroid Ltd. (St. Albans, UK).

III. METHODS

A. MOLECULAR BIOLOGY

1. Preparation of Competent *E. coli* Bacteria

Competent XL-1 *E. coli* were produced by two methods. A single colony was picked from a Luria-Bertani (LB) (1% w/v yeast extract, 0.5% w/v bacto-tryptone and 1% w/v NaCl) (1.2% w/v agar) which contained 10 µg/mL tetracycline. The colony was suspended in 4 mL LB media with 10 µg/mL tetracycline and grown overnight at 37°C in a shaking incubator (300 rpm). The overnight culture was subsequently transferred to 76 mL LB and grown another 1 to 2 hours. After chilling on ice for 10 minutes, the bacteria were centrifuged at 4500 x g for 5 minutes at 4°C and the supernatant was resuspended in 20 mL KMES buffer (20 mM KMES, 60 mM CaCl₂, 5 mM MgCl₂, 5 mM MnCl₂, pH 5.8). After incubating on ice for 1.5 hours, bacteria were centrifuged (4500 x g for 5 min. at 4°C) and resuspended in 4 mL KMES buffer. Competent bacteria prepared in this manner were stored up to two weeks at 4°C before being discarded.

A second method was used to produce frozen competent XL-1. A single colony was inoculated into 5 mL LB with 10 µg/mL tetracycline and grown overnight at 37°C and 225 rpm. The overnight culture was transferred into 500 mL LB + 20 mM MgSO₄. The culture was grown at 37°C/225 rpm until the OD₆₀₀=0.4 to 0.6 (3 to 6 hr.). Bacteria were pelleted (4500 x g for 5 min. at 4°C) and cells were gently resuspended in ice-cold, filter-sterilized buffer TFB1 (30 mM potassium acetate, 10 mM CaCl₂, 50 mM MnCl₂, 100 mM RbCl, 15% glycerol, adjusted to pH 5.8 with 1 M acetic acid). Cells were incubated on ice for 5 minutes and centrifuged (4500 x g for 5 min. at 4°C). Bacteria were gently resuspended in ice-cold, filter-sterilized buffer TFB2 (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15% glycerol, adjusted to pH 6.5 with 1 M KOH). After incubating on ice for 15 to 60 minutes, 100 µL aliquots were frozen in a dry-ice/ethanol bath and stored at -80°C for 1 year.

2. Transformation of Competent *E. coli*

To transform XL-1 bacteria, 100 µL of competent bacteria (prepared by either of methods described above) were thawed on ice, if necessary, and mixed with < 1 µg of plasmid DNA in an eppendorf tube. Cells were allowed to incubate on ice for 45 minutes, after which the bacteria were heat shocked for 2 minutes at 42°C and, finally, placed on ice for 2 minutes. Transformed bacteria were plated on LB agar + 50 µg/mL ampicillin and incubated overnight at 37°C.

3. Preparation of Plasmid DNA

To prepare small quantities (miniprep) of plasmid DNA (~20 µg), 4 mL of LB media containing 50 µg/mL ampicillin was inoculated with a single transformed bacterial colony. Cultures were grown overnight at 37°C with shaking at 300 rpm.

a. Rapid Minipreparation of DNA

Plasmid DNA was prepared by two methods. For most applications, DNA was prepared by a rapid alkaline lysis method (Zhou *et al.*, 1990a). An eppendorf tube with 1.5 mL of overnight culture was centrifuged at 14,000 rpm for 10 seconds. After decanting the supernatant, bacteria were resuspended in the residual media by vortexing, followed by addition of 300 µL of TENS (TE buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA) with 0.1 M NaOH and 0.5% SDS) and vortexing for 3 seconds. Next, 150 µL of 3 M sodium acetate (pH 5.2) was added and lysates were briefly mixed and cleared by spinning at 14,000 rpm for 5 minutes. The supernatant was transferred to a new eppendorf tube and plasmid DNA was precipitated by the addition of 0.9 mL ice-cold 95% ethanol. The DNA was allowed to precipitate for 15 minutes at -70°C, followed by centrifugation at 14,000 rpm for 5 minutes. After washing the DNA pellet with 1 mL of cold 70% ethanol and centrifuging (14,000 rpm for 5 min.), samples were allowed to dry for approximately 10 minutes. DNA was resuspended in TE buffer with 50 µg/mL RNase A.

b. QIAGEN Minipreparation of DNA

For applications requiring high purity plasmid DNA (eg. sequencing, preparation of plasmid DNA from *E. coli* strain HB101), the QIAGEN spin miniprep protocol was employed. Between 1.5 and 3 mL of overnight bacterial culture was transferred to an eppendorf, microfuged for 10 seconds, and resuspended in 250 µL resuspension buffer P1 (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/mL RNase A). After adding 250 µL lysis buffer P2 (200 mM NaOH, 1% SDS), bacteria were gently mixed by inversion. Subsequently, 350 µL neutralization buffer N3 was added and mixed gently, followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was applied to a QIAprep spin column and microfuged for 1 minute. With *E. coli* strain HB101, the column was washed with 0.5 mL buffer PB in order to remove nuclease activity. After washing the column with 0.75 mL buffer PE, plasmid DNA was eluted with 50

μ L 10 mM Tris-HCl, pH 8.5. The composition of buffers N3, PB and PE are the proprietary knowledge of QIAGEN.

c. Large-scale DNA Preparation

For larger quantities of plasmid DNA (maxiprep), a single colony was grown for 8 hours in 4 mL LB media + 50 μ g/mL ampicillin at 37°C/300 rpm. The initial culture was diluted 1:500 into 250-500 mL LB + 50 μ g/mL and grown overnight at 37°C/300 rpm. The bacteria were harvested (6000 \times g for 15 min.) and resuspended in 10 mL buffer P1. After adding 10 mL lysis buffer P2 and mixing, bacteria were incubated for 5 minutes at room temperature. Precipitation was carried out with 10 mL buffer P3 (3 M potassium acetate, pH 5.5) for 20 minutes on ice, samples were centrifuged (34,000 \times g) for 30 minutes, after which the supernatants were cleared by a second centrifugation for 15 minutes.

The resulting supernatant was applied to a QIAGENtip 500 column that had been previously conditioned with 10 mL equilibration buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100). The retained plasmid DNA was washed with 60 mL buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol) and finally eluted with 15 mL buffer QF (1.25 M NaCl, 50 mM MOPS, pH 8.5, 15% isopropanol). The DNA was precipitated by addition of 10.5 mL isopropanol and pelleted by centrifugation (35,000 \times g for 30 min.). Pellets were washed with 70% ethanol, centrifuged (35,000 \times g for 10 min.), air-dried briefly, and finally resuspended in 1.0 mL TE buffer.

The yield of DNA was quantified by measuring the UV absorbance at 260 nm of a 1:100 dilution of plasmid DNA ($\epsilon \approx 0.02$ OD \cdot cm 2 / μ g for double-stranded DNA). The ratio of A260/A280 was used to assess DNA quality with a ratio of 2:1 indicating acceptable DNA purity.

4. Restriction Enzyme Digests

For analytical or subcloning purposes, plasmid DNA was digested with various restriction endonucleases. Typically, ~1 μ g of DNA was mixed with 2 μ L of the appropriate NEB buffer (10x), 2 μ g BSA, and 1 to 5 U enzyme in a final volume of 20 μ L. Reactions were carried out for at least 1 hour at 37°C in the case of a full digest.

To visualize restriction digests, samples were mixed with 4 μ L loading dye (0.25%

bromophenol blue, 0.25% xylene cyanol FF, and 30% Ficoll). Samples and a 1 kb DNA ladder were run in an agarose gel (1% agarose in TAE buffer (40 mM Tris, 20 mM glacial acetic acid, and 1 mM EDTA) with 10 mg/mL ethidium bromide) submerged in TAE buffer, and DNA fragments were separated using 75 to 125 V for 0.5 to 1 hour. After electrophoresis, the DNA fragments were visualized using UV light.

5. Subcloning Techniques

Manipulation of plasmid or receptor DNA involved the restriction digest of a vector to remove either the entire coding sequence or a portion of the receptor, filling-in of 5' overhanging ends or digestion of 3' overhanging ends (blunting) if required, dephosphorylation of the vector, electrophoresis, and ligation of the receptor fragment into the vector (Sambrook *et al.*, 1989).

Blunting may be required to allow ligation at sites cut with incompatible enzymes. After heat inactivation of restriction enzymes (typically 70°C for 20 min.) followed by reannealing (37°C for 15 min.), 3'-overhangs were removed by addition of the Klenow fragment of DNA polymerase I (~1 U/μg DNA) and dilution to 50 μL with any NEB Buffer (1x), followed by incubation at 37°C for 30 minutes. To fill-in 5'-overhangs, Klenow (~1 U/μg DNA) and 33 μM of each dNTP were added and diluted in any NEB Buffer to a final volume of 50 μL after restriction enzyme inactivation. Fill-in reactions were carried out at 25°C for 15 minutes. Both 3'→5' exonuclease and 5'→3' polymerase reactions were terminated by addition of EDTA to 10 mM and heat inactivation (75°C for 10 min.) followed by reannealing of the DNA.

In order to reduce vector recircularization during subcloning, dephosphorylation of the 5' ends was carried out. After heat inactivation of restriction enzymes/Klenow, vector DNA was incubated with 0.5 μL (5 U) calf intestinal phosphatase (CIP) in 50 μL containing any NEB Buffer. After incubation for 60 minutes at 37°C, the reaction was terminated by addition of EDTA to 5 mM, followed by heat inactivation (75°C for 10 min.) and reannealing of the DNA. After digestion with CIP, DNA was extracted using phenol:CHCl₃:isoamyl alcohol (25:24:1). The aqueous phase was retained and DNA was precipitated by addition of 0.3 M sodium acetate, pH 5.2, and cold 70% ethanol, washed with 70% ethanol, dried, and resuspended in TE buffer.

Separation of DNA fragments was carried out by agarose gel electrophoresis (0.8% low-melt agarose in TAE buffer). Desired bands were cut from the gel and retained. Ligations were carried out “in gel” by mixing 10 μL 5X Ligase Buffer, 0.5 μL (5 U) T4 DNA ligase in 25 μL

H_2O . DNA-containing agarose was melted (80°C for 5 min.) and ~19 μL insert and 0.5 μL vector (approximately 4:1 molar ratio insert:vector) were added to the ligase mixture in a final volume of 50 μL . Ligations were carried out overnight at 16°C . Subsequently, 10 μL of the mixture was mixed with 100 μL competent XL-1 and transformed as described in Section III.A.2.

6. Sequencing

To sequence dsDNA, 2 - 4 μg of plasmid (prepared by either the QIAGEN maxiprep or miniprep methods) was denatured in 20 μL of 0.2 M NaOH and 0.2 mM EDTA at 37°C for 30 minutes. Denatured DNA was placed on ice and precipitated with 0.3 M sodium acetate (pH 5.2) and 70 μL of cold 95% ethanol. After precipitation (-70°C for 15 min.), DNA was pelleted (14,000 rpm for 15 min.), washed with cold 70% ethanol, and air dried.

DNA was resuspended in 4 - 8 μL primer solution (~10 ng/ μL), 4 - 0 μL water, and 2 μL 5x sequenase reaction buffer. If sequence close to the primer was needed, 1 μL of Mn Buffer (0.1 M MnCl_2 , 0.15 M isocitrate) was also added. Primers were annealed at 37°C for 30 min., followed by addition of 1 μL 0.1 M DTT, 2 μL 7-deaza dGTP labelling mix (1.9 μM 7-deaza GTP, 1.9 M dCTP, 1.9 μM dTTP), 1 μL [α - ^{35}S]dATP, and 2 μL Sequenase v 2.0 (3.25 U/mL). Extension reactions were carried out for 5 minutes at 37°C .

After incubation, 3.5 μL of the reaction solution was aliquoted into four tubes containing 2.5 μL of the termination mixes of ddATP, ddTTP, ddCTP, and ddGTP (each containing 80 μM 7-deaza-dGTP, 80 μM dATP, 80 μM dTTP, 80 M dCTP, 50 mM NaCl, and 8 μM ddNTP). Termination reactions were carried out at 37°C for 5 minutes followed by addition of 4 μL stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF).

Sequences were separated on a 6% acrylamide sequencing gel (6% acrylamide, 17.5 mM N,N'-methylene-bis-acrylamide, 6.82 M urea, 0.054% APS, 0.02% TEMED in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA)) which was pre-warmed for 1 hr. at 50 W. After denaturation (80°C for 2 min.), samples were placed on ice and 2 - 4 μL of each of termination reaction (G/A/T/C) was loaded in adjacent lanes. After running for 3 to 5 hr. at 50 W, the gel was soaked in a solution of 12% methanol and 10% acetic acid for 1 hr. The gel was transferred to Whatman No.1 filter paper and dried at 80°C using a gel drier (Bio-Rad). Dried gels were exposed film BioMax MR film overnight prior to developing.

7. Cloning of Epitope-tagged Dopamine Receptors

a. FLAG Epitope-tagged D_{4,4} Receptor (FD4.4)

The dopamine D4 receptor with four 16-amino acid repeats (D_{4,4}) was modified by the addition of an amino-terminal tag consisting of a cleavable signal sequence (MKTIIIALSYIFC-LVFA) followed by an epitope recognized by the M1 “FLAG” antibody (DYKDDDDA). To construct the modified dopamine D₄ receptor, the plasmid pBSSF β ₂, encoding a modified beta adrenergic receptor, was used (Guan *et al.*, 1992). The entire D_{4,4} coding sequence was excised from the vector pBD4.4 (Asghari *et al.*, 1994) by a partial *NcoI/XbaI* digest and ligated into the *NcoI/XbaI* site of pBSSF β ₂. The resulting plasmid, pBSSFD4.4, was subsequently digested with *HindIII/XbaI* to excise the a 1.6 kb fragment, which was cloned into the mammalian expression vector pRC/RSV to yield RSV SSFD4.4. The resulting construct was verified by sequence analysis.

b. FLAG Epitope-tagged D_{4,4} Receptor Mutant (FD4.4(M345A))

A point mutant was made at methionine 345 of the dopamine D_{4,4} receptor by Eline Korenromp using the QuickChange™ mutagenesis method (Stratagene, La Jolla, CA). Briefly, a *SmaI/HincII* fragment of the D_{4,4} cDNA was subcloned into pBluescript. To generate the point mutation, PCR was performed with the complementary oligonucleotides 5'-G CGC AAG GCC GCG AGG GTC CTG CCG-3' and 5'-CGG CAG GAC CCT CGC GGC CTT GCG C-3'. PCR cycling was carried out in a 10 μ L volume with 5 μ g BSA, 50 ng template DNA, 60 ng of each primer, 200 μ mol of each dNTP, and a mixture of 1.8 U Taq polymerase and 0.2 U pfu polymerase in Taq polymerase buffer. Prior to cycling, samples were denatured at 94°C for 60 seconds. After 25 PCR cycles (10 s at 94°C, 20 s at 65°C and 3 min. at 72°C), the samples were digested with *DpnI*, which is specific for the methylated template DNA.

The samples were separated by 0.8% low-melt agarose gel electrophoresis and a 3.4 kb product isolated. The DNA was 5'-phosphorylated with T4 polynucleotide kinase (5 U with 300 μ M ATP in T4 kinase buffer at 37°C for 30 min.) and ligated using 2 U DNA ligase for 3 hr. at 16°C. The ligation product was transformed into *E. coli* XL-1. Restriction enzyme digests and sequencing was performed to confirm that only the desired mutation was present. A *SmaI/HincII* fragment of the pBM345A construct was subcloned into the RSV SSFD4.4 to replace the wildtype sequence, generating RSV SSFM345A.

c. HA Epitope-tagged Dopamine D₄ and D_{2L} Receptors

Epitope-tagged dopamine D_{2L} and D₄ receptors were cloned by R. Vickery (University of California at San Francisco) in the following manner (Chu *et al.*, 1997). D_{2L} was excised from pcDNAI by a blunt *Nco*I/*Xho*I digest. The plasmid pBCSSHA δ , containing the δ -opioid receptor with an amino-terminal, cleavable signal sequence (MKTIIIALSYIFCLVFA) and the HA (hemagglutinin) epitope (DYPYDVPDYA), was digested with *Bam*HI to excise the opioid receptor. After blunting the vector and a *Xhol* digest, the D₂ sequence was ligated into the vector to create pBCSSHAD2L. Finally, the modified D_{2L} receptor was removed from pBC with a *Hind*III/*Xhol* digest and subcloned into pcDNA3 to produce pcSSHAD2L.

Using a partial *Nco*I/*Bam*HI digest, the dopamine D_{4,7} receptor was cut from pBD4.7 (Asghari *et al.*, 1994). The vector pBCSSHA δ was also digested with *Nco*I/*Bam*HI and the D_{4,7} fragment was ligated into the vector to create pBCSSHAD4.7. Finally, the tagged D₄ receptor was cut as a 1.8 kb *Hind*III/*Bam*HI fragment and subcloned into the multiple cloning site of pcDNA3 at *Hind*III/*Bam*HI. Epitope-tagged dopamine D_{4,4} receptor deletion mutants were subcloned by a similar strategy using pBD4(Δ 221-337) (*Eco*47III-*Dpn*1 deletion), pBD4(Δ 221-315) (*Eco*47III-*Tth*III1 deletion) and pBD4(Δ 254-315) (*Eco*NI-*Tth*III1 deletion) which have been described previously (Oldenhof *et al.*, 1998; Asghari *et al.*, 1994). The epitope-tagged deletion mutants produced 1.0, 1.1 kb and 1.3 kb *Nco*I/*Bam*HI fragments, which were subcloned further to yield pcSSHAD4(Δ 221-337), pcSSHAD4(Δ 221-315), and pcSSHAD4(Δ 254-315).

Cloning of additional dopamine D₄ variants and a pcDNA3 control were accomplished in our lab. A partial *Not*I/*Xba*I digest of the D_{4,2} and D_{4,4} receptors in pBluescript resulted in 1.2 kb and 1.3 kb fragments containing the repeat region of the third cytoplasmic loop. The vector pcSSHAD4.7 was completely digested with *Not*I/*Xba*I and the large vector fragment was ligated with the D_{4,2} or D_{4,4} fragments to produce pcSSHAD4.2 and pcSSHAD4.4. As a negative control, pcSSHAD4.7 was digested with *Hind*III/*Xba*I, blunted, and religated to produce pcDNA3-. All HA-tagged D₄ constructs were verified by restriction digests and sequencing of the 5' and 3' portions of the receptor gene.

B. TISSUE CULTURE

1. Growth and Maintenance of Mammalian Cells

Chinese hamster ovary (CHO) K1 cells were grown in supplemented α -MEM media

(2.5% FBS and 2.5% HS) as monolayers at 37°C in a humidified, 5% CO₂ atmosphere. Cells were subcultured by washing with 5 - 10 mL PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4), followed by dissociation using PBS containing 0.25% trypsin and 1 mM EDTA for 2 minutes. After removing the trypsin and stopping the reaction with media, cells were replated at 10-20% confluence. Freezer stocks of CHO K1 cells were prepared by resuspending cells in media + 10% DMSO and stored at -70°C. For long-term storage, cells were transferred to liquid N₂. Cells were collected for radioligand binding or Western blotting by scraping cells in PBS. Cells were pelleted (250 x g for 10 min. at 4°C) and frozen at -70°C.

To study the glycosylation of receptors, stable CHO cells were plated at either 1.5 x 10⁵ cells/mL (untreated) or 2.5 x 10⁵ cells/mL (treated) and grown overnight. The next day, cells were treated with 1 µg/mL tunicamycin. After incubating for 20 hr., the cells were collected and immunoprecipitation/western blotting was immediately carried out (Section III.E.2).

2. Stable cell lines

CHO K1 cell lines expressing receptor were produced by electroporation. A confluent 100 mm plate of wildtype CHO K1 was collected by treatment with trypsin and washed with 10 mL PBS. Cells were resuspended in 400 µL PBS (~2.5 x 10⁷ cells/mL) containing 20 - 40 µg plasmid DNA and incubated on ice for 5 minutes. After transferring to a chilled electroporation cuvette (2 mm gap), the cells were electroporated (150 V, 2280 µF, 48 Ω) using a BTX Electro Cell Manipulator 600 from Biotechnologies & Experimental Research Inc. (San Diego, CA). After incubating on ice for 10 minutes, cells were resuspended in 40 mL media, plated on 4 x 100 mm plates (1:1 to 1:10 dilution), and grown overnight. To select clonal CHO K1 lines expressing receptor, the media was replaced the following day with supplemented α-MEM containing 500 - 750 µg/mL G418. After approximately two weeks, surviving colonies were washed with PBS and fresh media was added. Clones were picked by scraping cells from surviving colonies and collecting in 50 µL media. Each clone was grown in media containing 500 µg/mL G418 to produce freezer stocks and tissue for radioligand binding analysis.

C. RECEPTOR PHARMACOLOGY

1. Radioligand Binding

Receptor expression was characterized by using saturation binding and drug competition

experiments. To carry out binding, cells were thawed in a 37°C water bath and resuspended in 5-15 mL Binding Buffer (120 mM NaCl, 50 mM Tris-HCl, 5 mM KCl, 5 mM MgCl₂, 1.5 mM CaCl₂, 0.5 mM EDTA, pH 7.4). Cells were mechanically disrupted by polytron (Pro Scientific Inc., Monroe, CT) for 15 seconds at maximum speed on ice. Cell membranes were pelleted by centrifugation (34,000 x g for 20 min. at 4°C) and resuspended by polytron (5 seconds at maximum speed, on ice) at 50 -200 µg/mL in Binding Buffer. Binding assays were carried out by adding 0.25 mL of membrane preparations to test tubes containing 0.25 mL [³H]spiperone (4 x final concentration in Binding Buffer) and 0.5 mL Binding Buffer containing unlabelled drugs (2x final concentration) if necessary. All binding conditions were assayed in duplicate. After vortexing briefly, tubes were incubated for 2 hours at room temperature. Membranes were harvested onto glass-fibre filters using a combicell harvester (Skatron Instruments Inc., Sterling, VA) and washed with 50 mM Tris-HCl, pH 7.5 (2 x 9 seconds). Filters were transferred to vials with 5 mL scintillation cocktail and incubated overnight. The next day, tubes were mixed by inversion and counted.

2. Saturation Binding

To identify clonal cell lines expressing dopamine receptors, membranes were incubated with 0.1, 0.5, and 1 nM [³H]spiperone. Non-specific binding was determined by the inclusion of 1 µM haloperidol. Once positive clones were identified, 12-point saturation binding plots were carried out with 0.01 to 1 nM [³H]spiperone. The receptor k_d (nM) and B_{max} (DPM) were determined by non-linear curve fitting using GraphPad Prism v2.0 (GraphPad Software Inc., San Diego, CA). Saturation binding data were fit to the equation $Y = (B_{max} \cdot (X - Y)) / (K_d + (X - Y)) + (X - Y) \cdot NS$, where X is the total amount of ligand (DPM), Y is the total binding (DPM), and NS is the non-specific binding constant. The non-specific binding constant was determined experimentally by co-incubating with 1 µM haloperidol or 10 µM (+)-butaclamol and fitting the data to the equation $Y = X \cdot (NS / (NS + 1))$. To calculate the B_{max} (fmol receptor/mg membrane protein), protein concentrations were determined using the BCA assay (Section III.E.3).

3. Drug Competition

The rank-order of drug binding was determined using ~500 pM [³H]nemonapride or ~300 pM [³H]spiperone incubated with receptor membranes and varying concentrations of cold drug.

Drug competition curves were plotted by fitting the data to a one-site competition curve ($Y = \text{Min} + (\text{Max} - \text{Min})/(1 + 10^{X-\log EC50})$), where X is the logarithm of the concentration, EC50 is the concentration of drug that blocks 50% of the radioligand binding, and Y is the response, using GraphPad Prism v2.0. The drug K_i values were determined using the Cheng-Prusoff equation ($K_{i, \text{drug}} = EC50/(1 + [\text{ligand}]/K_{d, \text{radioligand}})$) (Cheng & Prusoff, 1973).

4. Dopamine Binding

Measurement of the shift in receptor affinity for dopamine due to the presence of Gpp[NH]p was carried out using Binding Buffer without NaCl. After cells were disrupted and centrifuged as described in Section III.C.1, membranes were resuspended in 1 mL (0.3 - 1.5 mg/mL) in Binding Buffer (- NaCl). Membrane preparation (0.5 mL) was mixed with 0.5 mL Binding Buffer (- NaCl) \pm 400 μM Gpp[NH]p. After incubating at room temperature for 1 hour, membranes were diluted with 6 mL Binding Buffer (- NaCl) and competition binding using dopamine was carried out as described above. Dopamine binding data were fit to a one-site competition curve (Section II.C.3) and a two-site competition curve ($Y = \text{Min} + (\text{Max} - \text{Min}) * [F1/(1 + 10^{(X-\log EC50_1)}) + (1-F1)/(1 + 10^{(X-\log EC50_2)})]$), where EC50₁ and EC50₂ are the EC50's for the high and low affinity sites, respectively, and F1 is the fraction of high-affinity binding sites) using GraphPad Prism v2.0. The one-site and two-site fits were compared using an F test and the one-site model was selected if P<0.05 for the improvement of the fit using the two-site equation..

5. [³⁵S]GTP γ S Binding

Membranes prepared as in Section III.C.1 using Binding Buffer S (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 4 mM MgCl₂, 1.5 mM CaCl₂, 10 μM GDP). The membranes were resuspended in Binding Buffer S containing 0.1 - 0.2 nM GTP γ ³⁵S and 0.1 nM to 100 μM dopamine. After incubation (30 °C for 30 min.), the preparations were filtered onto GF/A filters and protein-bound [³⁵S]GTP γ S was measured by scintillation counting. The data was fit to a sigmoidal dose-response curve ($Y = \text{Min} + (\text{Max} - \text{Min})/(1 + 10^{\log EC50-X})$), where X is the logarithm of the concentration, EC50 is the concentration of drug that stimulates 50% of the GTP γ ³⁵S binding, and Y is the response, using GraphPad Prism v2.0.

D. FUNCTIONAL ASSAYS

1. Drug Incubations

a. Intracellular cAMP Assay

Functional assays were carried out after cells were incubated with agonists and antagonists. For cAMP assays, cells stably expressing recombinant receptors were plated on 6-well plates ($1.5 - 3.0 \times 10^5$ cells/well). When cells reached ~80% confluency they were washed with 2 mL HBBS (118 mM NaCl, 4.6 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, 20 mM HEPES, 0.3 mM IBMX, pH 7.2). Drug incubations were carried out in triplicate with 2 mL HBBS containing 10 μM forskolin in order to stimulate adenylyl cyclase. FLAG-tagged receptors were assayed with various concentrations of dopamine (0.1 nM to 30 μM). To test for antagonist effects, cells were incubated with 1 μM dopamine plus various concentrations of nemonapride (10 pM to 10 μM). Cells expressing HA-tagged receptors were treated with the following drugs to determine the effect on cAMP levels: a dopaminergic agonist (1 μM dopamine) or agonist and antagonists (1 μM dopamine and 1 μM haloperidol/5 μM haloperidol). After incubating for 15 minutes at 37°C, the drug solution was removed and 1 mL of ice-cold permeabilization buffer (0.05% v/v Triton X-100 in HBBS) was added to each well. Cells were scraped and transferred to eppendorf tubes. After incubating on ice for 10 minutes, samples were vortexed briefly and spun at 4°C for 5 minutes at 14,000 rpm. Supernatants were retained and stored at -70°C.

Functional desensitization experiments were carried out by pre-incubating cells with 1 μM quinpirole in media for 1 hour or 12 hours at 37°C in a humidified, 5% CO₂ atmosphere. After preincubation, cells were washed twice with 2 mL HBBS, followed by addition of HBBS containing 10 μM forskolin and 10 pM to 10 μM dopamine. After a 10 minute incubation at 37°C, samples were collected as described above.

b. MAPK Assays

In order to study the effect of drugs on the activity and phosphorylation status of the mitogen-activated protein kinases (MAPKs) ERK1 (p44) and ERK2 (p42), cells were plated on 6-well plates and grown to ~80% confluency and subsequently incubated overnight with α-MEM lacking serum. To study the role of G_{i/o} and PKC, cells were incubated for 20 hr. with PTX (200 ng/mL) or PMA (5 μM). The following day, the cells were washed with fresh α-MEM. To study

the transient activation of the MAPK pathway, cells were incubated with α-MEM containing various drugs for 5 minutes at 37°C. To study the timecourse of MAPK activation, cells were treated with 1 μM dopamine for 0 to 60 minutes at room temperature. Drug incubation was ended by washing the cells with 2 mL ice-cold PBS followed by the addition of 500 μL Lysis Buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 % sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, 1 mM PMSF) for MAPK functional assays or 100 - 200 μL SDS Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue) to assay the phosphorylation state of ERK1/2.

2. cAMP Radioimmunoassay (RIA)

To determine the concentration of cAMP, permeabilized cell solutions were diluted 15 - 25-fold in 50 mM sodium acetate, pH 6.2, and a 100 μL aliquot was transferred to a 5 mL polypropylene tube. Standards were prepared by diluting cAMP to 0.01 to 50 nM in sodium acetate, pH 6.2, and treating them in the same manner as the samples. The samples were modified by addition of 20 μL succinylation reagent (200 mg succinic anhydride dissolved in 1 mL anhydrous acetone, followed by addition of 360 μL triethylamine). After mixing, 2 mL of cold 50 mM sodium acetate, pH 6.2, was added. The diluted samples were vortexed and 100 μL was transferred (in duplicate) to a new 5 mL polypropylene tube.

cAMP antisera (100 μL of a 0.5 mg/mL solution prepared in 0.1% BSA) was added to the succinylated samples, mixed, and incubated 2 hours at 4°C. Subsequently, 100 μL of [¹²⁵I]cAMP (adenosine 3',5'-cyclic phosphoric acid, 2'-O-succinyl [¹²⁵I]iodotyrosine methyl ester, 0.045 μCi/mL in 50 mM sodium acetate, pH 6.2) was added to each, mixed, and incubated overnight at 4°C. The following day, 100 μL of 10% BSA was added to each tube, mixed, and precipitated by addition of 2 mL 95% ethanol (-20°C). After vortexing, tubes were centrifuged (3500 x g for 20 min. at 4°C). The supernatant was removed and the quantity of ¹²⁵I in the pellet was determined by gamma counting.

The cAMP standard curve was fit to a sigmoidal dose-response curve ($Y = \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{(\log EC_{50} - X) * N})$), where X is the logarithm of the concentration, Y is the response and N=hill slope) using GraphPad Prism v2.0.

3. MAPK Assays

a. MAPK Activity Assay

The activity of ERK1/2 was determined using a MAP kinase assay kit by New England Biolabs (Beverly, MA). After drug incubations were stopped by the addition of Lysis Buffer (Section III.D.1.b), the plates were incubated on ice for 5 minutes, scraped, and transferred to an eppendorf. The lysate was sonicated (4 x 5 seconds on ice) using a Branson Sonicator set at 50%. Lysates were centrifuged (14,000 rpm for 10 min. at 4°C) and the supernatant was retained.

To precipitate the active form of ERK1/2, 4 µL of rabbit polyclonal anti-phospho-MAPK antibody was added to 200 µL of cell lysate and the mixture was nulated (ie. gently rocked) overnight at 4°C. The next day, 20 µL of protein A-sepharose (50% v/v) was added to the lysate and the solution was nulated another 2 hours at 4°C. The sepharose beads were microfuged (30 seconds at 4°C) and washed twice with Lysis Buffer. The beads were then washed three times with Kinase Buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl₂).

The beads were resuspended in 50 µL Kinase Buffer containing 0.1 mM ATP and 1 µg Elk1-GST fusion protein, and the kinase reaction was carried out for 30 minutes at 30°C. The reaction was stopped by addition of 25 µL 3 x SDS Sample Buffer. The solution was denatured (100°C for 5 min.), microfuged for 2 minutes, and 25 µL was loaded onto a 10% Tris-glycine SDS-PAGE gel immersed in Running Buffer (24 mM Tris, 1.92 M Glycine, 10% SDS) and separated at 125 V for 1.5 - 2 hours.

After electrophoresis, proteins were transferred from the gel to a PVDF membrane by Western blotting in Transfer Buffer (12 mM Tris-HCl, 96 mM Glycine) at 25 V for 1 hour. The membrane was blocked for 2 hours in Blocking Buffer (TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20, 5% Carnation dry, non-fat milk, and 0.02% NaN₃) and incubated overnight in Primary Antibody Solution (1:1000 anti-phospho-Elk1 in TBS with 0.05% Tween-20, 5% BSA, and 0.02% NaN₃) at 4°C with gentle rocking. The next day, the membrane was washed three times with 15 mL TBS-T (TBS with 0.1% Tween-20) for 5 minutes each, and incubated with 10 mL Secondary Antibody Solution (1:2000 anti-rabbit-HRP in Blocking Buffer without NaN₃) for 1 hour at room temperature. Finally, the membrane was washed three times with 15 mL TBS-T for 5 minutes each and rinsed with TBS briefly. To

detect phosphorylated Elk1, the PVDF membrane was incubated with ECL+plus solution for 5 minute at room temperature. The blot was exposed to X-ray film for 15 to 120 seconds to visualize the activated MAPK substrate.

b. Phospho-MAPK Western Blotting

A second method used to measure the stimulation of the MAPK pathway was the direct detection of the phosphorylated forms of ERK1/2. After addition of Sample Buffer (Section III.D.1.b), cells were scraped, transferred to an eppendorf, and sonicated for 20 seconds. After boiling the lysate for 5 minutes, samples were microfuged 5 minutes and 20 µL was loaded onto a 10% or 8-16% Tris-glycine SDS-PAGE gel. After electrophoresis and transfer to PVDF (Section III.D.3.a), the membranes were incubated overnight with Blocking Buffer. The next day, the membrane was incubated with 10 mL Primary Antibody Solution (Blocking Buffer containing either rabbit polyclonal anti-phosphoMAPK(Tyr204) (1:1000) or mouse monoclonal anti-phospho-MAPK E10 (Thr202/Tyr204) (1:1000)). After incubating with gentle agitation for 1-2 hours at room temperature, the blots were washed with four 5 minute washes with 15 mL TBS-T. The blots were subsequently incubated for 1 hour with 10 mL of Secondary Antibody Solution (anti-rabbit-HRP (1:2000) or anti-mouse-HRP (1:4000) in Blocking buffer without NaN₃) at room temperature. After membranes were washed (see above), the phosphorylated forms of ERK1 and ERK2 were detected by chemiluminescence using ECL+plus as described in Section III.D.3.a.

E. RECEPTOR IMMUNOPRECIPITATION AND WESTERN BLOTTING

I. FLAG-Tagged Receptors

a. Immunoprecipitation

Cells (~5 x10⁷) stored at -70°C were thawed in a 37°C water bath and washed twice with 10 mL ice-cold TBS. Cells were resuspended in 1 mL NP-40 Lysis Buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 2 µg/mL Aprotinin, 2 µg/mL Leupeptin, 1 mM PMSF, 1 mM CaCl₂). The cells were disrupted by sonication on ice (4 x 5 seconds) and membrane proteins were solubilized by nucating for 1 hr. at 4°C. Lysates were centrifuged (14,000 rpm for 10 min. at 4°C) and the supernatant was retained.

After determining the protein concentration using the BCA assay (Section III.E.3),

samples were diluted to the appropriate concentration (0.25 - 3 mg/mL) in Lysis Buffer and 1 µg of mouse monoclonal M1 anti-FLAG antibody was added. Lysates were incubated with rocking for 1 hour at 4°C. After addition of 40 µL goat anti-mouse IgG-agarose (> 8 µg agarose-bound IgG in a 50% slurry of beads previously washed 3x with Lysis Buffer), the incubation was continued overnight. Immunoprecipitates were centrifuged (14,000 rpm for 1 min.) and washed three times with Lysis Buffer. The pellets were resuspended in 50 µL Receptor Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 0.05% bromophenol blue, 2.5% β-mercaptoethanol).

b. Western Blotting

The immunoprecipitates were heated (60°C for 2 min.), cooled on ice, and centrifuged (14,000 rpm for 2 min.). Samples (20 µL) were separated on an 8-16% Tris-glycine gel followed by transfer to PVDF membrane (see section III.D.3.a). Following transfer, the membrane was blocked for 1 hour with Blocking Buffer. The membrane was incubated with primary antibody (10 µg/mL mouse monoclonal M1 anti-FLAG antibody in Blocking Buffer) overnight with rocking. After washing the membrane (3 x 5 min. with 15 mL TBS-TC (TBS with 0.2% Tween-20 and 1 mM CaCl₂), the membrane was incubated with secondary antibody for one hour (1:6000 anti-mouse IgG-HRP in TBS-TC). The blot was washed (3 x 5 min. with 15 mL TBS-TC (TBS with 0.2% Tween-20 and 1 mM CaCl₂) and rinsed with 15 mL TBS. Detection was carried out by soaking the membrane in ECL solution for one minute and exposing the blot to film.

2. HA-Tagged Receptors

a. Immunoprecipitation

Cells (~ 5 x 10⁷) were washed twice with 10 mL cold PBS and resuspended in 10 mL cold TE (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 µg/mL aprotinin, 2 µg/mL leupeptin, and 1 mM PMSF). After sitting on ice for 5 minutes, the cells were disrupted by polytron set at maximum (2 x 10 seconds on ice). After removing cell debris (2000 x g for 3 min.), the cell membrane fraction was isolated by centrifugation (34,000 x g for 20 min.). The membranes were solubilized in 0.5 mL RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 µg/mL aprotinin, 2 µg/mL leupeptin, 1 mM PMSF, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). After incubation at 4°C for 1 hour with rocking, the solubilized

membranes were centrifuged (14,000 rpm for 10 min. at 4°C) and the protein concentration of the resulting supernatant was determined by the BCA protein assay.

After diluting to 0.8 mg/mL in 0.5 mL, the samples were precleared by incubating with 25 µL of Protein G-agarose (50% beads washed 3x with RIPA buffer) with rocking for 1 hour at 4°C. The beads were pelleted (14,000 rpm for 1 min.) and 0.5 µg of monoclonal rat anti-HA IgG 3F10 was added to the retained supernatants. After incubating overnight with rocking, the receptor/antibody complex was immunoprecipitated with 40 µL Protein G-agarose (50% beads washed 3x with RIPA buffer). The samples were incubated a further 3 hours and the beads were collected by centrifugation (14,000 rpm for 1 min.). After washing (3 x 0.5 mL RIPA buffer), the beads were resuspended in 80 µL Receptor Sample Buffer.

b. Western Blotting

After heating (60°C for 2 min.), samples were cooled on ice and microfuged (14,000 rpm for 2 min.). SDS-PAGE (4-20% Tris-glycine gel) and Western blotting were carried out as described in Section II.D.3.a, with the following modifications. The Primary Antibody Solution contained a 1:10 dilution of anti-HA hybridoma supernatant in TBS-T + 1% BSA, and the Secondary Antibody Solution consisted of a 1:4000 dilution of anti-mouse-HRP in TBS-T. Alternatively, detection was carried out using 200 ng/mL rat anti-HA IgG 3F10 in TBS-T + 1% BSA as a primary antibody. Blots were washed (15 min. followed by 3 x 5 min.) between steps. The Secondary Antibody Solution contained anti-rat IgG-biotin (1:10000) in TBS-T + 1% BSA. After incubating the secondary antibody for 1 hour at room temperature, the blot was washed and the Tertiary Probe Solution (1:10,000 Streptavidin-HRP in TBS-T + 1% BSA) was added. After washing, the HA-tagged receptor was detected by chemiluminescence using ECL+plus as described.

3. BCA Protein Assay

Protein concentrations were measured using the BCA assay. BSA standards (0, 50, 100, 200, 400, 600, and 800 µg/mL), prepared in the same buffer as samples, were diluted from a BSA stock solution (10 mg/mL BSA in 0.9% NaCl, 0.05% NaN₃). Binding preparations were assayed at 4x the final concentration, while solubilized samples for Western blotting were diluted 1:5 in H₂O. Duplicate 50 µL aliquots of H₂O blanks, standards and samples were mixed with

1.0 mL of BCA working reagent (50:1 BCA reagent A:BCA reagent B), vortexed, and incubated in a 37°C water bath for 30 minutes. After cooling to room temperature, the absorbance at 562 nm was measured. The blank values were subtracted from the sample and standard measurements and the unknown values were determined from the BSA standard curve.

IV. RESULTS

A. SELECTION OF STABLE CELL LINES

Wildtype and mutant D₄ and D_{2L} receptor cDNAs with an additional 5' sequence encoding a cleavable, membrane-targetted signal sequence and a FLAG- or HA-epitope tag were subcloned into the expression vectors pRc/RSV or pcDNA3. All of the expressed D₄ receptors had identical amino acid sequences except at the epitope tag and within the third cytoplasmic loop, as shown in Figure 5. After transfection into CHO K1 cells and selection with G418, between 10 and 66 individual clones were isolated for each construct. These were tested by 3-point radioligand binding with [³H]spiperone or [³H]nemonapride. Several positive clones were subsequently analyzed by saturation binding with [³H]spiperone to determine the receptor density, B_{max} (fmol/mg membrane protein), as shown with HAD4.4 in Figure 6.

A CHO cell line, CHO FD4.4-10, was chosen that expressed the FLAG-tagged D_{4.4} receptor. This cell line has a radioligand binding level comparable to that of a previously characterized cell line, CHO K1 RSV D4.4-7, which expresses an unmodified form of the receptor (Asghari *et al.*, 1995). With the HA-tagged receptors, a single clone expressing approximately 0.5 - 1.5 pmol/mg of receptor was chosen for further analysis. In the case of HAD4(Δ254-315), only a single clone with measurable specific binding was isolated (B_{max}=76 fmol/mg, K_d=0.069 nM). However, due to the low expression level, further pharmacological characterization of this clone was not carried out. In experiments with CHO K1 cells stably transfected with the pcDNA3 vector alone, no high affinity, saturable binding was detected, confirming that these cells do not express endogenous D₂-like receptors.

B. PHARMACOLOGY OF EPITOPE-TAGGED RECEPTORS

1. Saturation Binding

The wildtype FLAG-tagged receptor had normal [³H]spiperone pharmacology, as compared with data previously obtained with CHO K1 cells expressing untagged D_{4.4} (Table 4). Cells expressing HA-tagged wildtype and mutant dopamine D₄ and D_{2L} receptors had high-affinity [³H]spiperone binding with a B_{max} between 0.78 and 1.6 pmol/mg. K_d values were similar to those reported in the literature (Table 2). FLAG-tagged D₄ containing a point mutation at amino acid 345 of the D_{4.4} sequence (Met→Ala) did not bind this ligand. Specific binding was detected with [³H]nemonapride, however, which allowed selection of the clonal cell line CHO

	TM5			TM6		
HAD4(Δ221-337)	LLL	Y	WATFRGLQL			
HAD4(Δ221-315)	LLL	Y	WATFRGLQP			
HAD4(Δ254-315)	LLL	Y	WATFRGLQRWEVARRAKLHGRAPRRPSGP GPPSPTP	PAPRLP		
HAD4.2	LLL	Y	WATFRGLQRWEVARRAKLHGRAPRRPSGP GPPSPTP	PAPRLPQDPCGPDCAP	β	
F/HAD4.4	LLL	Y	WATFRGLQRWEVARRAKLHGRAPRRPSGP GPPSPTP	PAPRLPQDPCGPDCAP	β	PAPGLPRGPCGPDCAP
FD4.4 M345A	LLL	Y	WATFRGLQRWEVARRAKLHGRAPRRPSGP GPPSPTP	PAPRLPQDPCGPDCAP	β	PAPGLPRGPCGPDCAP
HAD4.7	LLL	Y	WATFRGLQRWEVARRAKLHGRAPRRPSGP GPPSPTP	PAPRLPQDPCGPDCAP	β	PAPGLPRGPCGPDCAP
HAD2L	LLVYI	KIYI	VL - RRR - - RKRVNTKRSSRAFRAHLRAPLK	GNCTHPEDMKLCTVI	MKSNGSF	PVNRRRV
HAD4(Δ221-337)						
HAD4(Δ221-315)						
HAD4(Δ254-315)						
HAD4.2						
F/HAD4.4					α/β	
FD4.4 M345A	η	ε	β	AAPSLPQDPCGPDCAP		
HAD4.7	AAPGLPPDPCGPDCAP	PAPGLPQDPCGPDCAP	PAPGLPRGPCGPDCAP	PAPGLPQDPCGPDCAP		
HAD2L	EAARRAQELEMEMLSSTSPPERTRYSPIPPSSHHQLTL					
HAD4(Δ221-337)				TGRERKAMRVL	PVVVG	
HAD4(Δ221-315)				VRAAAALPPQT	PPQTRRRRRAKIT	GRE
HAD4(Δ254-315)	ζ			VRAAAALPPQT	PPQTRRRRRAKIT	GRE
HAD4.2	PAPGLPPDPCGSNCAPP	DAVRAAAALPPQT	PPQTRRRRRAKIT	TGRERKAMRVL	PVVVG	
F/HAD4.4	PAPGLPPDPCGSNCAPP	DAVRAAAALPPQT	PPQTRRRRRAKIT	TGRERKAMRVL	PVVVG	
FD4.4 M345A	PAPGLPPDPCGSNCAPP	DAVRAAAALPPQT	PPQTRRRRRAKIT	TGRERKA	ARVL	PVVVG
HAD4.7	PAPGLPPDPCGSNCAPP	DAVRAAAALPPQT	PPQTRRRRRAKIT	TGRERKAMRVL	PVVVG	
HAD2L	KPEKNGHAKDHPKIAKI	FEI	QTMPNGKTRTS	LKTMSRRKLSQQKEKKATOMLAI	VLG	

Figure 5. Third intracellular loop amino acid sequence alignment for wildtype and mutant D₄ and D_{2L} receptors.

The boxed sequences of the D₄ receptor delineate the 16 amino acid polymorphic repeat regions, identified by the Greek letters α , β , η , ε , θ , and ζ . The boxed sequence in D_{2L} indicates the 29 amino acid insertion that is not present in D_{2S}.

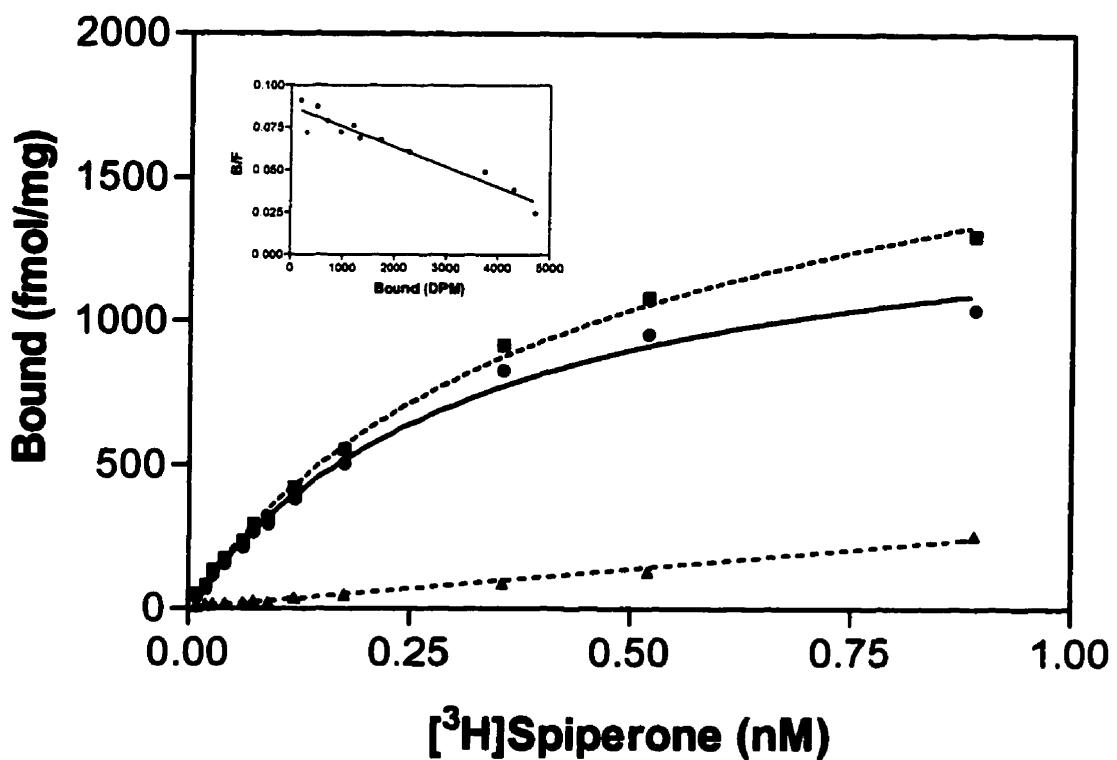


Figure 6. Saturation binding of [³H]spiperone to membranes from Chinese hamster ovary cells expressing HA-tagged D4.4.

Saturation radioligand binding was carried out as described in Section II.C.2 on membranes from CHO HAD4.4-29. A representative plot of three independent experiments is shown. The maximal specific binding, B_{max} (1500 fmol/mg membrane protein), and dissociation constant, K_d (0.33 nM), were determined by nonlinear curve fitting as explained in the methods. Data points are the mean of duplicate measurements and represent total binding (■), non-specific binding with 1 μ M haloperidol (▲), and specific binding (total - non-specific) (●). *Inset:* Scatchard transformation of the corresponding data. B , bound radioligand; F , free radioligand.

Table 4: [³H]Sipiperone dissociation constant (K_d) and receptor density (B_{max}) for HA-tagged wildtype and mutant D₄ and D_{2L} receptors.

Cell Line	K_d (nM) ± s.d.	B_{max} ± s.d. (pmol/mg protein)
CHO D4.4-7	0.094 ± 0.006	0.25 - 0.35
CHO FD4.4-10	0.164 ± 0.023	0.280 ± 0.064
CHO HAD4(Δ221-337)-7	0.196 ± 0.079	1.13 ± 0.10
CHO HAD4(Δ221-315)-8	0.145 ± 0.060	0.780 ± 0.148
CHO HAD4.2-35	0.161 ± 0.016	1.05 ± 0.59
CHO HAD4.4-29	0.246 ± 0.071	1.62 ± 0.16
CHO HAD4.7-1	0.160 ± 0.029	0.784 ± 0.100
CHO HAD2long-6	0.064 ± 0.011	1.30 ± 0.17

The equilibrium dissociation constant (K_d) and receptor density (B_{max}) were determined in cell membranes for receptors stably expressed in Chinese hamster ovary (CHO) cells by saturation binding with [³H]spiperone (0.01 - 1.0 nM) as described in Section II.C.2. Values are expressed as the mean ± standard deviation (N=3). Data for the untagged D₄ cell line CHO RSV D4.4-7 is from previous work in our laboratory (Asghari *et al.*, 1995).

FD4.4(M345A)-1. An accurate estimation of the receptor K_d and B_{max} could not be determined with this radioligand, possibly due to low expression or a lower affinity for nemonapride.

2. Antagonist Binding

To confirm that the antagonist pharmacology of FLAG-tagged D₄ was not altered by the addition at the amino-terminal sequence, competition binding experiments were carried out. Using the D₂ antagonists spiperone, nemonapride, haloperidol, clozapine, and raclopride, specific [³H]spiperone and [³H]nemonapride binding was displaced by unlabelled antagonists, as shown in Figure 7A and 7B. The FLAG-tagged D₄ receptor displayed the typical rank order of antagonist binding of the D₄ receptor: nemonapride > haloperidol > clozapine > raclopride. In addition, the derived inhibition constants (K_i), shown in Table 5 and 6, were close to values reported for this receptor in the literature (Seeman & Van Tol, 1994).

Both the wildtype and deletion mutant HA-tagged D₄ receptors had unaltered antagonist pharmacology (Figure 8). The rank order of competition for HAD2L was nemonapride > haloperidol > raclopride > clozapine, which showed the D₂ receptor's characteristic high-affinity for raclopride (Figure 8 and Table 5). The FLAG-tagged point mutant FD4.4(M345A) did not show high-affinity binding for spiperone, clozapine or raclopride using [³H]nemonapride as a probe. However, haloperidol did compete with [³H]nemonapride binding (Figure 7B). The EC₅₀ for haloperidol is lower than of the wildtype receptor, although the K_i of haloperidol at this mutant cannot be determined since its true affinity for [³H]nemonapride is not known.

3. Dopamine Pharmacology

Competition binding using [³H]spiperone was carried out in the absence of sodium to promote G protein coupling (Kenakin, 1996). Sodium acts allosterically at a conserved aspartate residue in the second intracellular loop (D80 in D₂) to reduce the affinity of agonists for the receptor, presumably by preventing interaction with G proteins (Neve *et al.*, 1991). The results obtained with FD4.4 indicated that the amino-terminal tag sequence did not affect the binding of dopamine (Figure 9 and Table 7). In addition, FD4.4 possessed both high-affinity and low-affinity dopamine binding sites, which is typical of receptors that can couple to G proteins. Pre-incubation of cell membranes with a non-hydrolyzable GTP analogue, Gpp[NH]p, resulted in a rightward shift in the dopamine competition curve. Therefore, the character of dopamine binding

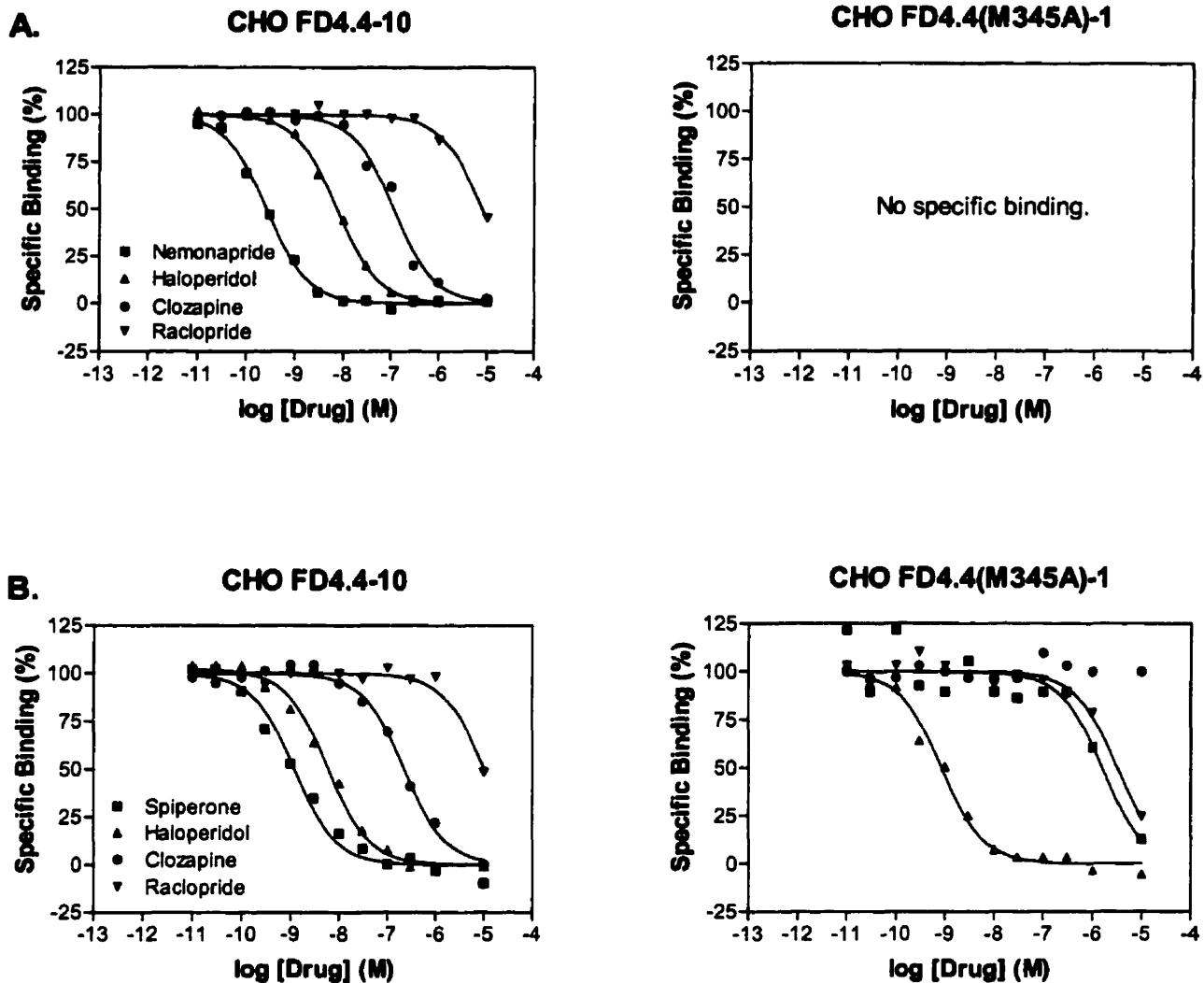


Figure 7. Antagonist competition of radioligand binding to FLAG-tagged wildtype and mutant D₄ receptors.

A. Competition of $[^3\text{H}]$ spiperone binding by the unlabelled antagonists nemonapride (■), haloperidol (▲), clozapine (●), and raclopride (▼).

B. Competition of $[^3\text{H}]$ nemonapride binding by the unlabelled antagonists spiperone (■), haloperidol (▲), clozapine (●), and raclopride (▼).

Binding was carried out using membranes from stably transfected Chinese hamster ovary (CHO) cells as described in the text (Section III.C.3). A representative curve of two independent experiments is shown in each case, and data points are the mean of duplicate measurements.

Table 5. Inhibition constants of antagonists for FLAG- and HA-tagged wildtype and mutant D₄ and D_{2L} receptors determined with [³H]spiperone.

Cell line	K _i (nM)			
	Nemonapride	Haloperidol	Clozapine	Raclopride
CHO D4.4-7	0.21	1.9	12	1520
CHO FD4.4-10	0.075 (0.020)	1.6 (0.6)	21 (15)	1300 (1400)
CHO FD4.4(M345A)	- ^b	- ^b	- ^b	- ^b
CHO HAD4(Δ221-337)-7	0.25 (0.08)	1.3 (0.2)	68 (24)	1700 ^a
CHO HAD4(Δ221-315)-8	0.26 (0.07)	1.2 (0.6)	47 (32)	1700 (200)
CHO HAD4.2-35	0.28 (0.22)	1.1 (0.3)	74 (87)	1400 (300)
CHO HAD4.4-29	0.30 (0.23)	1.2 (0.5)	71 (82)	1800 (1200)
CHO HAD4.7-1	0.23 (0.03)	1.4 (0.2)	41 (15)	1500 (200)
CHO HAD2L-6	0.10 (0.08)	0.61 (0.09)	230 (50)	1.7 (0.7)

^a N=1.

^b No specific [³H]spiperone binding.

Equilibrium inhibition constants (K_i) were determined with membranes from stably transfected Chinese hamster ovary (CHO) cells by competitive binding of unlabelled antagonist with [³H]spiperone (Section III.C.3). K_i values were calculated from the IC₅₀ using the method of Cheng-Prusoff (Cheng & Prusoff, 1973) and represent the mean of two independent experiments followed by the range between the two values in brackets, unless otherwise indicated. Data for the untagged D_{4,4} cell line CHO RSV D4.4-7 is from previous work in our laboratory (Sanyal, 1995).

Table 6. Inhibition constants of antagonists for FLAG-tagged wildtype and mutant D₄ receptors determined with [³H]nemonepride (YM09151-2).

Cell line	K _i (nM)			
	Spiperone	Haloperidol	Clozapine	Raclopride
CHO FD4.4-10	0.29 (0.01)	3.1 (3.4)	47 (15)	2900 (800)
CHO FD4.4(M345A)-1	1648 ^{a,b}	1.4 ^b (1.2)	- ^c	3200 ^{a,b}

^a N=1.

^b EC₅₀.

^c No competition curve.

Equilibrium inhibition constants (K_i) were determined with membranes from stably transfected Chinese hamster ovary (CHO) cells by competitive binding of unlabelled antagonist with [³H]YM09151-2 (Section III.C.3). K_i values were calculated from the IC₅₀ using the method of Cheng-Prusoff based on the K_d of wildtype D₄ for YM09151-2 (Cheng & Prusoff, 1973). Values represent the mean of two independent experiments followed by the range between the two values in brackets, unless otherwise indicated.

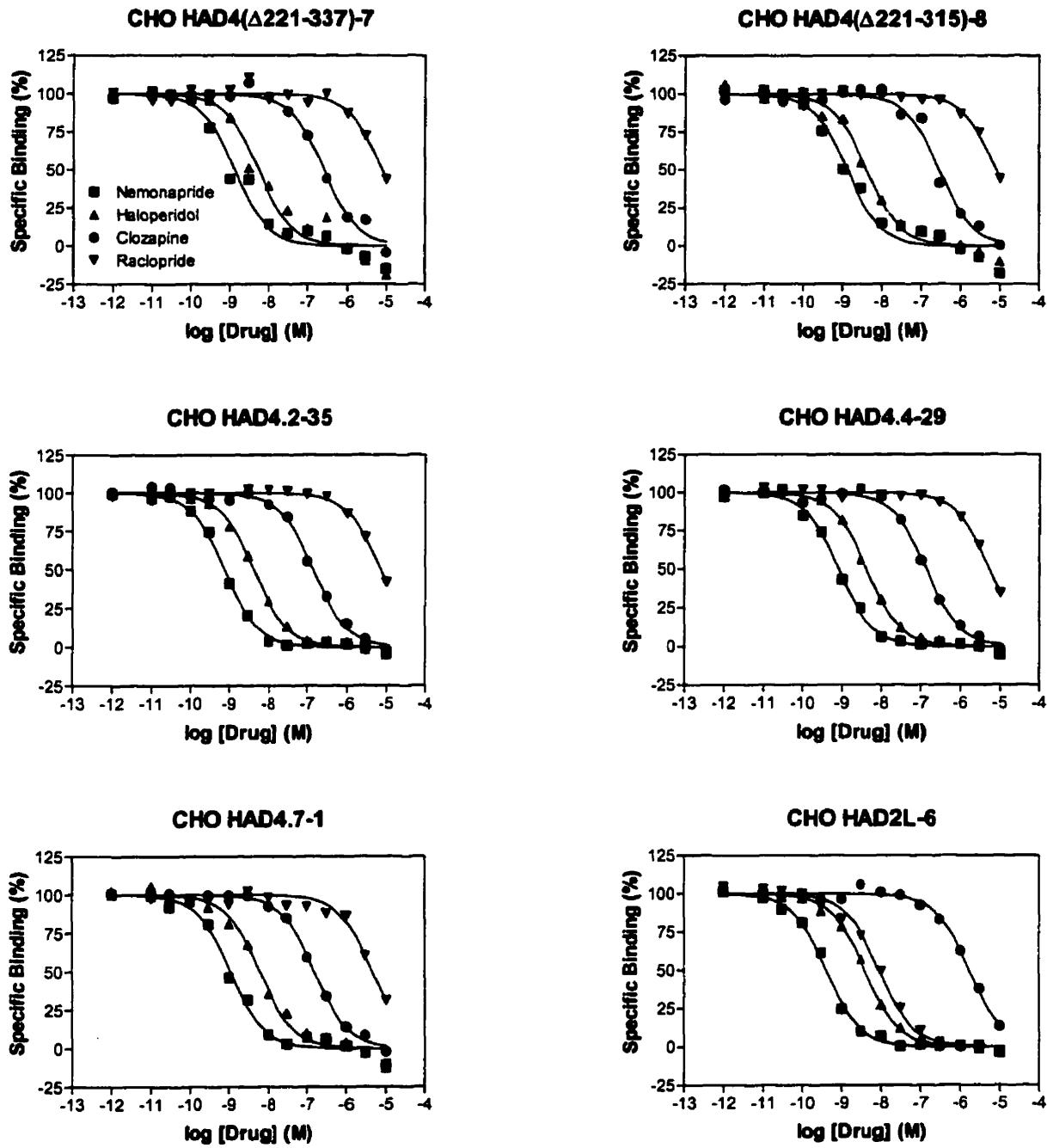


Figure 8. Antagonist competition of [³H]spiperone binding to HA-tagged wildtype and mutant D₄ and D_{2L} receptors.

Competition curves were determined by measuring the displacement of [³H]spiperone bound to membranes from stably transfected Chinese hamster ovary (CHO) cells by the unlabelled antagonists nemonapride (■), haloperidol (▲), clozapine (●), and raclopride (▽), as described in the methods (Section III.C.3). Representative curves of two independent experiments are shown, and data points are the mean of duplicate measurements.

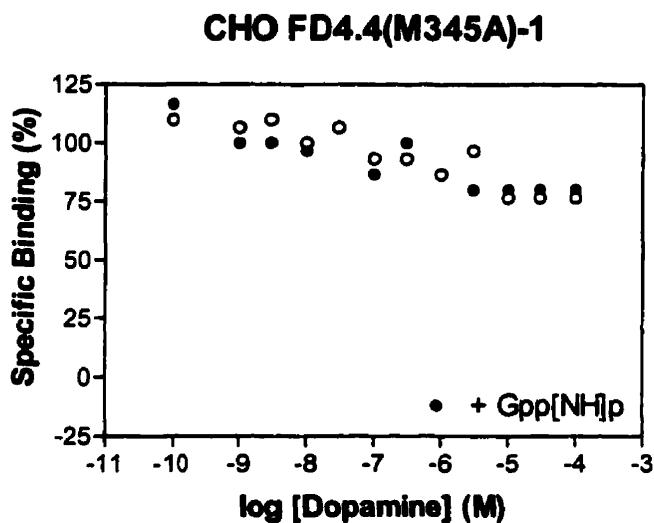
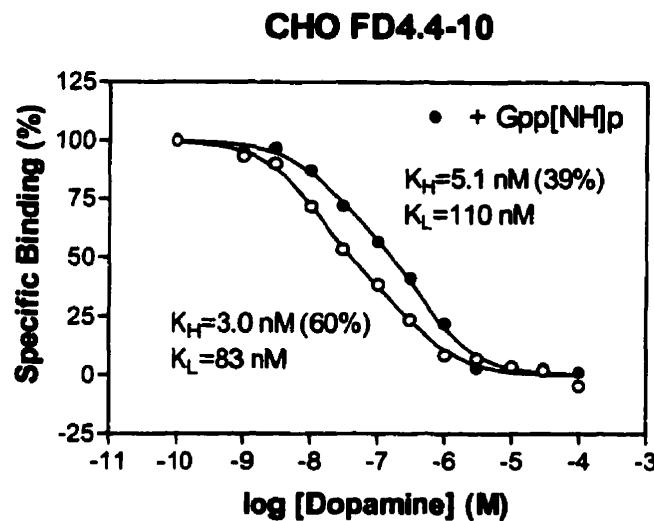


Figure 9. Dopamine competition curves for FLAG-tagged wildtype and mutant D₄ receptors.

Competition of [³H]nemanopride binding by dopamine was carried out with membranes from stably transfected Chinese hamster ovary (CHO) cells in the absence of NaCl (Section III.C.4). Membranes were preincubated for 1 hr. without (O) or with (●) 200 μ M Gpp[NH]p. Representative curves of two independent experiments are shown, and data points are the mean of duplicate measurements. Data was fit to 1-site and 2-site competition binding equations using GraphPad Prism, and the affinity constant(s) were calculated from the $IC_{50}(s)$ using the method of Cheng-Prusoff based on the K_d of wildtype D₄ for nemanopride (Cheng & Prusoff, 1973). The one-site and two-site fits were compared using an F test and the one-site model was selected if $P < 0.05$ for the improvement of the fit using the two-site equation.

Table 7. Dopamine binding properties of FLAG- and HA-tagged receptors.

Cell line	Disassociation constant (nM)		
	K _H	K _L	% High-affinity
CHO D4.4-7	1.16	60	47
CHO FD4.4-10 ^a	3.3 (0.5)	65 (37)	60 (0)
CHO FD4.4(M345A)-1 ^b	- ^c	- ^c	- ^c
CHO HAD4(Δ221-337)-7	9.5 (4.3)	-	100 (0)
CHO HAD4(Δ221-315)-8	3.5 (4.0)	270 (320)	59 (16)
CHO HAD4.2-35	1.4 (0.7)	140 (60)	69 (2)
CHO HAD4.4-29	0.83 (0.37)	47 (47)	65 (16)
CHO HAD4.7-1	0.73 (0.33)	200 (80)	74 (0)
CHO HAD2L-6	25 (33)	2200 (800)	31 (4)

^a Mean of experiments with [³H]spiperone and [³H]nemonapride (YM09151-2) (N=2).

^b [³H]nemonapride (YM09151-2).

^c No competition curve

High and low-affinity equilibrium inhibition constants of dopamine (K_H and K_L) were determined with membranes from stably transfected Chinese hamster ovary (CHO) cells by competitive binding with [³H]spiperone, unless otherwise indicated (Section III.C.4). Data was fit to 1-site and 2-site competition binding equations using GraphPad Prism, and the affinity constant(s) were calculated from the IC₅₀(s) using the method of Cheng-Prusoff based on the K_d of wildtype D₄ for spiperone/nemonapride (Cheng & Prusoff, 1973). The one-site and two-site fits were compared using an F test and the one-site model was selected if P<0.05 for the improvement of the fit using the two-site equation. Values represent the mean of two independent experiments followed by the range between the two values in brackets. Data for the untagged D_{4.4} cell line CHO RSV D4.4-7 is from previous work in our laboratory (Sanyal, 1995).

with epitope-tagged receptor appears to be identical to previous results with untagged D_{4,4} (Asghari *et al.*, 1995).

Experiments also demonstrated that HAD4(Δ221-315), HAD4.2, HAD4.4, HAD4.7, and HAD2L had normal dopamine binding (Figure 10). All these D₄ receptor variants had a similar ratio of high to low affinity sites (%High/Low≈70/30), but a lower percentage of HAD2L binding sites had high dopamine affinity (%High/Low≈31/69). Gpp[NH]p reduced the affinity of these receptors for dopamine. In the case of HAD4.4 and HAD2L, the shifted dopamine competition curves fit a one-site model, while HAD4(Δ221-315), HAD4.2, and HAD4.7 continued to fit to a two-site competitive binding equation, but with a lower percentage of high-affinity sites (Figure 10). The results for wildtype FD4.4 and HA-tagged receptors, shown in Table 7, are in general agreement with values cited by previous studies of D₄ and D_{2L} expressed in CHO K1 cells (Asghari *et al.*, 1994; Chio *et al.*, 1994). With the deletion mutant HAD4(Δ221-337), only a high-affinity dopamine site was present. In contrast, dopamine did not bind at all to the FLAG-tagged point mutant FD4.4(M345A) (Figure 9).

C. WESTERN BLOTTING OF EPITOPE-TAGGED RECEPTORS

1. Detection of FLAG-tagged Wildtype and Mutant D_{4,4} Receptors

The FLAG-tagged receptors FD4.4 and FD4.4(M345A) were immunoprecipitated from lysates of pharmacologically characterized CHO K1 stable cell lines (Figure 11). These samples were then separated by SDS-PAGE and Western blotting was carried out. Unfortunately, the use of the M1 antibody for both precipitation and blotting results in the presence of heavy and light chain antibody bands, which impair the visualization of the full-length D₄. However, wildtype FD4.4 resulted in bands with a molecular weights of 58, 51, and 37 kDa. Since the M1 anti-FLAG antibody only recognizes the FLAG epitope when it forms the amino terminal sequence, these results also confirmed that the signal sequence is properly cleaved after translation.

2. Detection of HA-tagged Wildtype and Mutant D₄ and D_{2L} Receptors

To verify that the expressed D₄ and D_{2L} receptors contain a recognizable HA epitope, immunoprecipitation and immunoblotting was carried on membranes prepared from CHO cell lines stably expressing receptors. Using a monoclonal rat anti-HA antibody and Protein G-agarose, receptors were isolated from cell membranes that were solubilized with RIPA buffer.

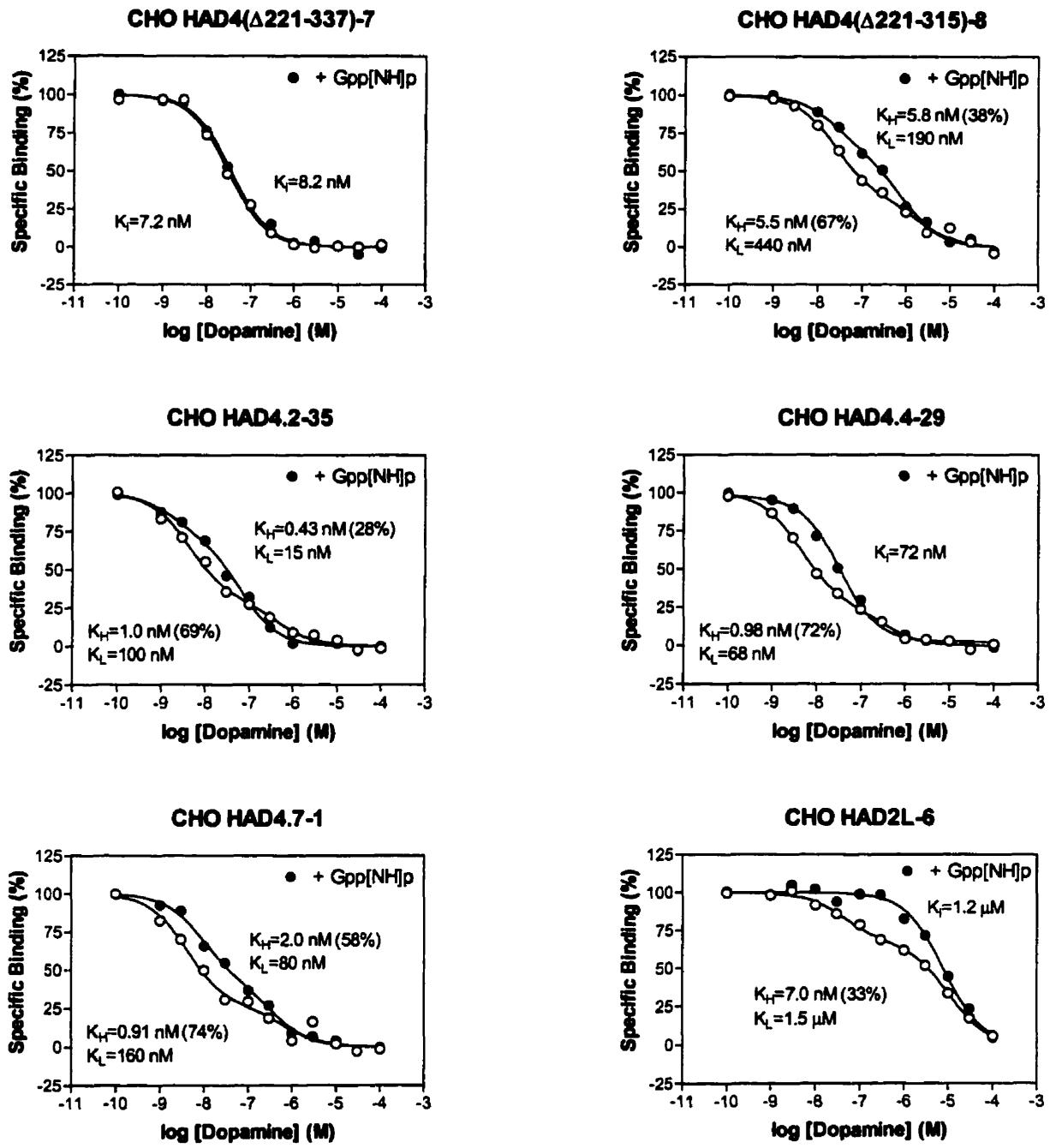


Figure 10. Dopamine competition curves for HA-tagged wildtype and mutant D₄ receptors. Competition of [³H]spiperone binding by dopamine was carried out with membranes from stably transfected Chinese hamster ovary (CHO) cells in the absence of NaCl (Section III.C.4). Membranes were preincubated for 1 hr. without (O) or with (●) 200 μM Gpp[NH]p. Data was fit to 1-site and 2-site competition binding equations using GraphPad Prism, and the affinity constant(s) were calculated from the IC₅₀(s) using the method of Cheng-Prusoff based on the K_d of wildtype D₄ for spiperone (Cheng & Prusoff, 1973). The one-site and two-site fits were compared using an F test and the one-site model was selected if P<0.05 for the improvement of the fit using the two-site equation. A representative curve of two independent experiments is shown in each case, and data points are the mean of duplicate measurements.

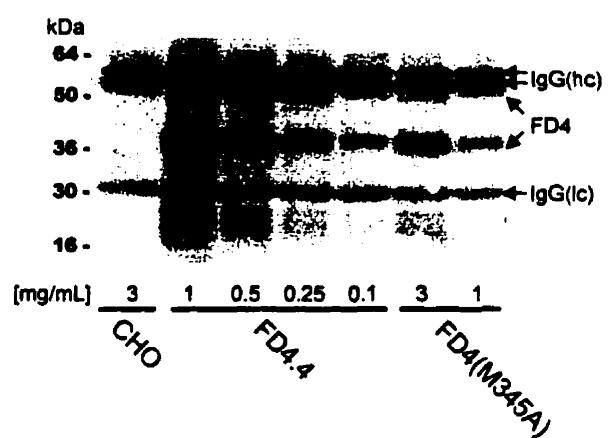


Figure 11. Western blot of FLAG epitope-tagged wildtype and mutant D₄ receptors. D_{4,4} receptors with an amino-terminal FLAG epitope stably expressed in CHO cells were solubilized in NP-40 lysis buffer. After determining the protein concentration of the lysate, samples were diluted in lysis buffer to the appropriate final concentration in a total volume of 750 µL. Receptors were immunoprecipitated with the anti-FLAG M1 monoclonal antibody/anti-mouse IgG-agarose. Following SDS-PAGE and transfer to PVDF, the blot was probed with the anti-FLAG M1 monoclonal antibody and anti-mouse IgG coupled to horseradish peroxidase.

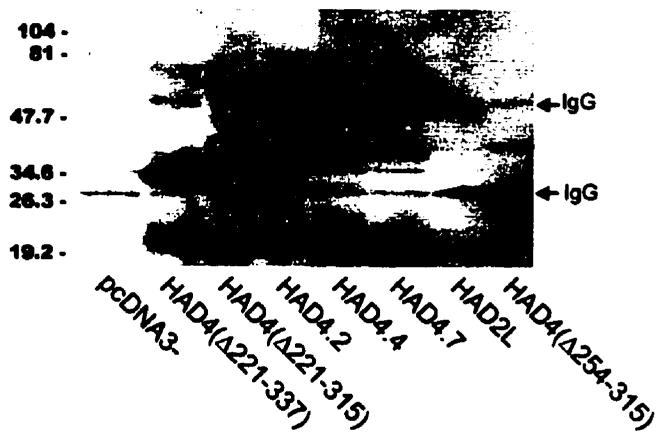
Following immunoprecipitation, proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The blots were probed with the monoclonal rat anti-HA antibody, anti-rat IgG-biotin, and a streptavidin-horseradish peroxidase conjugate. The blot illustrated in Figure 12A indicates that the deletion mutants HAD4(Δ 221–337) and HAD4(Δ 221-315) can be observed, with a major band near their predicted unglycosylated molecular weights of 33.2 and 35.7 kDa, respectively. Several bands appear for the wildtype HAD4.2, HAD4.4, and HAD4.7 receptors. The largest band in Figure 12A (53, 59, and 68 kDa for HAD4.2, HAD4.4, and HAD4.7) likely represents glycosylated receptor, since it appears diffuse and is larger than their predicted molecular weights of 42.1, 45.1, and 49.6 kDa, respectively. The second-largest band, clearly observable with HAD4.2 and HAD4.4, most likely represents unglycosylated, full-length receptor. An additional band of unknown origin (MW \approx 38 kDa) is also present in immunoprecipitates of all three wildtype D₄ receptors. The protein migration pattern of HAD4.4 is in good agreement with the results from Western blotting of FD4.4. A single band for HAD2L is visible at 55 kDa. The cell line CHO HAD4(Δ 254-315)-27, which expressed < 100 fmol/mg of receptor, also produced a faint immunoreactive band at 41 kDa, demonstrating the sensitivity of this method.

In order to determine which bands correspond to glycosylated receptor, the CHO stable cell lines were incubated overnight with 1 μ g/mL tunicamycin, an inhibitor of N-linked glycosylation.. After isolation of receptors by immunoprecipitation, the blot was probed with anti-HA hybridoma supernatant followed by anti-mouse IgG-horseradish peroxidase. Tunicamycin treatment resulted in a reduction in the molecular weight of several forms of the deletion mutants (Figure 12B). Immunoblotting consistently detected a stronger signal from the deletion mutants HAD4(Δ 221-337) and HAD4(Δ 221-315), and Figure 12B is composed from several exposures of varying duration. HA-tagged full-length receptors showed stronger immunoreactivity in the bands corresponding to unglycosylated receptor in the tunicamycin-treated cells. Thus the tagged D_{4.2}, D_{4.4}, and D_{4.7} receptors are predominantly glycosylated when expressed in CHO cells, with carbohydrates contributing 6 to 12 kDa to the apparent molecular weight of full-length receptors and 4 kDa to the deletion mutants.

D. FUNCTIONAL COUPLING OF EPITOPE-TAGGED RECEPTORS

1. Inhibition of Adenylyl Cyclase

A.



B.

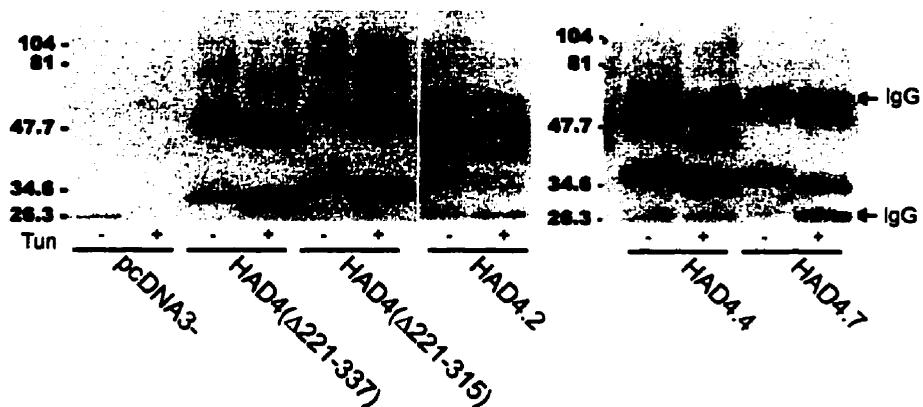


Figure 12. Western blot of HA epitope-tagged wildtype and mutant D₄ and D_{2L} receptors. Amino-terminal epitope-tagged receptors were immunoprecipitated with monoclonal rat anti-HA IgG/Protein G-agarose from the solubilized membranes of stably transfected CHO cells as detailed in Section III.E.2. The isolated receptors were separated by SDS-PAGE and transferred to PVDF membranes. **A.** Immunoblotting of HA-tagged D₄ and D_{2L} receptors using with monoclonal rat anti-HA antibody, anti-rat IgG-biotin, and streptavidin-horseradish peroxidase. **B.** Immunoblotting of HA-tagged D₄ receptors from CHO cells cultured in the absence (-) or presence (+) of 1 µg/mL tunicamycin. The blot was probed with anti-HA hybridoma supernatant followed by anti-mouse IgG conjugated with horseradish peroxidase.

To determine whether the tagged mutant and wildtype D₄ receptors couple to G proteins, the well-characterized ability of D₂-like receptors to inhibit adenylyl cyclase was assayed (Asghari *et al.*, 1995; Lajiness *et al.*, 1995). Functional coupling of FD4.4 and FD4.4(M345A) was determined by measuring the inhibition of forskolin-stimulated cAMP by varying concentrations of dopamine. As shown in Figure 13, dopamine mediated a reduction in the intracellular cAMP level in the cells expressing FD4.4, with an EC₅₀=70 nM. This is close to results from other studies of D₄ expressed in CHO cells, which measured an EC₅₀ of 12 to 37 nM (Asghari *et al.*, 1995; Chio *et al.*, 1994).

In order to determine whether the FLAG-tagged point mutant showed constitutive activity that can be interrupted by an antagonist, wildtype and mutant cells were exposed to 1 μM dopamine with increasing concentrations of nemonapride (Figure 13B). As expected, this antagonist reversed the inhibitory effect of dopamine (IC₅₀=29 nM). However, no effect on cAMP levels was noted with FD4.4(M345A). The nemonapride IC₅₀ for FD4.4 is near previous results with this assay carried out with CHO K1 expressing D_{4.4} (IC₅₀=7.0 nM) (Asghari *et al.*, 1995).

Receptor function of HA-tagged D₄ and D_{2L} receptors was determined similarly. With 1 μM dopamine, a statistically significant reduction in the concentration of intracellular cAMP was measured in CHO K1 cells expressing HAD4(Δ221-315), HAD4.2, HAD4.4, HAD4.7, and HAD2L (Figure 14). The effect of dopamine on cAMP levels was partially blocked by 1 - 5 μM haloperidol in cells expressing D₄ receptors. Haloperidol completely inhibited the response of HAD2L to dopamine. Neither the CHO K1 pcDNA3- control nor CHO K1 HAD4(Δ221-337)-7 showed significant alteration in forskolin-stimulated cAMP levels due to dopamine or haloperidol.

2. Stimulation of [³⁵S]GTPγS Binding

Dopamine was tested for the ability to stimulate the binding of [³H]GTPγS to Ga, which would indicate an increased rate of guanine nucleotide exchange and G protein coupling. The cell line CHO F4.4-10 shows a robust stimulation of [³⁵S]GTPγS binding in Figure 15, with the EC₅₀=390 nM. Despite their high expression level in CHO cells, the deletion mutants HAD4(Δ221-337) and HAD4(Δ221-315) stimulated less [³⁵S]GTPγS binding than the wildtype receptor. For the mutant with the larger deletion in the third cytoplasmic, HAD4(Δ221-337),

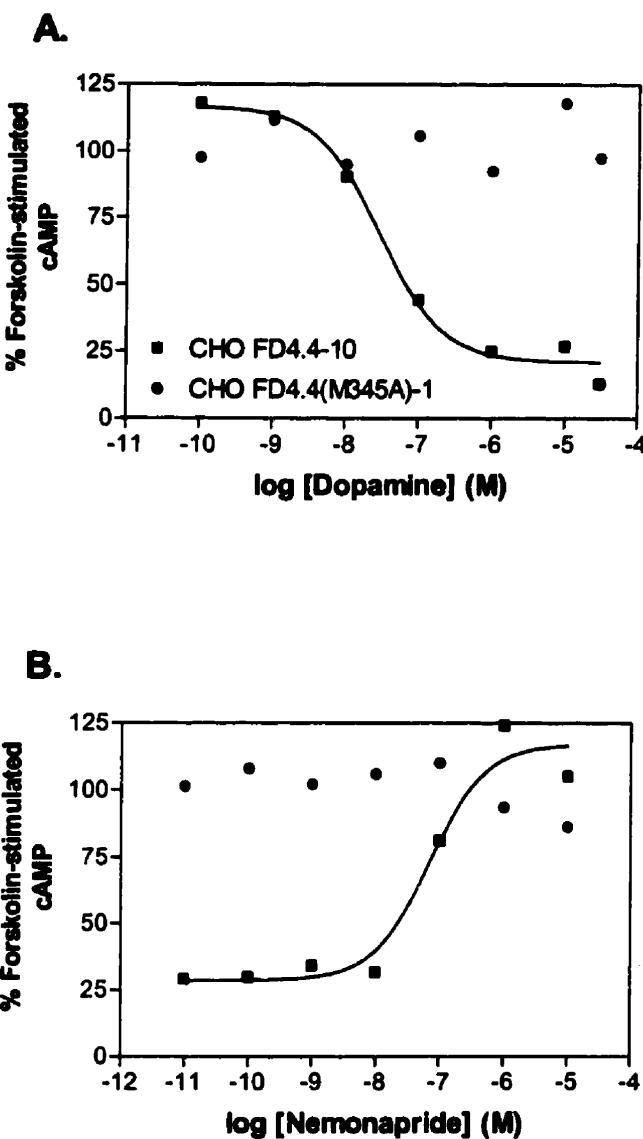


Figure 13. Inhibition of forskolin-stimulated cAMP by FLAG-tagged wildtype and mutant D₄ receptors.

Stably transfected cell lines CHO K1 FD4.4-10 (■) and CHO FD4.4(M345A)-1 (●) were treated with 10 μ M forskolin to activate adenylyl cyclase and stimulate cAMP levels. Cells were treated with drugs for 15 min. prior to collecting cell lysates, and the cAMP level was measured using a cAMP RIA (Sections III.D.1 and 2). A representative curve of two independent experiments is shown in each case, and data points are the mean of three measurements.

A. Inhibition of cAMP by 0.1 nM to 30 μ M dopamine.

B. Blockade of cAMP inhibition (1 μ M dopamine) by 0.01 nM to 10 μ M nemonapride.

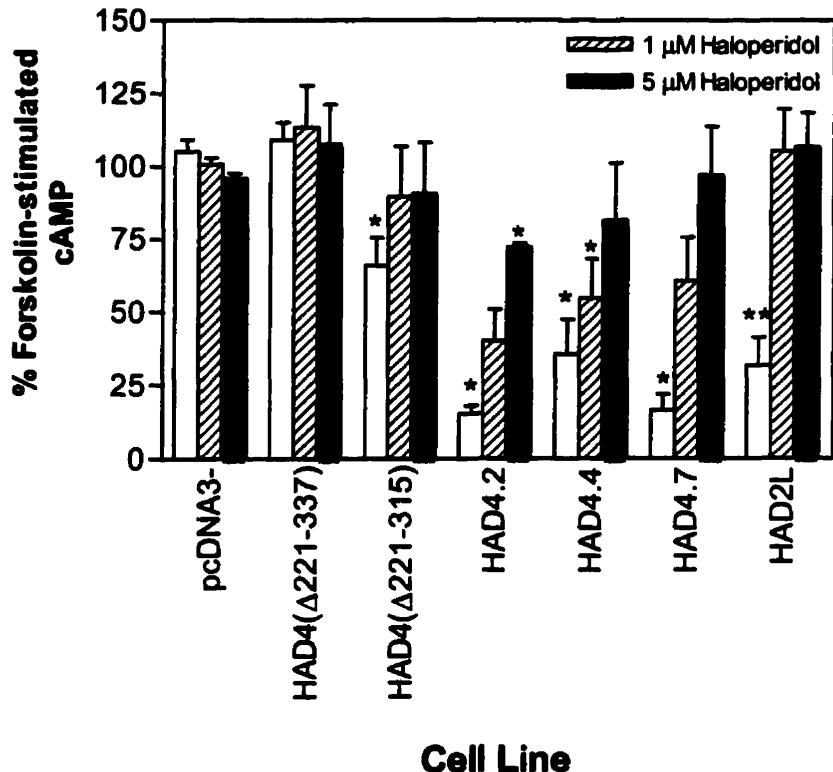


Figure 14. Inhibition of forskolin-stimulated cAMP by HA-tagged wildtype and mutant D₄ receptors.

Stably transfected CHO cells were treated with 10 μM forskolin to activate adenylyl cyclase and stimulate cAMP levels. Cells were treated with 1 μM dopamine (□) or with 1 μM dopamine + 1 μM/5 μM haloperidol for 15 min. prior to collecting cell lysates, and the cAMP level was measured using a cAMP RIA (Sections III.D.1 and 2). The results were standardized by setting forskolin-stimulated cAMP levels at 100% and represent the mean ± standard deviation for 3 to 5 independent experiments. Groups that showed a significant reduction in intracellular cAMP levels due to dopamine are indicated as follows: **, p<0.01; *, p<0.05, as determined using a paired t-test.

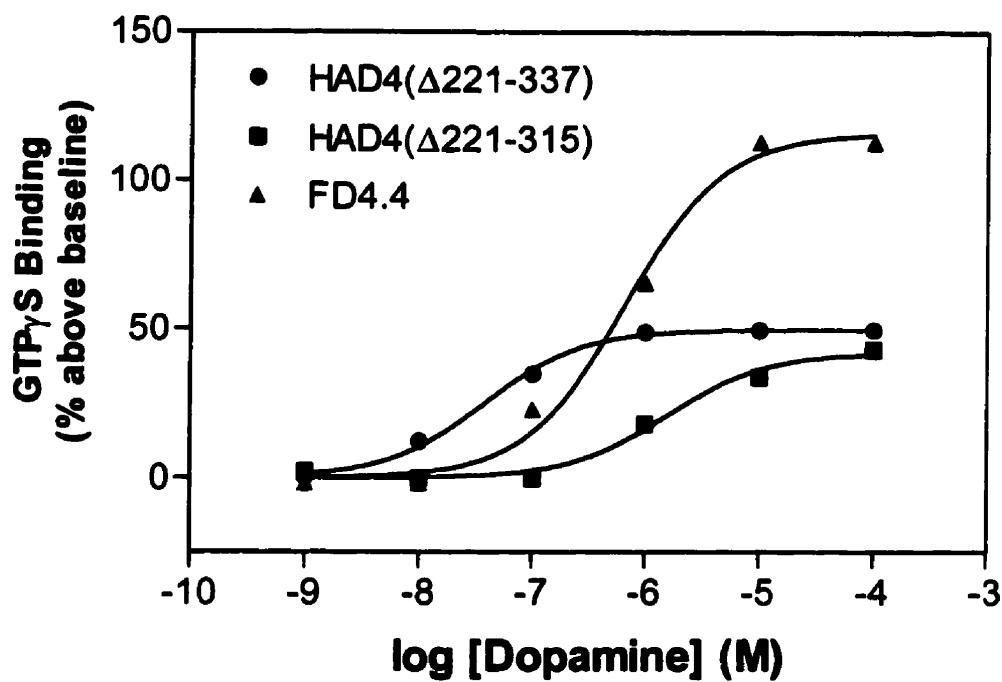


Figure 15. Dopamine stimulation of [35 S]GTP γ S binding to membranes from CHO cells expressing FLAG- and HA-tagged D₄ receptors.

Binding assays on cell membranes from CHO HAD4(Δ221-337)-7, CHO HAD4(221-315)-8, and CHO FD4.4-10 were carried out at 30 °C for 30 min.. with 0.145 nM [35 S]GTP γ S and between 1 nM and 100 μ M dopamine (Section III.C.5).

dopamine was more potent at stimulating guanine nucleotide exchange ($EC_{50}=77.6$ nM). Surprisingly, dopamine was less potent at stimulating [35 S]GTP γ S binding with the cell line CHO HAD4(Δ 221-315), with an $EC_{50}=2.2$ μ M.

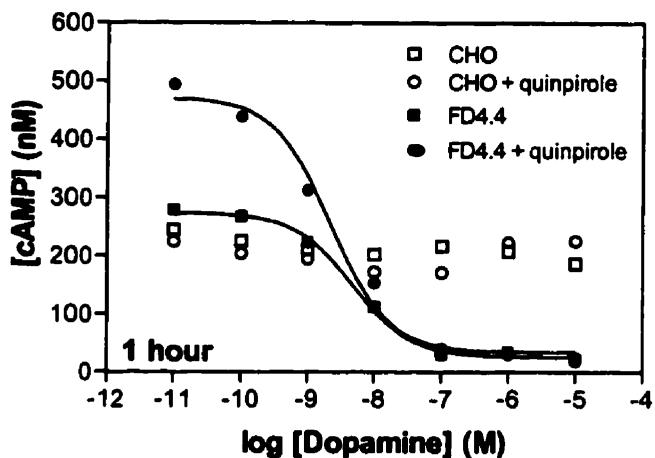
3. Sensitization of Adenylyl Cyclase after Quinpirole Preincubation

The cell line CHO FD4.4-10 was studied to determine whether dopamine D₄ receptors undergo desensitization due to extended exposure to 1 μ M quinpirole. Quinpirole has a similar affinity for D₂-like receptors as dopamine, but does not undergo rapid oxidation. After a 1 hr. or 12 hr. preincubation with quinpirole, no alteration in the response of CHO K1 control cells to forskolin or dopamine is observed compared with these cells treated with media + vehicle (Figure 16A and B). In contrast, a marked sensitization of the adenylyl cyclase response to forskolin was consistently observed with CHO FD4.4-10 preincubated with 1 μ M quinpirole. After a 12 hr. pretreatment, forskolin-stimulated cAMP levels were increased ~2.5-fold. Despite exposure to agonist for up to 12 hr., the potency and efficacy of dopamine-mediated inhibition of cAMP was not significantly changed. After 1 hr. of receptor stimulation with quinpirole, the EC_{50} of dopamine with HAD4.4 was 6.4 ± 3.4 nM (mean \pm s.d., N=3), compared with an $EC_{50}=7.3 \pm 4.8$ nM (mean \pm s.d., N=3) in cells that were not preincubated. After 12 hrs. preincubation, a reduction in the potency of dopamine was observed, with an $EC_{50}=11 \pm 10$ nM (mean \pm s.d., N=3), compared with an $EC_{50}=2.7 \pm 1.0$ nM for control CHO FD4.4-10 cells. However, due to experimental variation, the difference in dopamine EC_{50} of control vs. preincubated cells is not statistically significant at the 95% confidence interval. In addition, the efficacy of dopamine for reducing forskolin-stimulated cAMP levels was not changed after 1 hr. or 12 hr. preincubation with quinpirole. Dopamine continued to inhibit more than 75% of forskolin-elevated cAMP in both control and preincubated cells.

4. Activation of MAPK by Dopamine D₄ and D_{2L} Receptors

The ability of full-length D_{4.2}, D_{4.4}, D_{4.7}, and D_{2L} to stimulate the kinase activity of ERK1/2 was determined using the pharmacologically-characterized CHO cell lines stably expressing receptors. Figure 17A shows that 1 μ M dopamine dramatically increased the catalytic activity of MAPK as measured by the phosphorylation of Elk-1. In contrast, the control cell line CHO pcDNA3- did not respond to dopamine. The stimulation of MAPK activity in these lysates

A.



B.

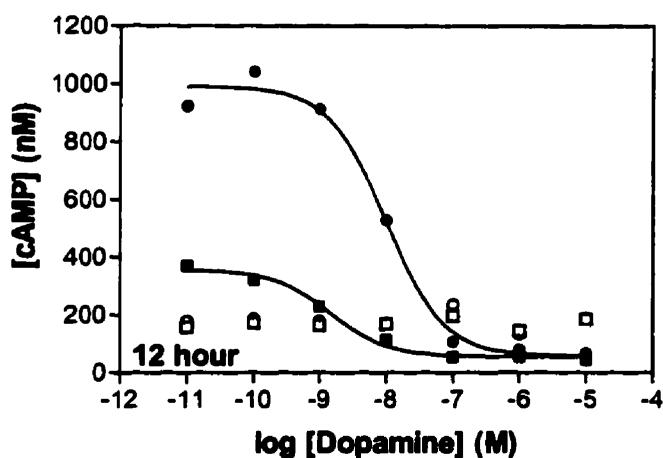


Figure 16. Sensitization of adenylyl cyclase activity in CHO cells expressing FD4.4.
Control cells and cells expressing FLAG-tagged D₄ were preincubated with media + vehicle (control) (□, ■) or with 1 μ M quinpirole (○, ●) for A. 1 hr. and B. 12 hr. (Section III.D.1). After preincubation, cells were washed twice with buffer and assayed for dopamine-mediated inhibition of forskolin-stimulated cAMP levels (10 μ M forskolin). Representative curves from three independent experiments are shown.

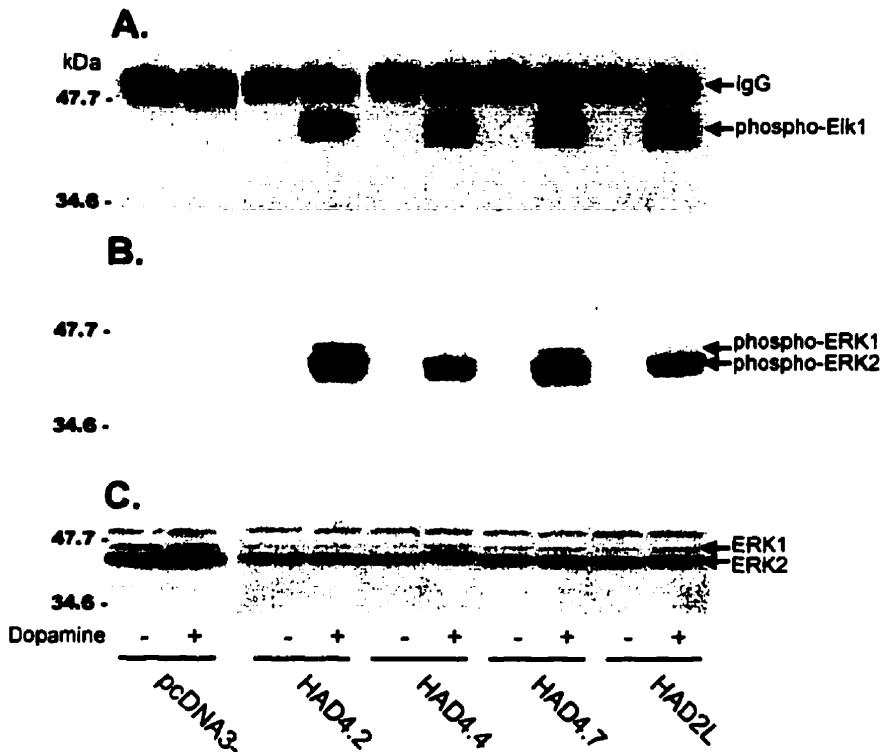


Figure 17. Stimulation of MAPK activity and phosphorylation by dopamine.

The activation of MAPK in serum-deprived CHO cells stably transfected with pcDNA3- (control) or HA epitope-tagged D₄ and D_{2L} was measured after a 5 min. incubation with dopamine (1 μ M) (Section III.D.1.b) **A.** Activity of MAPK after dopamine treatment. Phosphorylated ERK1 and ERK2 (p44 and p42 MAPK) was immunoprecipitated from cell lysates with anti-phosphoMAPK(Tyr204) antibody and Protein A-sepharose. MAPK catalytic activity was measured by a kinase assay with a recombinant MAPK substrate, Elk-1. The phosphorylated Elk-1 was detected by Western blotting with anti-phosphElk-1/horseradish peroxidase-coupled anti-rabbit antibody. **B.** Phosphorylation of ERK1/2 after dopamine treatment. Phosphorylated MAPK was detected directly from cell lysates by immunoblotting with anti-phosphoMAPK(Thr202/Tyr204)/anti-mouse IgG-horseradish peroxidase. **C.** Total ERK1/ERK2 levels in unstimulated and stimulated CHO cells. Total (ie. unphosphorylated + phosphorylated) ERK1/2 was measured in cell lysates by Western blotting with anti-p44/p42 MAPK antibody/anti-rabbit IgG-horseradish peroxidase. Western blotting was carried out as discussed in Section III.D.3.a, b.

corresponds to an increase in dually-phosphorylated ERK1/2 (Figure 17B), while total cellular ERK1/2 levels were unchanged (Figure 17C). ERK2 (p42 MAPK) phosphorylation by dopamine was more prominent, although this probably reflects the higher expression of this isoform in CHO cells that is apparent in Figure 17C.

Many reports have found that MAPK activation by many GPCRs is transient in CHO cells (Pullarkat *et al.*, 1998; Bouaboula *et al.*, 1997; Polakiewicz *et al.*, 1998), while other receptors such as the M1/M3 mAChR and the AT_{1A} angiotensin receptor can produce a sustained stimulation of MAPK in this cell line (Wotta *et al.*, 1998; Conchon *et al.*, 1997). To assess whether differences exist between the timecourse of MAPK activation by D₄ and D_{2L}, the CHO cell lines were treated with 1 μ M dopamine for up to 60 min. (Figure 18). In all cases, maximum MAPK stimulation occurred after approximately 5 min., followed by a gradual desensitization after longer exposure to agonist.

The pharmacology of D_{4,2} coupling to MAPK is similar to that of adenylyl cyclase inhibition. Figure 19 indicates that while no significant stimulation of ERK1/2 phosphorylation can be observed after a 5 min. incubation with 1 nM dopamine, 10 nM dopamine significantly activated MAPK in these cells. Therefore it appears that dopamine receptor-mediated high affinity binding of dopamine is stimulating this pathway.

The two deletion mutants which could be expressed at high levels, HAD4(Δ 221-337) and HAD4(Δ 221-315), were also tested to determine whether coupling to MAPK stimulation can be observed. The response of ERK1/2 was surveyed after exposure to 1 μ M quinpirole (Figure 20). While the full-length D_{4,7} produced a clear stimulation of phosphorylation, no response could be observed for either deletion mutant, although the rabbit polyclonal anti-phosphoMAPK antibody used in this experiment appears to be less sensitive than the mouse monoclonal antibody employed in Figures 18 and 19. MAPK activation in CHO HAD4.7 by the specific D₂-like receptor agonist quinpirole further verified that this response occurs via the D_{4,7} receptor, as did the partial inhibition by the D₂-like antagonist spiperone (10 μ M).

In order to determine whether activation of ERK1/2 by dopamine was mediated by a G_i/G_o-coupled or PKC-mediated mechanism, stimulation of MAPK phosphorylation was examined after incubation with PTX (200 ng/mL) or PMA (5 μ M) overnight (Figure 21). The phosphorylation state of ERK1/2 in control CHO pcDNA3- cells, which were stably transfected with empty vector, was not altered due to treatment with dopamine agonists or antagonists. In

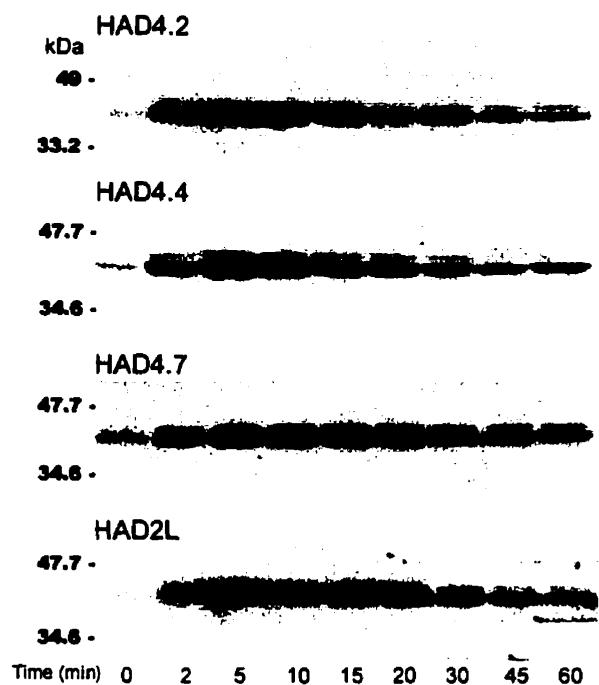


Figure 18. Time course of MAPK activation by dopamine.

Serum-deprived CHO cells stably expressing HA-tagged D_{4.2}, D_{4.4}, D_{4.7}, and D_{2L} were treated with dopamine (1 μ M) for up to 60 min. After drug incubation, phosphoMAPK(Thr202/Tyr204) level was measured in cell lysates by Western blotting as explained in Section III.D.3.b.

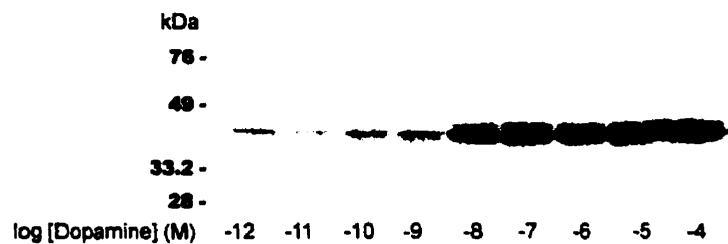


Figure 19. Dose-response of MAPK activation by dopamine.

The stable cell line CHO HAD4.2-35 was serum-starved overnight, followed by treatment with dopamine (1 pM to 100 μ M) for 5 min. After drug incubation, phosphoMAPK(Thr202/Tyr204) level was measured in cell lysates by Western blotting as explained in Section III.D.3.b.

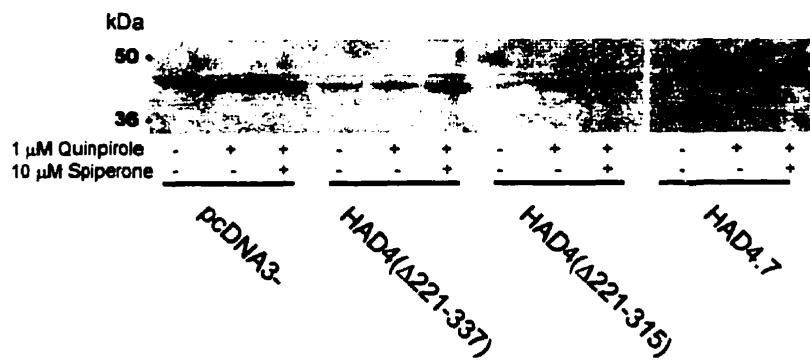


Figure 20. Impairment of MAPK activation in dopamine D₄ deletion mutants.

Stably transfected CHO cells incubated without serum overnight were treated for 5 min. with media alone, with quinpirole (1 μ M), or with quinpirole (1 μ M) and the D₄ antagonist spiperone (10 μ M). Stimulation of ERK1/ERK2 phosphorylation was detected by Western blotting with anti-phosphoMAPK(Tyr204) antibody as described in Section III.D.3.b.

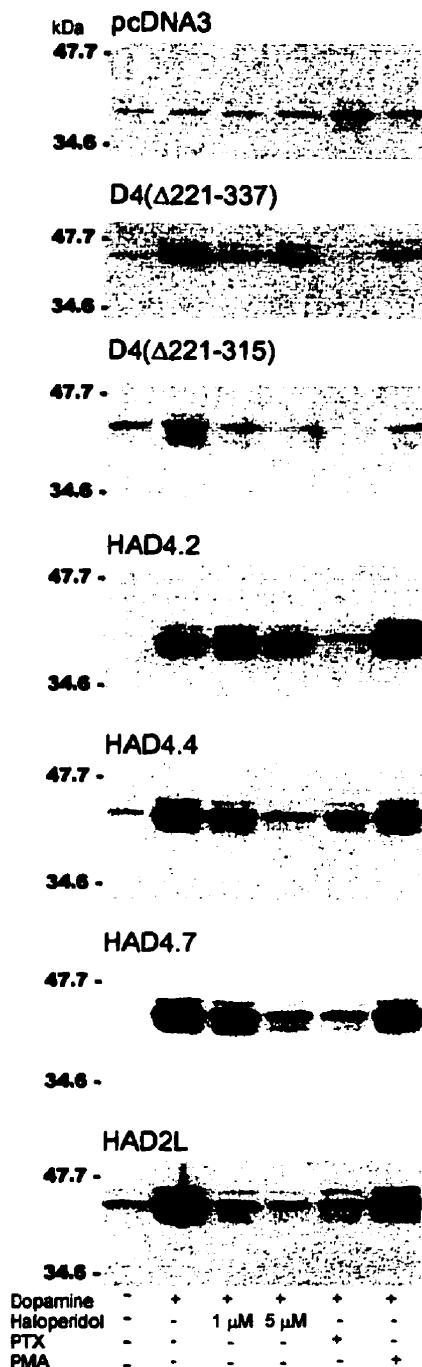


Figure 21. Haloperidol and pertussis-toxin sensitivity of MAPK activation by dopamine. CHO cells stably expressing pcDNA3 (control) or HA-tagged receptors were incubated with serum-free media for 18 hr. PTX (200 ng/mL) or PMA (5 μ M) was included with some cells to block stimulation of G_i/G_o and protein kinase C, respectively. MAPK activation by dopamine (1 μ M) was measured in the absence or presence of haloperidol (1 μ M, 5 μ M). After incubating with drugs for 5 min., phosphoMAPK(Thr202/Tyr204) levels were measured in cell lysates by Western blotting as explained in Section III.D.3.b.

contrast, the full-length D_{4,2}, D_{4,4}, D_{4,7}, and D_{2L} showed potent stimulation of MAPK that was partially inhibited in a dose-dependent manner by 1 - 5 µM haloperidol. The response in PTX-treated cells was almost eliminated, which indicates that the activation of this pathway by D₂ and D₄ is coupled through G_i/G_o. PMA pretreatment did not result in any noticeable inhibition of this response by D₄, and a potentiation of MAPK activation by HAD4.2 was consistently observed (N=3). Figure 21 hints that some weak dopamine receptor activity may be present in the cell lines expressing HAD4(Δ221-315) and HAD4(Δ221-315), although it is apparent that coupling in these receptors is significantly impaired.

V. DISCUSSION

A. THE POLYMORPHIC D₄ RECEPTOR

The enigma of the dopamine D₄ receptor's polymorphic third cytoplasmic loop has eluded explanation since its discovery (Van Tol *et al.*, 1992). With twenty different variants identified in the population, each containing between two and ten 16 amino acid repeats, it is clear that tremendous structural heterogeneity is possible in these receptors (Asghari *et al.*, 1994). Despite this fact, pharmacological and biochemical characterization of D_{4,2}, D_{4,4}, and D_{4,7} failed to find significant differences between these receptor forms (Van Tol *et al.*, 1992; Asghari *et al.*, 1994; Asghari *et al.*, 1995). Deletion of the entire repeat region (amino acids 221 to 315 of D_{4,4}) did not dramatically alter the ligand binding properties. The polymorphic region is also not essential for coupling to G proteins, since deletion of residues 254 to 315 of D_{4,4} resulted in a receptor that was capable of inhibiting forskolin-stimulated cAMP (Asghari *et al.*, 1995).

Many genetic association studies have attempted to find a relationship between the various alleles of D₄ and behavior. Several reports have emerged claiming an association between the personality trait of novelty seeking (impulsive, exploratory behavior) or substance abuse and the longer alleles of D₄ (Ebstein *et al.*, 1996; Benjamin *et al.*, 1996; Li *et al.*, 1997). However, other published reports failed to replicate these findings in their samples (Vandenbergh *et al.*, 1997; Gelernter *et al.*, 1997; Parsian *et al.*, 1997). While a small contribution to the variance in the trait of novelty seeking due to D₄ may or may not exist, the basic biochemical connection between the length of the exon III polymorphism and receptor function has not been resolved.

Examination of the amino acid sequence of the repeat region (Figure 5) shows that the third cytoplasmic loop is proline-rich, with numerous PPXP and PXXP sequences which are typical of SH3 binding domains (Cohen *et al.*, 1995). To study the hypothesis that the repeat region of D₄ may bind to SH3 domains, we tested whether the receptor could interact with the SH3 domains contained within the proteins Abl, Crk, p85 (PI3-kinase), PLC γ , spectrin, c-Src, and Grb2 (Oldenhof *et al.*, 1998). *In vitro*, full-length D₄ and the D₄ third cytoplasmic loop could interact with Nck-GST and Grb2-GST fusion proteins. In addition, rat D₄, D₃, β_1 -AR, and M4 mAChR, which all contain PXXP motifs, could interact with Grb2. Using saturation binding, the affinity constants (K_d) of the Nck and Grb2 interactions with a D_{4,4} fragment (amino acids 220-336) were 40 μ M and 10 μ M, respectively. While the deletion mutant D4(Δ 254-315) showed no

reduction in Grb2 binding, the loss of the amino terminal PXXP motif in D4(Δ 221-315) dramatically reduced the affinity ($K_d > 600 \mu\text{M}$), while deletion of both the amino and carboxyl SH3 binding domains (D4(Δ 221-337)) abolished binding. Similarly, both SH3 domains of Grb2 were required for maximal binding with D₄, indicating that a cooperative interaction with two SH3 domains occurs.

While this study came to the exciting conclusion that SH3 domains can bind to GPCRs, the implication of these interactions *in vivo* is unknown. Based on the hypothesis that additional proteins may associate with the D_{4,4} third cytoplasmic loop, we screened human and rat yeast two-hybrid expression libraries in an attempt to isolate the cDNAs of proteins which interact with this domain. Unfortunately, this strategy was unsuccessful, possibly due to the relatively low affinity of SH3 interactions as well as autoactivation of reporter genes by the GAL4-D4.4 fusion protein.

B. EPITOPE-TAGGED D₄ RECEPTORS

It has become clear that closely-related GPCRs can be differentiated by their trafficking and sequestration, as well as by their functional coupling. For example, among the homologous members of the α_2 -adrenergic receptor family, the α_{2A} -AR and α_{2B} -AR are expressed on the plasma membrane, while the α_{2C} -AR is predominantly located in the endoplasmic reticulum and cis/medial Golgi (Daunt *et al.*, 1997). In addition, while the α_{2B} -AR subtype undergoes internalization in response to agonists, the α_{2A} -subtype was not redistributed as effectively. Besides possible serine and threonine phosphorylation sites, additional sequences appear to direct trafficking. For example, mutation of a highly conserved NPXXY sequence in GPCRs has been shown to affect endocytosis (Barak *et al.*, 1994; Wang *et al.*, 1997; Böhm *et al.*, 1997b), although in the case of the β_2 -AR, this deficit could be overcome by β -arrestin overexpression (Ferguson *et al.*, 1996b). A pair of leucine residues at the carboxyl-terminal tail of the β_2 -AR are also required for agonist-induced internalization (Gabilondo *et al.*, 1997). Saunders *et al.* (1998) reported that multiple sequences within the α_{2A} -AR can direct this receptor to the basolateral surface of cultured kidney cells. In the D₂ receptor, alternate splicing leading to a 29 amino acid insert in the third cytoplasmic loop is now known to affect receptor processing, trafficking, and localization in neurons (Fishburn *et al.*, 1995; Khan *et al.*, 1998).

In order to investigate whether the repetitive 16 amino acid repeats, or the recently

identified SH3 binding domains flanking this sequence, are important in D₄ receptor trafficking, it was necessary to either 1) develop an antibody to the D₄ receptor or 2) add an additional epitope sequence that is recognized by a commercially available antibody. The former strategy allows the use of existing receptor constructs and cell lines, as well as providing a means of detecting D₄ *in vivo*. However, preparation of antibodies to GPCRs is a laborious task, and they are often of poor quality. In fact, success in producing antibodies to human D₄ was not reported until recently (Lanau *et al.*, 1997). Therefore, our approach was to add an epitope tag to the amino terminal extracellular domain of the D₄ receptor. A FLAG epitope and an HA epitope, which are recognized by the well-characterized monoclonal antibodies M1 and 12CA5, were utilized. These antibodies have been previously used to immunologically detect other GPCRs (Barak *et al.*, 1994; Chu *et al.*, 1997; Cao *et al.*, 1998). The epitope sequence was preceded by a cleavable, modified influenza hemagglutinin signal sequence, which was shown to double the production of functional β₂-AR and D₂ in Sf9 cells (Guan *et al.*, 1992; Grünwald *et al.*, 1996a).

The FLAG sequence was initially used to immunologically detect D_{4.4} and to determine whether the signal sequence or epitope tag altered the structure and function of the receptor. Results indicating that this receptor, FD4.4, was functionally unaltered (discussed below) led to the use of this FLAG-tagged construct in producing a D_{4.4} point mutant at amino acid 345 in the third cytoplasmic loop (M→A). The epitope sequence provides a second means of assessing receptor expression, along with radioligand binding. A technical limitation of the FLAG tag was subsequently discovered. The last amino acid of the complete FLAG tag, a lysine, is replaced by an alanine in FD4.4. While this does not affect recognition by the M1 anti-FLAG antibody, the useful M2 anti-FLAG antibody does not detect our receptor. While the M2 antibody has no metal requirement, the M1 antibody requires Ca²⁺ for epitope recognition. This problem led us to use the HA epitope.

Native D₄ receptors with two, four, and seven repeats, several D₄ deletion mutants, and the D_{2L} receptor were modified by addition of the HA sequence. We intended to characterize the ligand binding and functional coupling properties of these receptors, and subsequently utilize the epitope tag to study the expression, trafficking, and phosphorylation state of dopamine receptors. The trafficking properties and phosphorylation state of D₄ are presently unknown. In addition, the effect of two previously undescribed D₄ mutants, HAD4(Δ221-337) and FD4.4(M345A), were characterized in this study. While receptor binding was previously shown to be unaffected

by deletion of residues 221-315 of D_{4,4} (Asghari *et al.*, 1994), we expanded our knowledge of this mutant by measuring functional coupling in HAD4(Δ221-315).

C. RECEPTOR PHARMACOLOGY

1. Wildtype Receptors

While numerous receptors have been modified by additions to their amino terminus, changes in this domain can affect the receptor. For example, deletion of the initiation codon and the first 25 downstream nucleotides of D₄ results in expression of a truncated receptor by use of an alternate initiation site (Schoots *et al.*, 1996). While this cropped D₄ had normal antagonist binding, the K_d and EC₅₀ of dopamine was reduced more than 10-fold, although the receptor retained functional coupling. However, other experiments using site directed mutagenesis to replace potentially glycosylated asparagines in the M2 mAChR and H2 histamine receptor found that the pharmacology was insensitive to structural modifications in this domain (van Koppen & Nathanson, 1990; Fukushima *et al.*, 1995). Dixon *et al.* (1987) deleted residues 5-16 and 21-30 from the amino terminus of the β₂-AR without a significant change in receptor pharmacology. However, the need for caution in the use of epitope tagged receptors was recently emphasized in a report by Tolbert and Lameh (1998), who found that antibodies to an amino terminal epitope tag on the M1 mAChR resulted in the internalization of the receptor by clathrin-coated vesicles in the absence of agonists.

Transfection and expression of RSV SSFD4.4 in CHO K1 led to the isolation of a clonal cell line continuously expressing the receptor, CHO FD4.4-10. The B_{max} was close to the receptor density of a well characterized cell line, CHO D4.4-7, which expresses an unaltered receptor. Radioligand binding experiments determined that the antagonist and agonist pharmacology was unchanged compared with data from CHO D4.4-7. Therefore, it appears that addition of a cleavable signal sequence and an eight amino acid epitope sequence to the amino terminal extracellular domain did not affect receptor pharmacology.

Stable expression of wildtype, HA-tagged dopamine receptors (HAD4.2, HAD4.4, HAD4.7, HAD2L, and FD4.4) in CHO K1 cells resulted in the isolation of clonal cell lines with high-affinity, saturable [³H]spiperone binding. Clones expressing HA-tagged receptors at a density of ~1 pmol/mg were characterized. The antagonist binding affinities of these receptors were indistinguishable from previously published values determined for the unmodified receptors

in various cell lines (Table 2). The affinity of five antagonists ($[^3\text{H}]$ spiperone, nemonapride, haloperidol, clozapine, raclopride) agreed with earlier studies of untagged D_4 using CHO K1 cells, with the exception that clozapine's affinity was ~4-fold lower for the HA-tagged receptors (Asghari *et al.*, 1994; Asghari *et al.*, 1995). However, Hidaka *et al.* (1995) found the clozapine affinity constant for D_4 receptors, derived from $[^3\text{H}]$ spiperone competition experiments with $\text{D}_{4,2}$, $\text{D}_{4,4}$, and $\text{D}_{4,7}$, to be 49 to 56 nM, which is close to our results. Dopamine binding was also unchanged in the epitope-tagged receptors. While the K_L values are higher than those reported by Asghari *et al.* (1994), another study of human D_4 expressed in CHO cells found $K_H=3.0$ nM and $K_L=607$ nM (Chio *et al.*, 1994). Thus, the difference in dopamine affinity constants between wildtype D_4 and epitope-tagged D_4 are probably explained by variability between experiments and do not represent an altered pharmacology. All receptors except the largest deletion mutant showed a shift in affinity for dopamine after incubation with Gpp[NH]p. This GTP analogue is capable of uncoupling G proteins from receptors, resulting in a greater proportion of low-affinity dopamine binding.

2. Mutant Receptors

Three HA-tagged D_4 deletion mutants were also stably expressed in CHO K1 cells. Similar to previously reported problems expressing untagged $\text{D}_4(\Delta 254-315)$ (Asghari *et al.*, 1995), we were unable to isolate a clone expressing HAD4($\Delta 254-315$) at levels near that of the wildtype receptors, despite screening 46 separate stably transfected CHO K1 cell lines. Since the best expression that was attained was still more than 10-fold less than that of the wildtype receptors, and given that the pharmacology and functional coupling of the mutant were previously described, this mutant was not characterized further. The stable cell lines CHO HAD4($\Delta 221-337$)-7 and CHO HAD4($\Delta 221-315$)-8, with a B_{\max} close to 1 pmol/mg membrane protein, were studied. Despite the deletion of up to 116 amino acids from the third intracellular domain of $\text{D}_{4,4}$, mutant antagonist pharmacology was equivalent to that of the wildtype receptors. This may be unsurprising, since the site of ligand binding has been convincingly mapped to the transmembrane domains in bioamine GPCRs (Strader *et al.*, 1994). In fact, large deletions in the third cytoplasmic loop of the β_2 -AR, M1 mAChR, and M4 mAChR (consisting of 33 - 130 amino acids) did not significantly alter the affinity of ligands (Strader *et al.*, 1987; Shapiro & Nathanson, 1989; van Koppen *et al.*, 1994). The typical D_4 pharmacology of the mutants

indicates that there is no significant disruption in the overall tertiary structure. Therefore, they can provide a useful tool to delineate the role of these D₄ third cytoplasmic loop sequences in signal transduction, trafficking, and protein-protein interactions.

With regards to dopamine binding, both deletion mutants showed a reduced affinity for this agonist. In addition, dopamine binding by HAD4(Δ221-337) did not display guanine nucleotide sensitivity. While the failure of dopamine binding by this mutant to respond to Gpp[NH]p may indicate an inability of this mutant to activate G proteins or a lack of G protein coupling, that is not necessarily the case. For example, the D₃ receptor expressed in CHO cells has a high affinity for dopamine in the presence of GTP (Chio *et al.*, 1993). Despite this fact, the receptor was able to couple efficiently to G proteins. In addition, some dopamine agonists such as bromocriptine do not appear to discriminate between low and high states of the receptor, although they do result in G protein activation (Gardner *et al.*, 1997).

Surprisingly, the D_{4,4} point mutant M345A appears to lack high affinity [³H]spiperone binding and has a reduced affinity for [³H]nemonapride, although the K_d could not be determined from saturation binding with a stable CHO K1 cell line. These conclusions were supported by examination of a second CHO K1 D4.4(M345A) stable cell line as well as the transient expression of RSV-, RSV FD4(M345A), and RSV FD4.4 in HEK 293 cells (data not shown). In both cases, significant [³H]nemonapride binding but little [³H]spiperone binding was observed, while expression was confirmed by Western blotting. Using [³H]nemonapride as a probe, CHO FD4.4(M345A)-1 binding was displaced by haloperidol, but not spiperone, clozapine or dopamine. It appears that this mutation, located near the putative i3/TM 6 junction, may disrupt the structure of the receptor and abolish the binding of most ligands. The dramatic reduction in affinity is surprising, given that various chimeric D1/D2 receptors did not lose dopamine and antagonist binding (Kozell *et al.*, 1994). For example, the affinity of spiperone was only reduced 10-fold when the D₂ TM 5 to TM 6 sequence was replaced with the corresponding D₁ sequence (Kozell *et al.*, 1994). Interestingly, a chimeric D_{2L}/D₄ receptor (with the junction in the third TM domain) did not exhibit ligand binding (Shih *et al.*, 1997). When the reciprocal D₄/D_{2L} chimera was expressed, the ratio of the affinity constants (chimera/D₄ and chimera/D_{2L}) indicated that binding of antagonists and agonists was significantly altered in the chimera. A notable exception was haloperidol, where these ratios were close to unity. Various D₂/D₄ chimeras produced in our lab also had significantly reduced or abolished drug binding (Dr. H. Van Tol, personal commun-.

cation). Based on these observations, it appears that the D₄ receptor may be particularly sensitive to structural changes, leading to disruption of the ligand binding domain. This may explain the loss of binding in the HAD4.4(M345A) point mutant, since methionine 345 lies close to the predicted, yet undefined, location of transmembrane α -helix 6.

D. IMMUNOBLOTTING OF D₄ AN D_{2L} RECEPTORS

The utility of the epitope tag was proven by immunoprecipitation and immunoblotting of both wildtype and mutant dopamine receptors. Western blotting of the FD4.4 receptor was hampered by the presence of the M1 antibody IgG bands. However, it appears that glycosylated and unglycosylated full-length FLAG-tagged D_{4,4} is present. Since the expression of receptor in CHO FD4.4(M345A)-1 could not be estimated by radioligand binding, we used blotting to estimate the expression level. Based on Figure 11, it appears that the expression level is approximately 6-fold lower than that of the wildtype FD4.4 cell line.

Full-length glycosylated and unglycosylated HA-tagged receptors could be seen in immunoblots of D_{4,2}, D_{4,4}, and D_{4,7}, and the estimated molecular weights were 1 to 2 kDa larger than those reported by Lanua *et al.* (1997), as would be expected due to the additional sequence at the amino terminus. The molecular weight of the unglycosylated receptors are in good agreement with the predicted values, with the exception that HAD4.7 migrated at 56 kDa by SDS-PAGE, despite the predicted size of 50 kDa. A lower than expected electrophoretic mobility of the repeat region has been noted previously (Lanau *et al.*, 1997). The third cytoplasmic loop itself (amino acids 220 to 336 of D4.4), when expressed *in vitro*, has been shown to migrate at approximately 30 kDa compared to a predicted molecular weight of 20 kDa (Oldenhof *et al.*, 1998).

With HAD2L, it appears that the receptor is almost completely glycosylated when expressed in CHO cells, similar to previously reported observations (Fishburn *et al.*, 1995). The estimated molecular weight of D_{2L} (55 kDa) most likely represents glycosylated receptor, as other studies with transfected D_{2L} found that a 45 - 48 kDa species can be converted to a 39 - 40 kDa unglycosylated polypeptide by treatment with tunicamycin or N-glycanase (Fishburn *et al.*, 1995; Grünwald *et al.*, 1996a). It appears that recombinant D₂ receptors are not as extensively glycosylated as the native receptor from brain tissue. The initial purification and characterization of D₂ from the brain estimated the size of the receptor to be approx. 94 - 140 kDa using photo-

labelling and radiation inactivation (Bouvier *et al.*, 1986; Jarvie *et al.*, 1988). Cleavage of terminal sialic acid residues converted the native D₂ to a species of 50 - 54 kDa, which is close to the observed migration of D_{2L} in our study (Jarvie *et al.*, 1988; Clagget-Dame & McKelvy, 1989). The further removal of all N-linked sugars produced a species of 40 - 44 kDa which represents the peptide backbone of D₂.

Several interesting observations can be made from the detection of the D₄ protein. The apparent propensity for the deletion mutants to form denaturation-resistant oligomers is intriguing. The molecular weight of these bands (eg. 32, 55, and 79 kDa for unglycosylated HAD4(Δ221-315)) suggests they may represent monomer, dimer, and trimer forms of these receptors. This is similar to the reports of TM domain-mediated dimerization of the D_{2L} receptor (Ng *et al.*, 1996). We did not observe detectable dimerization of D_{2L} in these cells, however, and it remains to be seen whether our observations with the deletion mutants simply represent nonspecific aggregation. It is interesting to note that SDS-resistant dimers of GPCRs, initially discounted as an artefact, can be specifically inhibited by peptides derived from the trans-membrane domains (Hebert *et al.*, 1996; Ng *et al.*, 1996).

Secondly, a 38 kDa band immunoprecipitated from cells expressing D_{4,2}, D_{4,4}, and D_{4,7}. This protein product has been observed by others and was previously postulated to be a degradation product (Lanau *et al.*, 1997). When treated with tunicamycin, this form migrates at 34 kDa, while HAD4(Δ221-315) is found at 32 kDa. Based on the electrophoretic properties of the other receptor species, we estimate that this fragment is approximately 391 amino acids long. Given that we are detecting the amino-terminal tag, a peptide of this length would extend beyond the third intracellular loop of D_{4,4}. Since the sequence of each variant is of different lengths, the proteolysis site would have to be different in D_{4,2}, D_{4,4}, and D_{4,7} to produce a product with the same size. If these fragments are a degradation product with the same cleavage site, the largest peptide with an identical sequence from all three variants would be 264 amino acids. It is possible that the unusually high apparent molecular weight may be connected to the observation that the third cytoplasmic loop mobility is substantially lower than predicted. Another possibility is that the polymorphic region of the third cytoplasmic loop is excised post-translationally, producing a 32 kDa protein product with an identical primary sequence for all of the D₄ variants. While no examples of such a process are known in GPCRs, protein splicing has been observed in the *S. cerevisiae* vacuolar ATPase subunit (VMA), the *Drosophila* Hedgehog protein, and many

proteins from eubacteria and archaeabacteria (Chong & Xu, 1997; Perler, 1998; Perler *et al.*, 1997). It is also interesting to note that antibodies to mouse and rat D₄ detected bands that migrated at 36 and 41 kDa, respectively (Mrzljak *et al.*, 1996; Mauger *et al.*, 1998). Since the theoretical molecular weight of the rodent D₄ receptor is ~41 kDa, these results imply that the receptor was unglycosylated or that the actual primary sequence is shorter than is predicted by the cDNA sequence.

Finally, there is a contradiction between the equal [³H]spiperone B_{max} values of all HA-tagged receptors and the apparently higher expression of HAD4(Δ221-315) and HAD4(Δ221-337) when determined by immunoprecipitation/blotting. Direct Western blotting from membrane preparations did allow the detection of the major HA-tagged receptor species without the presence of IgG bands (data not shown). However, the high background present in these blots made visualization of lower intensity bands difficult. For unknown reasons, the deletion mutants HAD4(Δ221-337) and HAD4(221-315) were consistently easier to detect compared to the HAD4.2, HAD4.4, HAD4.7, and HAD2L. In contrast, the deletion mutant expression levels in CHO cells, as determined by radioligand binding, were equal to or less than that of the full-length receptors. The results from Western blotting may be an artefact arising from a more efficient electrophoretic transfer of the deletion mutants, or could indicate that a substantial portion of these mutants are not in a conformation that can bind the radioligand.

E. FUNCTIONAL COUPLING

1. Epitope-tagged D₄ and D_{2L} Receptors

The ability of full-length, FLAG-tagged D₄ to inhibit forskolin-stimulated cAMP with an EC₅₀ comparable to untagged D_{4.4} verified that these receptors are not functionally altered. Coupling can also be inferred from the ability of the GTP analogue Gpp[NH]p to promote the formation of a low-affinity dopamine binding site. This “GTP-shift” is known to correlate with functional coupling of GPCRs and is believed to occur in response to the dissociation of the heterotrimeric G protein from the receptor (Kenakin, 1996).

Comparing the percentage of HAD4.2, HAD4.4, and HAD4.7 receptors that have a high affinity for dopamine (~70%) with HAD2L (31%) appears to indicate that a higher proportion of D₄ receptors can couple to G proteins under these conditions. This may imply that the D₄ receptor’s interactions with G proteins are more promiscuous. Another notable deviation

between D₄ and D_{2L} receptors is the ~25-fold difference in the high affinity dissociation constant. The higher affinity of D₄ for dopamine is reflected in the reduced efficacy of haloperidol to reduce dopamine-mediated cAMP inhibition that was observed (see Figure 14).

We have replicated the recently reported finding that the dopamine D_{2L} receptor can transiently activate the MAPK pathway through a pertussis-sensitive G protein (Luo *et al.*, 1998b; Welsh *et al.*, 1998). In addition, we have shown that three isoforms of the D₄ receptor are all capable of activating ERK1/2 with a similar timecourse. The stimulation of ERK1/2 phosphorylation correlated with its catalytic activity, as measured by the phosphorylation of the substrate Elk-1. The specificity of the response to dopamine was confirmed by several lines of evidence: 1) No response to dopamine was seen in CHO cells stably transfected with the empty vector pcDNA3. 2) Stimulation of MAPK by dopamine or quinpirole is inhibited by the antagonists spiperone and haloperidol, indicating the involvement of D₂-like receptors. 3) The dose-response of MAPK activation by dopamine in CHO HAD4.2 has an estimated EC₅₀ was ~10-100 nM. This is in agreement with the inhibition of cAMP in CHO cells, where an EC₅₀ of 14 - 37 nM was reported, depending on the number of the repeat sequences (Asghari *et al.*, 1995). 4) D₄-mediated activation of MAPK involved G_i/G_o. Pertussis toxin is known to block coupling through G_i/G_o by catalyzing the ADP-ribosylation of the α -subunit (Neer, 1995). Long-term exposure to the phorbol ester PMA down-regulates PKC and prevents its subsequent activation (Hepler *et al.*, 1988). ERK1/2 phosphorylation in response to dopamine was almost completely blocked by pertussis toxin, indicating that signal transduction involved G_i/G_o. PKC downregulation by PMA did not appear to greatly affect MAPK stimulation by D_{4,4} or D_{4,7}, although a small reduction is observed with D_{2L}. In contrast, an increase in dopamine-stimulated ERK1/2 phosphorylation after PKC downregulation was consistently observed with D_{4,2}. This may be a characteristic of the clonal CHO cell line itself, and needs to be confirmed in additional clones or in transiently transfected cells.

A recent study found that dopamine can lead to apoptosis by the sustained activation of another MAPK cascade, the SAPK/JNK pathway, in HEK 293 cells (Luo *et al.*, 1998a). This activity was not dependent on the presence of dopamine receptors, required a relatively high dopamine concentration ($\geq 100 \mu\text{M}$), and was prevented by anti-oxidants. However, D₂ can also stimulate a transient increase in ERK and JNK activity at a low dopamine concentration (100 nM) (Luo *et al.*, 1998b). Here, we have confirmed that dopamine's effects on CHO cells stably

transfected with D₄ are not due to oxidative stress, and MAPK activation represents a previously undocumented signal transduction pathway activated by D₄ receptors. This may be unsurprising, given the reported ability of D₂ to stimulate mitogenesis via the activation of MAPK and the now well-characterized ability of many G_i-coupled receptors to stimulate ERK1/2 (Lopez-Illasaca *et al.*, 1997). Yet little is known about the ability of particular G $\beta\gamma$ dimers to stimulate this pathway, although G $\beta_1\gamma_2$ and G $\beta_1\gamma_5$ are known activators (Luttrell *et al.*, 1995; Clapham & Neer, 1997). For example, brain specific G β_5 does not activate MAPK, but does stimulate PLC- β_2 , when expressed with G γ_2 in COS cells, demonstrating that not all G $\beta\gamma$ dimers can activate this kinase cascade (Zhang *et al.*, 1996b).

The stimulation of ERK1/2, and possibly other MAPKs such as JNK and p38, provides a mechanism for the previously observed effects of dopamine in cells expressing D₄. Dopamine stimulation or potentiation of AA release may be mediated by MAPK, since phosphorylation of cPLA₂ by MAPK is required for full activation of this enzyme (Lin *et al.*, 1993). The mitogenic effect of dopamine in CHO 10001 cells expressing D₄ receptors has been measured by increased [³H]thymidine incorporation (Lajiness *et al.*, 1995). This is most likely a result of MAPK stimulation, since dominant-negative RasN17 inhibited both ERK1/2 phosphorylation and [³H]thymidine incorporation in cells expressing D_{2L} (Luo *et al.*, 1998b). Depending on the cell type, duration of the signal, and the pathways activated, MAPKs can also induce differentiation (Seger & Krebs, 1995). The D₄ receptor is known to induce branching extension in neuronal MN9D cells (Swarzenski *et al.*, 1994), and similar neurite outgrowth in PC12 cells has been attributed to the MAPK pathway (Qui & Green, 1992). This change in morphology also appears to require the activation of p38 MAPK, suggesting that D₄ activates this kinase as well (Morooka & Nishida, 1998).

2. Desensitization of the Dopamine D₄ Receptor

To determine whether D₄ undergoes desensitization, we used the approach of Bates *et al.* (1990). Using Ltk⁻ fibroblasts, they found that the EC₅₀ of D₂-mediated inhibition of cAMP by dopamine was significantly increased (~4-fold) after a 1 hr. preincubation with 1 μ M quinpirole, although longer treatment did not increase this effect. In addition, the efficacy of dopamine stimulation of D₂ was not altered, since >90% of forskolin-stimulated cAMP production was inhibited. Others have observed a 5-fold decrease in potency and a 25-30% decrease in efficacy

after exposure to 100 μ M DA for 24 hrs. (Zhang *et al.*, 1994), and a dose- and time-dependent increase in the EC₅₀ and decrease in the efficacy of dopamine, with 50% of the maximum desensitization occurring after preincubation with 2-3 μ M dopamine (Barton *et al.*, 1991). Boundy *et al.* (1995) found biphasic desensitization of the cAMP response in HEK 293 cells expressing D_{2L}, where the EC₅₀ increased >10-fold after a 1.5 hr. treatment with 5 μ M quinpirole, but was unchanged after a 16 hr. treatment.

Since 1 μ M quinpirole is sufficient to produce maximal inhibition of cAMP by transfected D₄ receptors (EC₅₀=4.8 nM) (Chio *et al.*, 1994), we tested the ability of a 1 hr. or 12 hr. pretreatment with this dose of agonist to desensitize the subsequent response of the receptor to dopamine. In contrast to reports with D₂, we failed to find a statistically significant reduction in the EC₅₀ of dopamine. If D₄ desensitization does occur under these conditions, the assay chosen to measure the change in response does not appear to be sensitive enough to small changes in EC₅₀. It is also noted that desensitization may require higher concentrations of agonist than are required for maximal response. For example, in one study the dose of quinpirole required to decrease the number of D₂ receptors in the G protein-coupled state by 50% was ~7 μ M, with a t_{1/2}=15 min. (Boundy *et al.*, 1995). Another report using dopamine pretreatment found that desensitization of cAMP inhibition had an EC₅₀=2 μ M but a t_{1/2}=5hrs. (Zhang *et al.*, 1994). It is known that GRKs preferentially phosphorylate active (agonist occupied) receptors, and therefore are more effective at higher (μ M) concentrations of agonist (Pitcher *et al.*, 1998a). In contrast, lower agonist concentrations may activate signalling pathways, allowing heterologous desensitization. For example, the β_2 -AR is phosphorylated and desensitized by PKA at nanomolar concentrations of the agonist isoproterenol, while β ARKs are active at micromolar concentrations of this drug (Hausdorff *et al.*, 1989). Several G_i/G_o-coupled receptors, including the M2 mAChR and δ -opioid receptor are phosphorylated and desensitized by GRKs (Pals-Rylaarsdam & Hosey, 1997; Kovoor *et al.*, 1997). It is possible that a reduction in dopamine potency at D₄ may require a higher dose of agonist in order to make the receptor a suitable substrate for GRKs. The physiological significance of such concentrations may be questionable, given that nanomolar concentrations of agonist can elicit the maximum functional response. However, microdialysis studies have found that extracellular dopamine levels in the nucleus accumbens of the rat reach 0.25 μ M after a single electrical pulse to the ascending dopaminergic fibres. with four pulses producing 1 μ M dopamine (Garris *et al.*, 1994). Given the much smaller volume of the synaptic

cleft, Garris *et al.* (1994) estimate that the local dopamine concentration at the receptor may reach 1.6 mM.

While the potency of dopamine at transfected D₄ receptors was not altered after quinpirole preincubation, changes in cell physiology were detected which may compensate for the presence of a tonic inhibitory activity. We observed a sensitization of the response of adenylyl cyclase to forskolin after pretreatment. Similar observations have been made with recombinant D₂ receptors (Bates *et al.*, 1990; Boundy *et al.*, 1995), as well as with cell lines endogenously expressing D₂ and the α₂-AR (Ivins *et al.*, 1991; Jones *et al.*, 1987). This effect appears to be time-dependent, as a greater sensitization was observed after 12 hrs. In studies with D₂, this response was found to be blocked by antagonists and PTX, but was not due to low cAMP levels since addition of 8Br-cAMP, a membrane-permeable cAMP analogue, actually increased sensitization (Bates *et al.*, 1990). This report proposed that the sensitization of adenylyl cyclase activity (both basal and forskolin-stimulated), in response to G_i/G_o activation, is a mechanism of increasing the absolute concentration of cAMP in the cell. In terms of the dopamine dose required to reduce cAMP levels to a particular concentration, the combined effect of decreased potency and AC sensitization can reduce the effectiveness of agonists at D₂ significantly (~66-fold). While the EC₅₀ of dopamine at D₄ may not be significantly changed after preincubation for 12 hrs., the concentration required to reduce cAMP levels to 200 nM was increased from 1.6 nM to 50 nM (see Figure 16).

3. Mutant D₄ Receptors

a. D_{4,4} Deletion Mutants Δ221-315 and Δ221-337

Since the antagonist binding and expression level of the deletion mutants HAD4(Δ221-337) and HAD4(Δ221-315) were equivalent to that of the full-length D₄ receptor, experiments were carried out determine whether functional coupling was compromised. The second intracellular loop, third intracellular loop, and the carboxyl-terminus of GPCRs are recognized to interact with G proteins, although the precise involvement of each varies among receptors (Bourne, 1997). For example, O'Dowd studied 19 point and deletion mutants of the β₂-AR to map regions critical to G protein coupling to the C-terminal portion of i3 as well as the membrane-proximal region of the cytoplasmic tail (O'Dowd *et al.*, 1988). Experiments with α₂/β₂, D₁/D₂, and D₂/M1 mAChR chimeras have clearly shown that the specificity of G protein

coupling is primarily dictated by the i3 loop (Kobilka *et al.*, 1988; Kozell *et al.*, 1994; England *et al.*, 1991). In general, there is a lack of conservation in the sequence of the i3 loop among GPCRs from different families. In contrast, highly conserved residues in the i2 loop are crucial for G protein activation. These sequences, such as the DRY motif, can function to activate varying classes of G proteins. The recent systematic mutation of these residues has indicated that ordered clusters of activating and inactivating mutations are spaced 3-4 residues apart, implying an α -helical structure (Burstein *et al.*, 1998). Their conclusion was that two faces of the i2 loop α -helix, 100° apart, allow the receptor to switch conformation from the inactive to active state. The i2 loop is also involved in selectivity for G_s (Nasman *et al.*, 1997), while D_1/D_2 chimeras showed that the i2 loop, as well as the i3 loop, are required for maximum activation of G_i (Kozell *et al.*, 1994). Unlike the ubiquitous role of i2 and i3 in G protein activation, the importance of the C-terminus is receptor dependent. Thus, while deletion of 7 residues in this region of the β_2 -AR reduced coupling 50% (Hausdorff *et al.*, 1989), chimeras seem to indicate that this region does not contribute to specificity of G protein-receptor interaction in the α_2 -AR or D_1 receptor (Kobilka *et al.*, 1988; MacKenzie *et al.*, 1993).

In our study, deletions were made in the central portion of the i3 loop. HAD4(Δ 221-337) represents the removal of 84 ($D_{4,2}$) to 164 ($D_{4,7}$) amino acids from the third cytoplasmic loop. A smaller deletion, HAD4(Δ 221-315), removed 62 to 142 residues. Deletion studies indicate that large portions in the i3 loop are not required for efficient G protein coupling of receptors such as the M1 mAChR (Shapiro & Nathanson, 1989). Our lab has previously demonstrated that a 61 residue deletion in the i3 loop of $D_{4,4}$ (including all but 6 residues of the first repeat) resulted in a receptor that could inhibit adenylyl cyclase (Asghari *et al.*, 1995). Deletion, point mutation, chimera, and peptide studies have all determined that the regions critical for G protein activation are at the amino- and carboxyl-terminal ends of i3. These regions are thought to be in close proximity in the folded receptor. In particular, a positively charged region close TM 6 appears to be crucial for effector activation since soluble peptides derived from this region of the M4 mAChR and the α_2 -AR can activate G_i or G_o (Okamoto & Nishimoto, 1992; Wade *et al.*, 1996). The importance of this basic region was confirmed by deletions proximal to TM 6 in the β_2 -AR and M3 mAChR (Cheung *et al.*, 1990; Kunkel & Peralta, 1993). Okamoto and Nishimoto (1992) proposed that a motif, consisting of a C-terminal BBXXB or BBXB sequence and at least two N-terminal basic residues, was crucial for G_i activation in many GPCRs. In fact, the single TM

IGF-II receptor activates G_i by such a sequence. However, peptide studies with α₂-AR suggest that the BBXB sequence has a modulatory role, while arginines located 15-20 residues from TM 6 are crucial for G protein coupling (Wade *et al.*, 1996). The D₂ and D₄ receptors have very basic sequences at the amino terminal end of i3 (BBBBBBBXXXBBXXB and BXXXBXXXXBBXB for D₂ and D₄, respectively, where B denotes a basic residue (R, K, H), and X denotes any other amino acid). Strongly basic sequences are also present in the carboxyl-terminal portion of the i3 loop. The role of these basic residues may be to interact with the negatively charged surfaces of the G protein α and β subunits in the switch II region, which has been identified in the crystal structure of Gα_{i1}β₁γ₂ (Wall *et al.*, 1995).

The differing functional response of the two D₄ deletion mutants confirms the importance of the arginine rich membrane proximal sequence of i3. Functional coupling of the HAD4(Δ221-315) deletion mutant was established by the induction of low-affinity binding using Gpp[NH]p and by inhibition of forskolin-stimulated cAMP levels. In contrast, these effects were not seen with the HAD4(Δ221-337) receptor. Since both mutants had the same remaining N-terminal portion of i3, functional coupling to cAMP inhibition in the HAD4(Δ221-315) mutant must be due to the additional VRAAALPPQTRRRRAKI sequence at the carboxyl-terminal portion of i3 (refer to Figure 5). The dopamine competition curve, which showed this mutant had only a slightly lower affinity for the agonist, implies that this mutant is more retarded in G protein activation than binding. Since the BBXB/BBXXB sequence is deleted in both mutants, it does not appear to be essential for coupling to G_i, although the functional response of HAD4(Δ221-315) was blunted by >50% (Figure 14). This impairment was similar in magnitude to that seen in a M4 mAChR deletion mutant lacking 131 residues of i3 (van Koppen *et al.*, 1994). However, this mutant had all but 7 carboxyl-terminal residues of i3 deleted, while retaining a large amino-terminal segment. It is possible that the amino-terminal arginine-rich region of i3, which is deleted in HAD4(Δ221-315), may underlie the loss in efficacy. Perhaps the reciprocal deletion, in which the carboxyl-terminal segment of i3 is deleted but the amino-terminal sequence is retained, may have a similar phenotype. It should be noted that the actual G protein activated by D₄ may, in fact, be a member of the transducin family, based on the recent findings that PTX-sensitive G_{i2} mediates cAMP inhibition by this receptor (Yamaguchi *et al.*, 1997). Western blotting has identified Gα_{i2} and Gα_{i3} in CHO K1, although it is not known whether Gα_{i2} is expressed in these cells (Gerhardt & Neubig, 1991; Dell'Aqua *et al.*, 1993). D₄

coupled to inhibit cAMP in MN9D, but not in CCL1.3 cells, while both express only the G α_{i2} -subtype of G α_i (Tang *et al.*, 1994). Therefore, the G protein coupling of D₄ in these cells may involve G α_{i3} or G α_{i2} .

Interestingly, the HAD4(Δ 221-315) mutant did not appear to be more effective than the larger deletion mutant in stimulating the phosphorylation of MAPK. Both appeared to have some residual activity when stimulated with DA, although the effect was not observed using quinpirole. Several possible explanations would account for this observed lack of stimulation by HAD4(Δ 221-315). It is possible that this deletion, which allows coupling to AC inhibition, may abolish coupling to other G proteins which are mediating the MAPK response. This seems unlikely, since many G ι -coupled GPCRs activate MAPK by way of G $\beta\gamma$. Another possibility is that other structural elements of the third cytoplasmic loop that are required to MAPK activation may be deleted. These could include sequences that are required for endocytosis, which is required for MAPK activation by the β_2 -AR (Daaka *et al.*, 1998), or the recently described SH3 binding domains which can interact with adapter proteins that are involved in signalling, such as Grb2 and Nck (Oldenhof *et al.*, 1998). It is interesting to note that Grb2 is required for endocytosis of the EGF receptor, apparently via formation of an EGFR-Grb2-dynamin complex (Wang & Moran, 1996). Finally, it is possible that the difference is due to the "all-or-none" character of MAPK pathway. For example, in *Xenopus* oocytes stimulated with progesterone, the MAPK cascade acts like an cooperative enzyme with a Hill coefficient of 35 to control cell fate (Ferrell Jr. & Machleider, 1998). This is in contrast to the graded response of adenylyl cyclase to G α_i .

It appears that both deletion mutants can couple to at least some G proteins. This evidence comes from measuring the dopamine-stimulated increase in [³⁵S]GTP γ S binding to G proteins. With respect to D4(Δ 221-337), this coupling probably represents a G protein other than G ι /G α_{i2} . It is possible that deletions may allow binding of G proteins to the mutant which do not interact with wildtype receptors. The potency of dopamine at this mutant is higher in the GTP γ S assay, as well as in radioligand binding assays with Gpp[NH]p. The lack of a low-affinity agonist site has been previously observed in functionally defective β_2 -AR deletion mutants as well as with constitutively active α_{1B} -AR (Strader *et al.*, 1987; Kjelsberg *et al.*, 1992). Strader suggested that the increase in agonist binding affinity at uncoupled receptors was due to a reduced energy barrier resulting from deletion of the receptor's activating region. The lack of a

significant antagonist effect in the cAMP assay seems to indicate that HAD4(Δ 221-337) is not constitutively active.

Based on the [35 S]GTP γ S experiment, dopamine appears to have a reduced potency at the mutant HAD4(Δ 221-315). This may contribute to the blunted response observed in the cAMP inhibition assay, since the experiment was carried out using 1 μ M DA. A dose-response curve for cAMP inhibition with this mutant may reveal that these deletions do not affect the maximum inhibition, but instead shift the response to dopamine to the right. However, the reduced [35 S]GTP γ S stimulation by dopamine in cell lines expressing mutant receptors compared with CHO FD4.4 indicates that dopamine efficacy is also lower. This experiment may indicate that dopamine does not catalyze the 35 S labelling of as many G proteins in the deletion mutant cell lines compared to the cell line expressing FD4.4.

b. D_{4,4} Point Mutant M345A

We mutated a methionine located close to TM 6 in the i3 loop of D₄. Selection of this site was based on the alignment of conserved amino acids in TM 6 with those of other bioamine receptors. This residue is in a position equivalent to that of mutations in the α_{1B} - and α_2 -adrenergic receptors which lead to spontaneous activity. (Kjelsberg *et al.*, 1992; Ren *et al.*, 1993). In the α_2 -AR (G_i-coupled), mutation of T348 to five other amino acids (F/A/E/C/K) led to increased basal activity in each case. Even more dramatically, all 19 possible substitutions at A293 of the α_{1B} -AR led to constitutive activity. These receptors exhibited an enhanced affinity for agonists, as did a β_2 -AR CAM (Pei *et al.*, 1994). A second rationale for mutating this site was an unconfirmed report indicating that this position in D₄ may undergo post-translational modification by adenylylation (Kramer *et al.*, 1996).

In contrast to the CAM adrenergic receptors, no increase in forskolin-stimulated cAMP was noted due to nemonapride in the cell line CHO FD4.4(M345A)-1, suggesting this receptor is not constitutively activated. In fact, ligand binding appears to be disrupted and functional activity is abolished with the FD4.4(M345A) mutant. To explain why this may occur, it is useful to examine the predicted secondary structure of the third intracellular loop in this region. As early as 1987, Strader *et al.* (1987) proposed that the region of i3 preceding TM 6 may form an amphiphilic helix. 2D 1 H NMR and CD experiments on peptides from this region of the β_2 -AR provide evidence that these activating residues are part of an α -helix (Jung *et al.*, 1995). An i3

C-terminal peptide from the 5-HT_{1A} receptor also forms a positively charged, amphipathic α -helix in solution (Varrault *et al.*, 1994). Burstein *et al.* (1995) used random mutagenesis to define tolerated substitutions at the carboxyl-terminus of i3 in the M5 mAChR. He proposed that the TM 6 α -helix is extended one turn into the cytoplasmic loop, based on the pattern of radical tolerated mutations (4 residues apart), as well as the exclusion of helix-breaking prolines and glycines in this region of receptors having a wildtype phenotype. Emphasizing the importance of this α -helical domain, Högger *et al.* (1995) found that mutations in this region of the M1 mAChR were not additive in effect, since multiple point mutations were less defective than the equivalent individual point mutants. This was thought to be due to conformational compensation due to multiple alterations.

We found no evidence that FD4.4(M345A) has constitutive activity that can be blocked by the antagonist nemonapride. In contrast, this receptor appears to be structurally impaired, most likely due to disruption of the TM 6 α -helix by the M-A transition. It is possible that this residue is located in a different position in the proposed amphipathic helix than the analogous T/A residue of the adrenergic CAMs, accounting for the different phenotype seen with this D₄ mutant.

F. Localization of Epitope-tagged D₄ Receptors

As mentioned previously, residues in the central region of i3 play a role in receptor trafficking. The amino-terminal epitope-tagged receptors can provide a means to determine whether the polymorphic repeats or flanking sequences are involved in receptor localization. The stable CHO cell lines described here will be examined using immunofluorescence microscopy to determine the effect of deleting the repeat region and the SH3 binding domains. In experiments using transiently transfected HEK 293 cells, it was found that the full-length HAD4.7 receptors were stably expressed on the cell surface (Oldenhof *et al.*, 1998). The deletion mutant HAD4(Δ 221-337) appears to undergo rapid internalization, since a 20 min. exposure to antibody results in significant intracellular fluorescence. The turnover rate of HAD4(Δ 221-315) was intermediate to that of the large deletion and full-length receptor. While these results may argue against a direct role for SH3 binding in receptor internalization (since Grb2 did not bind to HAD4(Δ 221-337)), they suggest that the deletion does indeed affect trafficking. One can speculate that perhaps the SH3 binding domains function to interact with

cytoskeletal components, thereby preventing spontaneous internalization. Further immuno-fluorescence experiments will be required to determine the response of the D₄ receptor to agonist and antagonist binding.

VI. SUMMARY AND CONCLUSIONS

This thesis has presented data on the characterization of D₄ and D_{2L} dopamine receptors containing an amino-terminal epitope tag. Both FLAG- and HA-tagged receptors demonstrated ligand binding and functional coupling that matched what has been found with native receptors. Previously observed differences between D₄ and D_{2L}, such as the D₄ receptor's higher affinity for dopamine and lower affinity for raclopride, were observed. Using monoclonal antibodies to the epitope tag, immunoblotting was carried out, confirming a previous finding that D₄ is present in glycosylated and unglycosylated forms (Lanau *et al.*, 1997). The longer alleles of D₄ migrated slower in SDS-PAGE, confirming that this region of the receptor is not spliced out at the mRNA level. Therefore, we have demonstrated that these epitope-tagged proteins can provide a useful tool to investigate the location and trafficking of receptors in transfected cells.

Our knowledge of signal transduction pathways that are activated by D₄ has been furthered by this research. Three isoforms of the D₄ receptor stimulate a transient increase in ERK1/2 phosphorylation and catalytic activity in CHO cells, similar to observations made with D_{2L} in CHO and rat C6 glioma cells (Luo *et al.*, 1998b; Welsh *et al.*, 1998). This novel transduction pathway is dependent on G_i/G_o and independent of PKC. The results provide a partial biochemical pathway connecting D₄ activation with known cellular responses such as PLA₂ potentiation, differentiation, and mitogenesis (Lajiness *et al.*, 1995; Swarzenski *et al.*, 1994). Future studies of these processes should incorporate experiments utilizing MAPK inhibitors (eg. the G $\beta\gamma$ sequestering agent β ARKct, the PI3-kinase inhibitor wortmannin, or dominant negative RasN17) in order to determine whether the effects are mediated by this pathway. The finding that dopamine, as well as many other neurotransmitters such as epinephrine, acetylcholine, serotonin, and enkephalins, can activate MAP kinases suggests that G protein-coupled receptors may have an important role in processes previously attributed to growth factor receptors. The recent discovery that the MAPK pathway is involved in LTF/LTP suggests that this signal transduction system may be an important target for GPCRs *in vivo* (Bailey *et al.*, 1997).

Analysis of mutant receptors has produced insight into the nature of the third intracellular loop of D₄. Two D₄ deletion mutants were stably expressed at levels comparable to full-length receptors. Despite removal of much of the large intracellular loop, antagonist binding was unperturbed. A previously described deletion in D_{4.4}, Δ254-315, indicated that the repeat region

was not required for activation of G_i (Asghari *et al.*, 1995). Our research has shown that deletion of the arginine-rich region amino-terminal to the polymorphic repeat sequence of D_{4,4} (Δ 221-315) significantly impairs but does not abolish functional coupling to cAMP inhibition. However, deletion of both the amino- and carboxyl-terminal, cationic regions (Δ 221-337) abolishes this response as well as the shift in agonist binding due to a guanine nucleotide. Thus, two sequences contributing to G protein coupling/activation in the D₄ dopamine receptor have been located flanking the variable 16 amino acid repeats.

In contrast to the tolerated deletions, a point mutation located at methionine 345 in the putative extension of the TM 6 α -helix of D_{4,4} reduced or abolished binding of many typically high-affinity ligands such as spiperone and dopamine. Receptor expression was confirmed by Western blotting, specific [³H]nemonapride binding, and the ability of unlabelled haloperidol to compete with the radioligand. In contrast to CAM adrenergic receptors containing mutations at an analogous site, the D4(M345A) mutant did not show spontaneous activity that could be blocked by an antagonist. From this result, it can be hypothesized that the M345A mutation disrupts the secondary structure in this region. It is possible that this residue is located at a different position in the proposed α -helix, thereby producing a loss-of-function mutation as opposed to a constitutive activity.

VII. FUTURE RESEARCH

By defining the pharmacological and functional properties of several epitope-tagged dopamine receptors, we have established that they can act as a useful tool in future experiments. The ability to detect and isolate receptors with readily available antibodies may allow us to determine whether interactions with the third cytoplasmic loop of D₄ occur *in vivo*. Immunoprecipitation of D₄, followed by Western blotting to anti-Grb2, anti-Nck, or anti-phosphotyrosine antibodies could provide evidence that the receptor is part of a multiprotein complex in the cell. The availability of deletion mutants will provide an excellent means of determining whether interactions are dependent on the SH3 binding domains recently identified in the D₄i3 loop (Oldenhoef *et al.*, 1998).

Preliminary experiments seem to suggest that there may be a role for the central portion of the third cytoplasmic domain in receptor trafficking. It is also possible, however, that the high rate of internalization observed with D4(Δ221-337) may be due to the deletion of important sequences outside this repeat region. It will be interesting to determine whether a difference in trafficking between D_{4,2}, D_{4,4}, and D_{4,7} can be observed using immunofluorescence microscopy. Colocalization of the internalized mutant receptors with endocytic markers such as clathrin will identify the location of internalized receptors.

D₄ contains fewer potential phosphorylation sites than the D₂ receptor, which has been previously labelled with ³²P; *in vivo* (Ng *et al.*, 1994b). Initial experiments using the FLAG-tagged D_{4,4} receptor did not produce any evidence of receptor phosphorylation. However, this experiment was flawed since many common phosphatase inhibitors were not included in the buffers. These compounds (eg. EDTA, sodium pyrophosphate, sodium fluoride) precipitated with Ca²⁺ in the buffer, which was required to form the epitope recognized by the M1 antibody. The HA tag will solve this technical problem, since this epitope does not require a chelated metal to be recognized by antibodies. Therefore, in the future we plan to determine whether receptor phosphorylation occurs in another D₂-like receptor, D₄, despite the reduction in kinase consensus sites.

Transgenic mice in development in our lab are being developed to express an HA-tagged D₄ receptor that is under control of the endogenous promoter but that can be conditionally knocked-out. This animal will allow us to determine whether compensatory developmental mechanisms in the previously described D₄ knock-out mouse masked the true role of the

receptor in the adult animal. The presence of an epitope tag will allow an immunohistochemical determination of both the D₄ receptor expression pattern as well as the success of the conditional knock-out in eliminating D₄ in adult animals. The work presented here demonstrates that this modification does not alter the structure or function of the receptor.

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