Annu. Rev. Pharmacol. Toxicol. 1993. 32:281–307

MOLECULAR DIVERSITY OF THE DOPAMINE RECEPTORS

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KEY WORDS: Parkinson's disease, schizophrenia, D_1 D_2 D_3, D_4 D_5, G protein-coupled receptors

INTRODUCTION

The Dopaminergic System and Its Relationship to Human Diseases

Our current understanding of the relationship between the dopaminergic system and human brain disorders is based on two fundamental discoveries: dopamine-replacement therapy can alleviate Parkinson's disease (1–3) and, secondly, many antipsychotic drugs are dopamine receptor antagonists (4–7). These discoveries have guided two major directions in dopamine-related basic research and drug design: to activate dopamine receptors left understimulated by the degeneration of the afferent dopamine-secreting cells and to prevent dopamine from binding to its receptor, according to the hypothesis that schizophrenia is the result of dopamine receptor overactivity (5, 8). Since blockade of the dopamine receptors (antipsychotic therapy) can lead to a state similar to that resulting from dopamine depletion (Parkinson's therapy) and higher doses of dopamine can cause psychoses, the therapies of disorders resulting from dopamine imbalances are associated with adverse side effects. The ideal drug(s) that will treat one disorder without affecting the other has thus far not been found. However, the search for such a drug has led to the design of several dopamine receptor ligands that, in turn, have increased our understanding of the dopaminergic system. In particular, these studies have

0362-1642/93/0415-0281$02.00

led to the proposition that the etiology of movement disorders and psychoses involve different neuronal pathways and that the dopamine receptors may differ in these pathways. Here we review the latest developments regarding the dopamine receptors.

The dopaminergic system comprises three principal neuronal pathways: the nigrostriatal, the mesocorticolimbic, and the tuberoinfundibular. The nigrostriatal pathway contains the neurones of the substantia nigra, which synthesize dopamine and neurones of the striatum that respond to it. Degeneration of this pathway leads to Parkinson's's disease, underscoring its role in the control of locomotion. The mesocorticolimbic pathway, composed of neurones of the ventral tegmental area that connect with those of the limbic forebrain, is thought to be involved in emotional stability, contributing to the etiology of schizophrenia, and to be the desired site of action of the neuroleptics. The tuberoinfundibular pathway originates in the neurones of the hypothalamus. The dopamine secreted by these neurones into the portal blood is transported to the pituitary to regulate prolactin secretion from the pituitary. This pathway influences lactation and fertility.

The dopaminergic system relies on the interaction of dopamine with several receptors. In 1979 two were known and characterized as the D_1 and D_2 receptors (9). These receptors can been differentiated pharmacologically using D_1 and D_2 receptor-selective agonists and antagonists. Of therapeutic interest, most of the commonly prescribed neuroleptics bind the D_2 receptor with high affinity. These two receptors exert their biological actions by coupling to and activating different G protein complexes. The D_1 receptor interacts with G_s complexes resulting in the activation of adenylyl cyclase and in an increase in intracellular cAMP levels. The D_2 receptor interacts with G_i complexes to inhibit cAMP production. These biological activities placed the two dopamine receptors in the superfamily of G protein-coupled receptors, a feature of utmost importance for their molecular characterization. The anatomical distributions of these two receptors overlap in the CNS, yet their quantitative ratios differ significantly in particular anatomical areas. It is noteworthy with respect to mental disorders, that both D_1 and D_2 receptors are present in the nigrostriatal and mesocorticolimbic pathways.

For ten years, the two-subtype classification could accommodate most of the activities attributed to the dopaminergic system. This has changed with the definitive demonstration of the existence of several other dopamine receptors. These receptors have been reviewed recently (10–12). How the existence of new dopamine receptors will modify our previous conceptions of the dopaminergic system cannot be totally foreseen at this time. This review discusses the first ramifications that the dopamine receptor heterogeneity has brought on our understanding of the dopaminergic system.

HETEROGENEITY OF THE DOPAMINE RECEPTORS

Molecular Characterization of the Dopamine Receptors

The molecular characterization of the dopamine receptors originated with the recognition that G protein-coupled receptors are evolutionarily related (13–15). The discovery of their heterogeneity results from the application of this concept.

The existence of a G protein-coupled receptor supergene family was proposed on the basis of two receptor sequences, the rhodopsin and β_2-adrenergic receptors. The rhodopsin receptor transmits light signals to the brain through its interaction with a specific G protein, known as transducin. The β-adrenergic receptors transmit adrenergic stimulation in heart and lung tissues by interacting with another G protein, known as G_s. When the molecular structure of the β_2-adrenergic receptor was determined (16), its putative topology was found to be similar to that of the rhodopsin receptor. Both receptors were proposed to contain seven (putative) transmembrane domains in which several conserved amino acid residues are found. These similarities led to the concept that all receptors that couple to G protein to induce second messenger pathways might share these common structural characteristics. This concept was rapidly strengthened by the cloning of the acetylcholine-muscarinic2 and of the neuropeptide-substance K receptors (17–20).

An important outcome of the concept that G protein-coupled receptors share sequence similarities was the development of technical approaches applicable to the cloning of any G protein-coupled receptors without previous knowledge of the receptor's peptide sequence or of its biological activity (21). These approaches, referred to as "homology approaches," rely on the use of DNA probes encoding sequences expected to be conserved among G protein-coupled receptors and can be technically divided into: (*a*) The low-stringency screening approach, which uses DNA fragments (>300bp) as hybridization probes to identify homologous sequences under hybridization conditions of reduced stringency, and (*b*) the PCR (polymerase chain reaction)-based homology approach, which uses oligonucleotides, complementary to short (<50b) highly conserved sequences, as primers to amplify related cDNAs in polymerase chain reactions. The first approach led to the characterization of the D_2, the second was used for the D_1 dopamine receptors.

The D_2 dopamine receptor was cloned using the hamster β_2-adrenergic receptor coding sequence as hybridization probe (22). A rat brain cDNA was identified via genomic and cDNA screenings and shown to encode a protein featuring the characteristics expected for a G protein-coupled receptor. This cDNA was transfected into eukaryotic cells and led to the expression of a receptor with the pharmacological profile and biological activity of the

dopamine D_2 receptor found in the brain and pituitary (22–24). In particular, the cloned receptor presented the expected affinity for neuroleptics and its stimulation inhibited adenylyl cyclase activity and prolactin secretion. This receptor is encoded by a gene (DRD_2) located on human chromosome 11q23 (25).

The PCR-based approach was used to clone the D_1 receptor from rat striatum (26, 27) and mouse NS20Y neuroblastoma cells (28), although the low-stringency screening approach was also successful (29). The resulting partial clones were used to screen human and rat DNA libraries. The sequences derived from these clones share the characteristics expected of G protein-coupled receptors in general and of the catecholamine receptors in particular (26). These putative receptors were expressed by DNA transfection and were shown to bind D_1 receptor ligands and to stimulate adenylyl cyclase activity, the two hallmarks of the D_1 receptor. The human D_1 receptor gene (DRD_1) is located on chromosome 5q35.1 (27, 30).

The success of the homology approach led to the search for other dopamine receptors. The D_3 receptor was originally identified by using a DNA fragment of the D_2 receptor as probe under low stringency hybridization conditions (31). Subsequent PCR extension and genomic library screening led to the isolation of a cDNA that encodes a receptor most closely related to the D_2 receptor. When expressed in eukaryotic cells, this receptor was shown to bind D_2 but not D_1 ligands. Although its ability to affect second messenger systems has not been demonstrated, its structure and binding characteristics permitted its classification as the D_3 receptor and has been assigned to chromosome 3q13.3 in human (DRD_3) (32).

By analyzing the mRNAs of human neuroepithelioma SK-N-MC cells with D_2 receptor cDNA probes under conditions of low stringency, another D_2-related mRNA was detected (33). The corresponding cDNA and gene analyses led to the characterization of the D_4 receptor. The D_4 receptor, when expressed in COS-7 cells, binds D_2 antagonists with a pharmacological profile distinct from, but reminiscent of, that of the D_2 receptor. Although the D_4 receptor can couple to G proteins, it has not been conclusively shown to modulate adenylyl cyclase activity. Its gene (DRD_4) is located at the tip of the short arm of the human chromosome 11p15 (34).

Finally, the D_1 receptor clone was used as a hybridization probe to identify D_1-related genes. A human D_5 and a rat D_1b receptors were subsequently characterized (35–37). They display the same pharmacological profile, reminiscent of that of the D_1 receptor and are able to stimulate adenylyl cyclase activity. On the basis of their sequences, the D_5 and D_1b receptors are human and rat equivalents of the same receptor, respectively. The human D_5 receptor maps to 4p15.1-p15.3 (38).

Three "unexpected" dopamine receptors, D_3, D_4 and D_5, were discovered

through the application of homology screening techniques. The existence of dopamine receptors different from the canonical D_1 and D_2 receptor had been proposed over the past decade but had been refuted when receptors were recognized to exist in two affinity states (39, 40). One definitive outcome of the cloning of receptors is to make them physical entities. The cloned D_3, D_4 and D_5 receptors had not been previously characterized in detail.

COMMON FEATURES OF THE DIFFERENT DOPAMINE RECEPTORS

The action of dopamine was, for the past decade or more, interpreted through its interactions with only two receptors. The discovery of the D_3, D_4, and D_5 receptors immediately raised the question whether the activities of these new receptors had been masked by those of the classical D_1 and D_2 receptors. The search for features common to both the new receptors and the classical ones can help resolve that possibility. The data presented here allows us to divide the dopamine receptors into two subfamilies, the D_1-like (D_1,D_5) and the D_2-like (D_2,D_3,D_4) subfamilies (Table 1).

Gene Organization

The genomic organization of the dopamine receptors supports the notion that they derive from the divergence of two gene families, which can be divided into the D_1-like and D_2-like receptor genes (Figure 1). The D_1-like receptor genes do not contain introns in their protein-coding regions, whereas the D_2-like genes do (26, 27, 31, 33, 41). Such a gene organization differentiating two receptor subfamilies (D_1-like and D_2-like) has also been described for other G protein-coupled receptor gene families, including the serotonin (5HTla-like and 5HTlc-like) receptors (42). Strengthening this notion, several introns in the D_2-like receptor genes are located in similar positions (Figure 1). It is noteworthy that two introns (after transmembrane domain IV and at the 3′ half of the third cytoplasmic loop) were found in the D_2 and D_3 receptor genes to correspond almost precisely to intron positions found in the opsin genes (41). These introns are, however, absent in the D_4 receptor gene. Finally, two features of interest have been described regarding the genomic organization of the D_2 receptor gene: it contains an unusually long intron (250 kb) dividing its mRNA 5′-untranslated region; 150 kb downstream of the D_2 gene is the N-CAM gene (J. H. Eubanks, M. Djabali, L. Selleri, D. K. Grandy, O. Civelli, et al, submitted).

Sequence and Topology

The overall topology of the five dopamine receptors is predicted to be highly similar. They should contain seven putative membrane-spanning α helices,

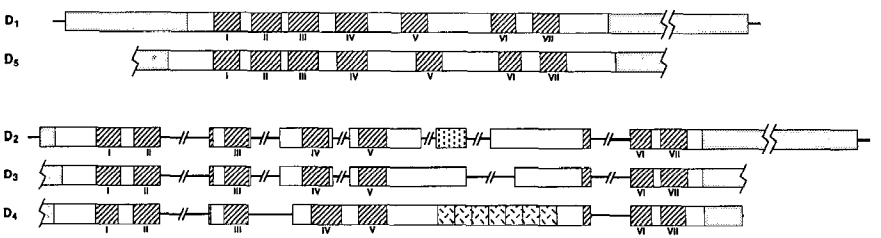


Figure 1 Genomic organization of the human dopamine receptor genes (data from refs. 31, 33, 36, 41, 74). Lines indicate introns, boxes exons; striped boxes with Roman numerals show the location of the putative transmembrane domains, shaded boxes those of the untranslated region of the corresponding mRNA; the pointed exon in the D_2 receptor gene is the alternatively spliced exon differentiating D_2s from D_2s (41). The seven repeats found in some human genes are outlined in hatched boxes in the D_4 receptor gene (91).

hallmark of the G protein-coupled receptors (44). By homology to the rhodopsin and adrenergic receptors, each of the dopamine receptor polypeptides should have its amino and carboxy termini located outside and inside the cell, respectively. The seven transmembrane domains would form a narrow dihedral hydrophobic cleft surrounded by three extracellular and three intracellular loops. The receptor polypeptides are probably further anchored to the membranes through palmitoylation of a conserved Cys residue found in their C-tails (347 in D_1, the C-terminus in D_2-like receptors) (45).

The dopamine receptors contain consensus sequences for glycosylation sites in the N-terminal domain, in addition the D_1-like subtypes have potential glycosylation sites in their first extracytoplasmic loop. D_1 and D_2 receptors are known to be naturally glycosylated (46) and the cloned D_2 receptor also has been shown to be glycosylated when expressed in an heterologous cell (47). It is noteworthy that these experiments have also shown that the D_2 receptor exhibits a different glycosylation pattern when isolated from different cells and that, since the differently glycosylated receptors have the same pharmacological profile, the glycosylated moiety does not affect ligand recognition.

Ligand Binding

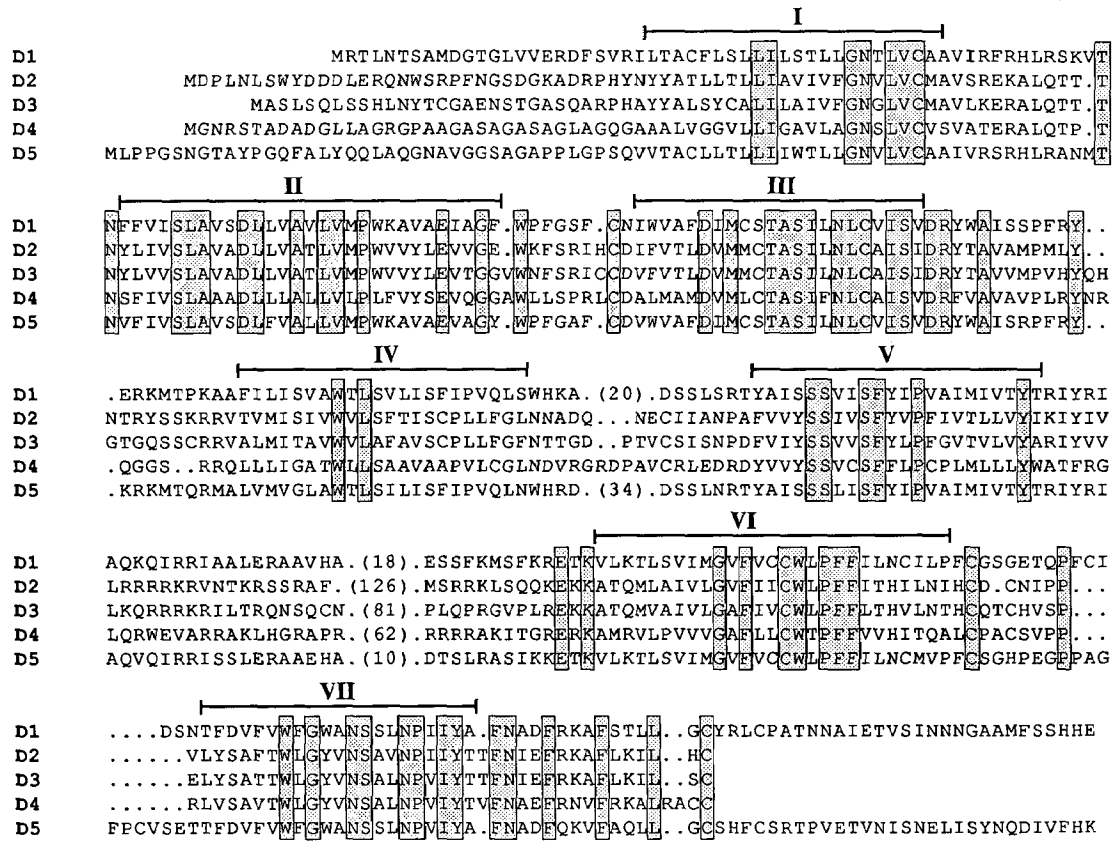
Analogous to the mode of ligand recognition by the rhodopsin and adrenergic receptors, the binding of dopaminergic ligands must involve each receptor's hydrophobic core. This view is supported by the fact that the highest degree of sequence identity is found in the hydrophobic domains. Within their transmembrane domains, the amino acid sequences of the dopamine receptors are 31% identical (Figure 2). This percent increases to 75% and 52% if they are divided into D_1-like and D_2-like receptors, respectively.

The mechanism by which dopamine binding to the receptor induces G protein activity is unknown but most likely involves a cascade of intramolecular reactions. In particular, charged and conserved amino acid residues found in transmembrane domains should participate in dopamine recognition. Indeed, molecular models confirm that the charged residues of the α helices face the inside part of the hydrophobic cleft (48). An Asp (103 in D_1, and 114 in D_2) in transmembrane III and two Ser (199–202 in D_1 and in D_2) in transmembrane domain V could interact with the amine and the hydroxyl groups of dopamine, respectively (48–50). This model was recently tested experimentally and proven valid although the two serine residues in transmembrane domain V differentially affect agonist binding (51). In addition, the dopamine-receptor interaction might be stabilized by the interactions of two Phe residues (Phe 203–289 in D_2, in transmembrane V and VI) with the benzene ring and by three aromatic residues (Trp 284, Phe 288, Phe 617) which could form an aromatic cluster around the aspartate ammonium ion pair (48). Furthermore, in transmembrane domains II and VII, Asp 70(D_1) or 80(D_2) and Asn 324(D_1) or 390(D_2) might be involved in agonist binding (52, 53). Indeed, mutations of the Asp 80 in the D_2 receptor lead to receptors with different ligand affinities and impaired in their potency to inhibit adenylyl cyclase (54). Finally, two Cys residues found in extracytoplasmic loops (96–186 in D, and 107–182 in D_2) might form a disulfide bond that could affect ligand binding (49).

The cloned dopamine receptors, when expressed by transfection, exhibit binding profiles differentiating them into the D_1-like and D_2-like subfamilies. The D_1-like receptors bind with high affinity D_1 and not D_2 antagonists. A prototypic ligand for the D_1-like receptors is the benzazepine SCH23390 (Kis $<$ 1nM). On the other hand they bind the butyrophenone spiperone with low affinity (Kis in the μM range). In contrast, the D_2-like receptors bind efficiently spiperone (Kis $<$ 1nM) and not SCH23390 (Ki for D_2 in the μM range); they also recognize most of the neuroleptics. While there is presently no ligand that differentiates the D_1 from the D_5 receptor, several D_2 antagonists can distinguish the different D_2-like receptors (see below). At the structural level, 21 amino acid residues differentiate D_1-like from D_2-like receptors in the transmembrane domains, and these might participate in the selective recognition process (Figure 2).

G Protein Coupling and Desensitization

The receptors' interactions with G proteins involve the cytoplasmic loops (55, 56). The D_2-like receptors have a large third cytoplasmic loop and a short C-terminal tail, whereas the D_1-like receptors have a relatively short third cytoplasmic loop and a long C-tail. Since both receptor domains have been implicated in G-protein coupling, their relative homology suggests that



receptors of the same subfamily might couple to the same set of G proteins. While this is true for the D_1-like receptors whose stimulation leads to an increase in cAMP levels, it remains to be shown for all the D_2-like receptors.

In their cytoplasmic domains, each of the dopamine receptors contains several consensus sites for phosphorylation by cAMP-dependent protein kinase or protein kinase C (22, 26). Several of these sites are present in the third cytoplasmic loop. Biochemical studies have shown that phosphorylation of such residues may attenuate G protein-receptor interactions and that these residues are directly involved in the homologous and heterologous mechanisms of desensitization (57). Since D_2 receptors are subject to desensitization when transfected into heterologous cells (47) while D_1 receptor desensitization occurs in striatal slices (58), model systems can now be established to test whether dopamine receptor phosphorylation affects desensitization.

The data discussed in this section show that the dopamine receptors can be divided into two subfamilies, the D_1-like and D_2-like subfamilies. That the D_1 and D_2 receptors are quantitatively predominant, particularly in the central nervous system (see below), may explain why most of the activities attributed to dopamine could be accounted for by the simpler two-receptors system (9). On the other hand, the novel D_3, D_4, and D_5 dopamine receptors each have their raison d'être, which might be found by searching for their specific features.

SELECTIVITIES ASSOCIATED WITH THE DIFFERENT DOPAMINE RECEPTORS

The studies made possible by the use of dopamine receptor clones have allowed the discoveries of distinctive features of the different receptor subtypes. This section presents some of these results, in particular those that have helped us reevaluate our understanding of the dopaminergic system and of its roles in brain disorders (Table 1).

Selectivities in Pharmacological Profiles

Thus far, no selective ligand able to differentiate the D_1 from the D_5 receptor has been described. The salient feature of the D_5 receptor is that it binds dopamine with a higher affinity than does the D_1 receptor (35, 36). On the other hand, the pharmacological profiles of the D_3 or D_4 receptors are reminiscent of, yet distinct from, that of the D_2 receptor. Most neuroleptics were developed as D_2 receptor antagonists and have a higher affinity for the D_2 than the D_3 or D_4 receptors. This implies that most of the neuroleptics are still acting predominantly at D_2 receptors in the human brain. However, the few exceptions that have been described are striking. One may help differen-

Table 1 Particularities of the different dopamine receptor subtypes.

	D_1-LIKE		D_2-LIKE		
	D_1	D_5	D_2	D_3	D_4
GENE and mRNA					
Chromosome	5q	4p	11q	3q	11p
Selective gene expression		Human pseudogenes 2p, 1q	Alternative splicing D2S/D2L		Polymorphism in human
Intron in coding seq.	NO		YES		
PROTEIN					
Seq. identity in TM (%)	100		45		
PHARMACOLOGY					
Prototypic antagonist	SCH23390		SPIPERONE		
Selective antagonist			Haloperidol	AJ76, UH232	Clozapine
BIOLOGY					
Guanylnucl. sensitivity	yes	yes	yes	no	yes
Adenylyl cyclase	activation	activation	inhibition	?	(?)
Other pathways	$IP3^{\uparrow}$, Ca channel		$IP3^{\uparrow}$, K channel		
LOCALIZATION					
Respectively high	caudate-putamen nucl. accumbens olfact. tubercle	hippocampus hypothalamus	caudate-putamen nucl. accumbens olfact. tubercle	olfact. tubercle hypothalamus nucl. accumbens	frontal cortex medulla midbrain
Selective	amygdala	parafascicular nucl. kidney	substantia nigra zona incerta pituitary, adrenal	islands of Calleja	mesolimbic system heart

tiate pre- from postsynaptic receptors, and the other could impact our understanding of the action of an atypical neuroleptic.

The Dopamine Presynaptic Receptors

The receptors harboring a D_2-like pharmacology have been subdivided into pre- and postsynaptic receptors (59). The postsynaptic receptors convey dopamine messages in the postsynaptic cells by inducing a second messenger system, e.g. by decreasing intracellular cAMP levels. The presynaptic or autoreceptors are present on the cells that secrete dopamine. Their stimulation by dopamine is thought to lead to an inhibition of impulse flow, co-transmitter release, and dopamine synthesis and release, thereby regulating dopamine production via a feed-back mechanism. Whether differences exist between pre- and postsynaptic receptors is controversial.

The D_3 receptor binds two antagonists with a higher affinity than does the D_2 receptor (31). These compounds, UH232 and AJ76, are classified as selective for the presynaptic receptors and are the only ligands known to date to be more selective for the D_3 than the D_2 receptor. In addition, dopamine was found to bind the D_3 receptor with a 20-fold higher affinity than the D_2 receptor, a characteristic expected for autoreceptors. Furthermore, the presence of D_3 receptor mRNA in the substantia nigra, a center of dopamine production, supports the hypothesis that the D_3 receptor may be a presynaptic receptor. Note also that the D_2 receptor mRNA is the predominant dopamine receptor mRNA in the subtantia nigra (60) and that, like the D_3 receptor,

6-OHDA lesions show D_2 presence in the dopamine-secreting neurons (31, 61–64). Therefore, both the D_2 and the D_3 receptors are autoreceptors. Whether they are present in the same cells and whether stimulation of the D_3 receptor affects the presynaptic neuron differentially than does the D_2 receptor remain to be determined.

The D_4 Receptor Connection to Schizophrenia

Except for clozapine, the D_4 receptor has a lower affinity for neuroleptics than does the D_2 receptor. Clozapine is an "atypical" neuroleptic, i.e. a neuroleptic in which actions are not accompanied by adverse motor control side effects. In schizophrenia therapy, clozapine is administered at a concentration tenfold lower than its affinity constant for the D_2 receptor, indicating that clozapine may not be primarily acting at the D_2 receptor. Since the D_4 receptor binds clozapine with a tenfold higher affinity than does the D_2 receptor (33), it could be classified as the specific clozapine target. A corollary is that antagonism of dopamine binding to the D_4 receptor could be an important step in the prevention of psychoses. Compared to the D_2 gene, the D_4 gene is expressed at low levels, suggesting that D_4 receptor-mediated activities are difficult to detect and thus were lost in measurements of D_2 receptor reactivity. Moreover, preliminary data on the tissue distribution of the D_4 mRNA shows that it is most abundant in the frontal cortex, midbrain, amygdala, and medulla, areas associated with psychotic etiologies, and at very low level in the striatum, the site of motor control (S. Watson, personal communication). Thus, the lack of extrapyramidal side effects observed with clozapine treatment may be a reflection of D_4 receptor localization in the CNS. These observations point to the D_4 receptor as an important molecule in balancing emotional control and may serve as a basis for understanding atypical neuroleptic actions.

Selectivities in Biological Activities

The predominant biological activities associated with D_1 and D_2 receptor stimulation are the activation and inhibition, respectively, of adenylyl cyclase activity. The D_2 receptor in lactotroph cells also induces opening of K+ channels and affects phosphoinositide hydrolysis upon dopamine binding (65). The establishment of cell lines expressing individual dopamine receptors through DNA transfection has permitted definitive analyses of each receptor's potential to induce second messenger systems.

Second Messenger Pathways Induced by the D_1 and D_2 Receptors

The ability of dopamine receptors to induce different second messenger pathways has thus far been studied in D_1 and D_2 receptors (66, 67). The D_1 and D_2 receptors induce two types of signal transduction pathways, one

obligatory and several cell-specific. The obligatory pathway is detected in every cellular environment. In D_1 or D_2 receptors it is stimulation or inhibition of adenylyl cyclase, respectively. But dopamine also induced additional and sometimes different signal transduction pathways in Ltk-fibroblast, GH4C1 somatomammotroph, 293 kidney, CHO ovarian, and C6 glioma cells through its interaction with the D_1 and D_2 receptors.

In every cell studied, dopamine stimulation of the D_1 receptor increases cAMP production. In addition, in GH4C1 cells, the D_1 receptor potentiates

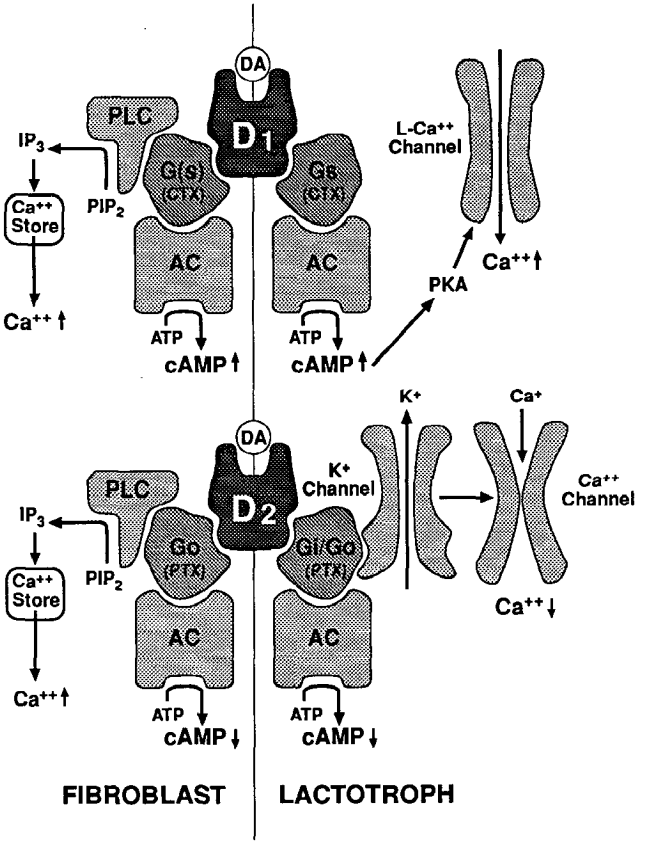


Figure 3 Signaling pathways of the D_1 and D_2 dopamine receptors in the mouse fibroblast Ltk- and the rat somatomammotroph GH4C1 cells. Data are taken from (66, 67). DA represents dopamine, CTX and PTX means cholera toxin- and pertussis toxin-sensitive, respectively. G = G protein; PLC = Phospholipase C; AC = Adenylyl cyclase; PIP2 = Phosphoinositol bisphosphate; IP3 = Inositol triphosphate. The direction of arrows indicates whether the second messenger increases or decreases.

activation of L-type voltage-dependent calcium channel in a cAMP-dependent manner (Y. F. Liu, O. Civelli, Q. Y. Zhou, P. R. Albert, submitted). In Ltk-cells, D_1 receptor stimulation leads to an increase in cytosolic free calcium concentrations ($[Ca^{++}]_i$) by mobilization of the intracellular calcium. This effect correlates with an increase in phospholipase C activity (PLC) and is cholera-toxin sensitive (67). The D_2 receptor, while inhibiting adenylyl cyclase activity, does not affect phosphoinositide (PI) hydrolysis in GH4C1 cells and induces a decrease in $[Ca^{++}]_i$ mediated by a hyperpolarizing effect, mostly due to activation of K^+ channels. In Ltk-cells, the D_2 receptor stimulation leads to an increase in $[Ca^{++}]_i$ due, in part, to the release of calcium ions from intracellular stores following the rapid stimulation of PI hydrolysis and, in part, to influx from extracellular medium. This surprising induction in PI hydrolysis is not due to the presence of nonphysiological concentrations of D_2 receptors in the cells. PI hydrolysis is induced by dopamine in Ltk-cells containing low levels of D_2 receptor (G. Gatti, C. Muca, E. Chiaregatti, D. K. Grandy, O. Civelli, et al, submitted). In addition, in CHO cells, D_2 receptors mediate the potentiation of arachidonic acid release by a mechanism that involves protein kinase C and that is independent of the concurrent adenylyl cyclase inhibition (69).

These data show that dopamine receptors can potentially induce different second messenger pathways in different cellular environments. The obligatory signaling pathway is always induced while the others are cell-dependent. This dual ability has been described for other G protein-coupled receptors (70–72) and points to the importance of the cellular environment in the outcome of receptor stimulation.

The Lack of Coupling of the D_3 Receptor to G Protein

The search for the second messenger pathways induced by D_3 receptor stimulation has led to the surprising conclusion that the D_3 receptor does not seem to link to G proteins (31). The binding of dopamine to the D_3 receptor expressed into CHO or COS-7 cells is not modulated by the addition of guanylnucleotides, which differentiate the high- from the low-affinity state of G protein-coupled receptor and is accepted as an indication of effective G protein to receptor coupling. In addition, the D_3 receptor was unable to affect adenylyl cyclase activity. One possibility is that the D_3 receptor associates selectively with G proteins absent in the test cells. Since, in general, G protein-coupled receptors find G proteins to couple to in CHO and COS-7 cells, the possibility that the D_3 receptor does not couple to G protein might be entertained. In view of its possible autoreceptor nature (see above), it could, for example, act as a scavenger to modulate dopamine release from the presynaptic membrane but would not directly affect intracellular chemistry. However, since the binding of somatostatin to one of its specific receptor

has also been recently found to be insensitive to the addition of guanylnucleotides (73), it is our understanding of guanylnucleotides action that may need to be revised.

Regulation of D_1 and D_2 Receptor Gene Expression

Therapies directed at disorders involving the dopamine receptors are administered over long periods of time. In animals these drug treatments modify the levels of dopamine receptor. It has been suggested that these changes may account for some of the side effects of the drugs. The availability of dopamine receptor clones allowed pilot studies to define which step in the expression of the receptor is affected by these drug-induced changes. Furthermore, genomic elements are also beginning to be characterized that recognize the transcriptional factors modulating dopamine receptor expression.

Regulation of Expression of the D_1 Receptor Gene In Vitro

The D_1 promoter sequences of the rat and human D_1 receptor genes have been determined (74, 75). The D_1 gene contains a small intron that interrupts the 5′ untranslated region of its mRNA. The region upstream of the start of transcription does not contain the canonical CAAT or TATA sequences, is G+C rich, and contains multiple potential sites for transcription factors.

Genomic sequences located upstream of the start of transcription (cap site) were evaluated for their ability to direct transcription of the receptor mRNA. It was found that the 735 bases upstream of the cap site were sufficient to confer transcriptional activity and that this activity was cell-specific. Cells endogenously expressing D_1 receptor could express D_1 receptor using this DNA fragment as the promoter, whereas others could not. In addition, it was observed that the D_1 gene promoter is induced in response to cAMP (75), suggesting the existence of an autoregulatory mechanism in D_1 gene expression. Activation of the D_1 receptor by dopamine increases intracellular cAMP level which leads, among other results, to receptor desensitization and enhancement of D_1 gene transcription. Since the D_1 receptor undergoes a very fast turnover (76), the increase in de novo protein synthesis is used as a compensatory mechanism to maintain a sustained dopamine activation. Such a mechanism has been proposed for the β_2-adrenergic receptor and might be shared by stimulatory receptors (77, 78).

Modulation of D_2 mRNA levels in vivo

The importance of the D_2 receptor in schizophrenia and Parkinson's disease led to two lines of experiments analyzing changes in D_2 receptor mRNA levels.

Chronic neuroleptic administration, the traditional antipsychotic treatment, increases striatal dopamine D_2 receptor binding sites in rats (79). This increase

can account for the behavioral supersensitivity of the drug treatment. Whether the mechanism of this increase involves transcription has been analyzed by measuring D_2 mRNA levels by Northern blot analysis, solution, and in situ hybridization in rats subjected to long-term haloperidol administration. Several reports have concluded that the D_2 mRNA density in the basal ganglia is not affected by this treatment and have suggested that the increases in binding sites may be the result of increased protein stability (80, 81). However, the opposite has also been reported. Chronic haloperidol treatment increases striatal D_2 mRNA levels significantly, doubling it in some cases (82). These conflicting findings need to be reevaluated with regard to the method of mRNA detection and the drug regimen. It is noteworthy that chronic haloperidol treatment differentially affects D_2 mRNA levels in the pituitary, increasing them in the intermediate but not in the anterior lobe (83).

Denervation and degeneration by 6-hydroxydopamine of dopamine neurones have been used as a model for parkinsonism in rats. While the levels of striatal D_2 mRNA increase by about 30% after denervation (84), conflicting results were found after 6-OHDA treatment. Two studies found that the 6-OHDA treatment increases striatal D_2 mRNA by in situ hybridization (63, 64), while one found no change by Northern blot analyses (85). Here again, the methods of detection and the drug paradigms need to be carefully considered. The definitive answer to these questions may be obtained only after these paradigms have been reproduced in cell lines.

Selectivity in Tissue Distribution

One advantage to the organism of an heterogeneous population of dopamine receptors is that it permits selective tissue-specific expression. This would imply that distinct receptor subtypes are expressed in different tissues. Since antibodies against all the different dopamine receptors are not currently available, our knowledge of their tissue distribution comes primarily from in situ hybridization experiments. In the central nervous system, the five dopamine receptors each overlap but also exhibit some striking location differences. In the periphery, the different receptors are mostly expressed in a tissue-specific fashion.

CNS Distribution

The D_1 and D_2 receptor mRNAs are present in all dopaminoceptive regions of the rat brain (60, 61, 86–90). High levels of D_1 and D_2 mRNAs are present in the caudate-putamen, nucleus accumbens and olfactory tubercule, lower levels in the septum, hypothalamus and cortex. Regions where D_2 but no D_1 mRNAs were detected are the substantia nigra and ventral tegmental area, where the D_2 mRNA is expressed at high level, and the hippocampus. Conversely, the amygdala contains D_1 mRNA but little if any D_2 mRNA.

The tissue distribution of the D_1 and D_2 mRNAs in the CNS supports their participation in the different aspects of dopaminergic neurotransmission that have been described on the basis of ligand binding and receptor autoradiography experiments.

Since the tissue distribution of the different dopamine receptors overlap in the CNS, some selectivity may be attained quantitatively rather than qualitatively. First, (as judged from mRNA band intensities (31)) D_3, D_4, and D_5 mRNAs are one to two orders of magnitude lower in abundance than D_1 or D_2 mRNAs (31, 33, 37). Moreover, the relative abundance of the D_1 mRNA is striatum > amygdala > thalamus > mesencephalon > hypothalamus = medulla, that of the D_2 is striatum > mesencephalon > medulla > hypothalamus > hippocampus (22, 26). By comparison, the relative abundance of the D_3 receptor mRNA is olfactory tubercule-Islands of Calleja > nucleus accumbens = hypothalamus > striatum > substantia nigra, it is absent in the hippocampus (31), while that of the D_4 mRNA is medulla = amygdala > midbrain = frontal cortex > striatum > olfactory tubercule > hippocampus (33). So, relative to the D_1 or D_2 receptors, the D_3 and D_4 receptors are more selectively associated with the "limbic" brain, a region that receives its dopamine input from the ventral tegmental area and is associated with cognitive, emotional, and endocrine functions. The location of the D_5 receptor mRNA, on the other hand, is a matter of controversy. The distribution of the D_5 receptor mRNA was first reported to overlap that of the D_1 mRNA (35), but two studies have subsequently conflicted with this view (37, 91). These authors found that the tissue distribution of the D_5 mRNA tissue is highly restricted. The D_5 mRNA is found only in the hippocampus, the hypothalamus, and the parafascicular nucleus of the thalamus and thus might be involved in affective, neuroendocrine, or pain-related aspects of dopaminergic function (91). That the D_5 receptor mRNA is present at a very low level and thus can be easily masked by the predominant D_1 receptor mRNA probably explains the discrepancies in tissue distribution.

One important question unresolved before in situ hybridization experiments was that of the cellular colocalization of the D_1 and D_2 receptors (60). In brain regions where both mRNAs exist, the amounts of each are approximately equal. Analysis of sequential thin sections reveals that D_1 and D_2 mRNA are colocalized in 26–40% of all caudate-putamen cells and in about 50% of all dopamine receptor mRNA-positive cells.

Peripheral Distribution

The D_1 and D_3 receptor mRNAs are practically absent outside of the CNS (26, 31), although the presence of D_1 mRNA in the parathyroid gland, a prototypic location of the D_1 binding site, has not been analyzed thus far. The D_2 receptor mRNA is expressed at high level in the pituitary (22) where

its physiological role in regulating hormone secretion is well-known. No D_1, D_3 or D_5 receptor mRNA was detected in the pituitary where the D_4 mRNA exists, albeit at low level (92). Finally, the D_2 mRNA is also present in the adrenal gland (H. H. M. Van Tol, J. R. Bunzow, O. Civelli, unpublished data).

A major question about the peripheral dopamine receptors concerns the identity of the receptors present in the kidney and the cardiovascular system; do they differ from those of the CNS as indicated by pharmacological analyses (94)? The peripheral dopamine receptors are of therapeutic importance since their stimulation is used to improve kidney function in case of shock and low cardiac output. Both D_1- and D_2-like activities have been described in the kidney and in the heart (40, 94). The D_5 receptor mRNA is expressed, albeit at low level, in the kidney (J. H. Meador-Woodruff, D. K. Grandy, unpublished data). Whether it is the expected D_1-like receptor or yet another one has not been demonstrated. None of the cloned D_2-like receptor mRNAs is present in the kidney. On the other hand, the D_4 mRNA is expressed in the heart (96) and might account for the expected D_2-like reactivity reported for this tissue. The D_1-like receptor mRNAs do not exist in any significant amount in the heart. These data open the possibility that the D_4 and D_5 receptors are the only dopamine receptors present in the kidney and the heart, an hypothesis that must be investigated pharmacologically and physiologically.

In summary, the different dopamine receptors exhibit specificity in their tissue distribution in the periphery. In the CNS, they often share tissue locations and, possibly, individual neurones as in the case of the D_1 and D_2 receptors, although selectivity in cellular distributions has also been found. Furthermore, some selectivity in receptor reactivity may also be gained quantitatively, as suggested by the relative abundance of the subtypes. This abundance indicates that interactions between different subtypes, such as those described between the D_1 and D_2 receptors (97–100), may be an important factor in the regulation of dopamine actions.

Genomic Polymorphism and Alternative pre-mRNA Maturation

Although the human genome as seen now contains five dopamine receptor genes, it encodes a higher number of mRNA species. This increase in complexity results from the discovery that polymorphism and alternative splicing events play a role in dopamine receptor gene expression and leads to the existence of more than five different receptor binding sites. Alternative splicing events and genomic polymorphism have been described for other genes and shown to be physiologically important in several cases (101–104).

Two Alternative Forms of the D_2 Receptor

That different dopamine receptors can be expressed from a single gene was first demonstrated by the existence of not one but two dopamine D_2 receptor cDNAs (41, 105–111). These two forms differ in 29-amino acid residues located in the putative third cytoplasmic loop of the receptor. They are generated by an alternative splicing event that occurs during the maturation of the D_2 receptor pre-mRNA (41, 106, 111). This event was demonstrated by the discovery of an 87-bp exon encoding the additional amino acid residues. The 29-amino acid addition contains two potential glycosylation sites but thus far nothing is known about their physiological importance, if any (41). The two D_2 receptor forms are neither species- nor tissue-specific. They exist in human, rat, bovine, mouse, and frogs; they coexist in all tissues analyzed but at a highly variable ratio. The shorter form is the least abundant, its concentration is very low in the pituitary but it represents about half of the D_2 receptor mRNA in the pons or medulla (107, 111).

The presence of a 29-amino acid addition in the third cytoplasmic loop should a priori not affect ligand recognition. Although this was shown for D_2 receptor antagonists whose affinities for the two forms are the same (41, 107), it remains to be shown for agonists. Due to its location in the third cytoplasmic loop, however, the addition was expected to affect G-protein coupling and consequently second messenger systems. It has been shown that both forms can inhibit cAMP accumulation (106). Whether they do so with different efficiencies has been analyzed in two reports. In one report, CHO lines shown to express the same quantities of receptors were established. In these cells, the short form can maximally inhibit cAMP production by up to 85% while the long form can only reach 64% (112). In the other report, a human choriocarcinoma line, JEG-3, was established that expresses β_2-adrenergic receptors (113). Stimulation of this receptor was measured via the cascade of events that leads from the increase in adenylyl cyclase activity to the increase of CREB (cAMP-responsive element-binding protein) binding to CRE. This activation was monitored using a reporter gene containing the CRE sequence linked to CAT (chloramphenicol acetyltransferase) gene, whose activity can be measured biochemically. These cells were transiently transfected with either of the two D_2 receptor form cDNAs and inhibition of cAMP production was reflected by corresponding inhibition in CAT activity. In these assays, the short form of the D_2 receptor elicited a stronger effect on adenylyl cyclase inhibition than the long form (74% versus 61% at 10 μM dopamine) and a lower affinity (EC50 58 nM versus 72 nM). Part of these latter findings might be accounted for by the discovery that the 87 bases differentiating the two receptor forms have a unusually high intrinsic CREB-binding property (M. Martin, J. R. Bunzow, unpublished data). In any case, the merging concept

from these studies is that the short form of the D_2 receptor requires less dopamine to be stimulated and, as a result, cells expressing a higher level of the short form will be activated first. Thereafter, the differences in ratio between the two forms might reflect some physiological differences.

Alternative splicing events have also been shown to occur during the maturation of the D_3 dopamine receptor pre-mRNA (115, 116). These resulting mRNAs would direct the translation of truncated receptor proteins. Indeed, upon transfection of the truncated cDNAs, no dopamine-binding activity was detectable (115). It is thus possible that, in vivo, the truncated mRNAs are products of abnormal posttranscriptional processing, possibly a minor event detectable by the advent of PCR.

Polymorphism in the Human D_4 Receptor Gene

Analysis of different human D_4 receptor cDNA sequences demonstrated that the D_4 receptor can exist in at least three variants (Figure 1). These variants differ in the number of 48 base-pair repeats contained in their putative third cytoplasmic loop (92). cDNAs harboring 2, 4, and 7 repeats have been identified from the neuroepithelioma cell line SKN-MC, pituitary, or substantia nigra. These and two more variant alleles have been detected in the genomes of different individuals, showing that a genetic polymorphism is responsible for the generation of the D_4 receptor variants. These repeats are not present in the rat gene, making the polymorphism possibly specific to humans. When expressed by DNA transfection, the variants containing 2, 4, and 7 repeats bind clozapine with equal affinities in the presence of sodium chloride. In the absence of sodium ions, however, the variants containing 2 and 4 repeats had a six- to eightfold lower dissociation constants for clozapine, while the affinity of the variant containing seven repeats was practically unaffected (92). Although the effects that the sodium ions have on receptors are not understood, these data indicate that the variants can behave differently with respect to the mechanism of ligand recognition. The presence of the repeats in the third cytoplasmic loop also suggests differences in G-protein coupling. Furthermore, the discovery of this polymorphism in the human population may enhance understanding of affective disorders at the molecular level.

The D_5 Receptor Pseudogenes

The D_5 receptor gene is particular among the G protein-coupled receptors in that it is associated with two pseudogenes in the human genome (36). The three D_5 related genes are found on different chromosomes (117). Only one gene (DRD_5, Chromosome 4 q15.1-q15.3) codes for the active receptor, the two others contain an 8 base-pair insertion that leads to a frame-shift. Since it was demonstrated by expression that the insertion is not part of an intron,

the two other genes (DRD_5P1, Chromosome 2 p11.1-p11.2, and DRD_5P2 Chromosome 1 q21.1) are genuine pseudogenes. Two facts are intriguing about the D_5 pseudogenes: they are embedded in more than 5000 bases that are practically identical on the three chromosomes; and they appear to be specific to human. The rat D_5 gene, which has the same sequence as the D_1b gene, has no pseudogene (117) and some monkeys have only one (118). Together, these data suggest that the evolution of the D_5 pseudogenes is a very recent event that may be restricted to the primates. The D_5 pseudogenes could serve as markers to elucidate the evolutionary process that led to dopamine receptor heterogeneity.

DOPAMINE RECEPTOR AND GENETIC LINKAGE TO HUMAN DISORDERS

Finally, the dopamine receptor clones have also been used to test for possible linkage between the receptor and human neuropsychiatric disorders. Several disorders associated with the malfunctioning of the dopamine system have a genetic component. The identification of restriction length polymorphisms (RFLPs) in the human dopamine receptors genes (25, 30, 119) has permitted their use as probes for linkage analyses of members of these families. Thus far, most studies have involved the D_2 receptor and have used a Taq1 RFLP found downstream of the poly A adenylation site of the D_2 receptor (25). Schizophrenia, Tourette syndrome, and manic depression have been found not to be directly associated with the RFLP of the D_2 receptor gene locus (120–123). Furthermore, the D_2 receptor peptide sequences of 14 schizophrenics and 4 controls have been found to be identical (124). Several similar studies are being conducted on D_3 and D_4 receptor genes. In contrast, linkage between the D_2 receptor and severe alcoholism has been reported (125). However, this finding has been a matter of controversy (126) and will undoubtedly be the subject of many more studies.

CONCLUSIONS AND PERSPECTIVES

The identification of "unexpected" dopamine receptor subtypes has had a tremendous impact on our understanding of the dopaminergic system. The diversity of the physiological activities attributed to dopamine can now be analyzed knowing that a greater number of dopamine receptors are involved. The availability of receptor clones, receptor antibodies, and expressed receptor proteins will permit in-depth studies of the circuitry of the dopaminergic system and of the mechanisms regulating it at the genomic and cytoplasmic level. It will also allow us to decipher the physical structure of the receptors and permit the design of highly specific ligands. Eventually, therapies for

disorders associated with malfunction of the dopaminergic system should benefit from the discovery of the receptor heterogeneity. The possibility that the D_3 and D_4 receptors are preferential targets of some neuroleptics (such as the atypical ones) stands as a first example. Our renewed understanding of the dopaminergic system will perhaps shed light on the molecular basis of human psychoses and Parkinson's disease.

Whether other dopamine receptor subtypes exist is still unclear. Heterogeneity is common among G protein-receptor families but the number of members in each family varies (20, 127, 128). This variability indicates that one neurotransmitter can interact with a variety of receptors, a fact that helps support, at a molecular level, the complexity of synaptic transmissions. Interestingly, it seems that each family is composed of two major subtypes distinguishable in the type of obligatory signaling pathways that they induce. There are indications that other receptors in the dopamine receptor family may exist, thus far not cloned (40). A D_1-like and a D_2-like receptor have been detected through expression of their corresponding mRNA or gene but have not been sequenced (129, 130). Since the identification of a new receptor necessitates determination of its sequence, these and other previously detected novel dopamine receptor-like activities remain putative until associated with a defined molecule.

ACKNOWLEDGMENTS

We thank Hubert Van Tol and to Qun-Yong Zhou for many discussions, our lab colleagues and outside collaborators for their contributions to the work discussed in this review, and, in particular, S. Watson, A. Mansour, J. Meador-Woodruff, Huda Akil, J. Gelernter, M. Caron, M. Litt, S. Sommer, J. Ebanks, G. Evans, Y. F. Liu, P. Albert, J. Meldolesi, and L. Vallar for their invaluable help. We also acknowledge J. Tasnady for preparing the manuscript and J. Shiigi for illustrations. The work from our laboratory was supported in part by grants from the National Institute of Health (DK37231, MH45614, MH48991)

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Annual Review of Pharmacol. Toxicol.
Volume 33, 1993

CONTENTS

(*Continued*)