The Dopamine D4 Receptor

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INTRODUCTION

The observation that receptors which mediate their signals through heterotrimeric guanosine triphosphate (GTP)-binding proteins (G-proteins) share a considerable amount of sequence similarity has resulted in the rapid molecular characterization of this receptor superfamily. Moreover, it became clear that several receptor families consisted of more receptor subtypes than originally anticipated from pharmacological studies. The dopamine (DA) receptor family, which was originally thought to comprise two receptor types (D1 and D2), includes to date five different DA receptor genes (Bunzow et al. 1988; Dearry et al. 1990; Sokoloff et al. 1990; Sunahara et al. 1990, 1991; Tiberi et al. 1991; Van Tol et al. 1991; Zhou et al. 1990). The major difference between the D1-like receptors D1 and D5/D1B lies in their distribution, but pharmacologically and functionally these two receptors are almost indistinguishable (Sunahara et al. 1991; Tiberi et al. 1991). The D2-like receptor family includes two alternatively spliced forms of the D2 receptor (Dal Toso et al. 1989; Giros et al. 1989; Grandy et al. 1989; Monsma et al. 1989), and the D3 (Sokoloff et al. 1990) and D4 (Van Tol et al. 1991) receptors. Apart from clear differences in distribution profiles, the D2-like receptors also display pharmacological differences. The cloned D2 receptor is able to inhibit adenylyl cyclase (Albert et al. 1990), and is also able to activate several other signal transduction pathways (Elsholtz et al. 1991; Kanterman et al. 1991; Vallar et al. 1990). Moreover, the two alternatively spliced forms of the D2 receptor are not identical in their functional activity (Hayes et al. 1992; Montmayeur et al. 1993).

Because of the increased complexity of the DA receptor system, the role of this system in self-rewarding behaviors has become potentially more complex and might need a reevaluation of the roles played by the individual receptor systems. The observation that D3, D4, and D5/D1b receptors are relatively more concentrated in the limbic system than the D1 and D2 receptors suggests a role for these newly recognized receptors in the etiology of addiction. This is illustrated by the observation that dopamine agonists with higher potencies for D3 receptors seem to be relatively more effective in decreasing cocaine self-administration in rats (Caine and Koob 1993).

MOLECULAR CHARACTERIZATION OF THE D4 RECEPTOR

After the initial cloning of the D1 and D2 receptors, it was speculated that there were several other DA receptor types. These speculations were based on several observations, particularly that dopamine receptors from different tissues or brain areas displayed pharmacological and functional profiles that were not in agreement with the predominant D1 and D2 receptor types described for brain tissue (Anderson et al. 1990; Nisoli et al. 1992; Sokoloff et al. 1984).

The search for novel DA receptor subtypes was based on the presumed homology between these receptor subtypes with the D1 and D2 receptors. The search was begun by scanning several tissues and cell lines for the presence of ribonucleic acid (RNA) species that would hybridize to a D2 receptor probe encoding the putative transmembrane regions VI and VII under lower stringency conditions, but not under high stringency conditions. Using this approach, D2 positive hybridizing RNA species (but not D2 itself) were identified in mouse neuroblastoma NB41A3 and N4TG1, hamster kidney cells BHK-21, and the human neuroepithelioma SK-N-MC. In the subsequent screening of a cDNA library from the cell line SK-N-MC the author succeeded in the isolation of a partial cDNA clone (750 bp) that displayed good homology with the D2 receptor and could detect a 5.2 kb RNA species in the SK-N-MC cell line and rat brain (Van Tol et al. 1991). Screening of several human and rat brain cDNA libraries did not result in the isolation of full-length clones, although several similar partial cDNAs were isolated. However, genomic clones from human and rat were isolated that encoded the entire coding region for this putative receptor (Asghari et al. 1994; O'Malley et al. 1992; Van Tol et al. 1991) (figure 1). Pharmacological analysis established that the isolated clones were indeed novel DA receptor subtypes (see below), which were called the DA D4 receptor (Van Tol et al. 1991). Based on the homology with the D2 and D3 receptors, the entire coding sequence for the D4 receptor was identified in a 4.5 kb genomic DNA fragment.

The coding sequence is interspersed by three introns for which the donor/acceptor splice junction sites were conserved at identical positions as seen for introns in the D2 and D3 receptors. Introns 2 and 3 were positively identified by comparative analyses of the genomic clones with

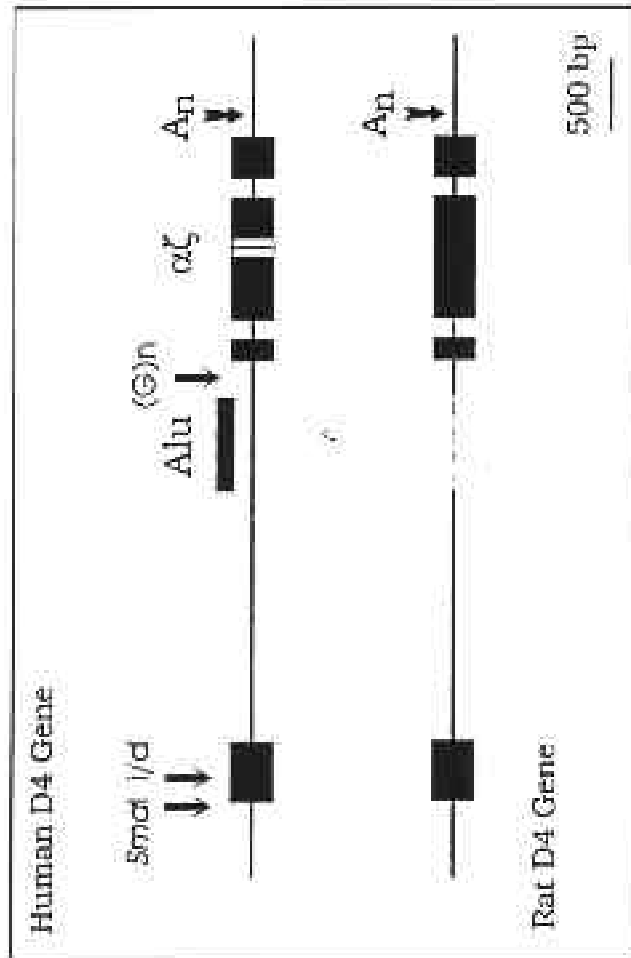

FIGURE 1.

FIGURE 1. *Genomic organization of the human D4.2 and rat D4 receptor genes. The coding regions are represented by the blocks and the noncoding regions by the connecting line. The repeat units alpha and zeta are indicated as two white boxes in the coding block. The position of the polyadenylation site is indicated with An and an arrow. An Alu sequence has been recognized within the first intron of the human gene that is not present within the rat gene. The dotted line within the first intron of the rat gene indicates where the Alu sequence is inserted in the human gene. Apart from the 48 bp repeat units in the third exon, three other polymorphic markers are indicated: (1) a polymorphic Smal site located immediately upstream from the initiation codon (Petronis et al. 1994a, 1994b), (2) an insertion/deletion polymorphism of 4 amino acids in the first exon (Catalano et al. 1993), and (3) a polymorphic G Mononucleotide repeat within the first intron (Petronis et al. 1994a, 1994b).*

the isolated partial D4 cDNA clones. The position of all three introns was confirmed by exon trapping, a technique through which a full-length D4 cDNA clone was also isolated (Van Tol et al. 1992).

Translation of the coding sequence into amino acid sequence revealed that the isolated D4 receptor clones encode a polypeptide with seven stretches of hydrophobic amino acids that could span the membrane. These putative transmembrane spanning domains display the highest amino acid sequence identity with the putative transmembrane regions of the D2 and D3 receptors, especially transmembrane domains 3, 6, and 7,which have 60percent to 80 percent sequence similarity to the homologous domains in the D2 and D3 receptors. The overall sequence similarity between the D4 and the D2 and D3 receptors is about 40percent. The sequence similarity is least preserved in the intra- and extracellular loops and tails, which supports the notion that the transmembrane domains form the ligand-binding pocket, as has been shown for adrenergic receptors. This is also supported by the fact that an Asp residue in transmembrane 3 and two Ser residues in transmembrane5, which have been identified as crucial amino acids for catecholamine binding in several catecholaminergic receptors, are conserved in the D4 receptor (Strader et al. 1988, 1989). In analogy to the models created for other G-protein-coupled receptors, the amino terminus is located extracellularly and contains one putative N-linked glycosylation site. The carboxy terminus ends in a Cys, as is seen for D2 and D3 receptors, and can serve as a potential substrate for palmitoylation (O'Dowd et al. 1989). Within the third cytoplasmic loop of the human D4 receptor there are only a limited number of residues that potentially can be used for phosphorylation, in contrast to the rat D4 receptor where there are several more such sites (Asghari et al. 1994; O'Malley et al. 1992; Van Tol et al. 1991). An unusual structural feature of the human D4 receptor is the presence of a polymorphic 16 amino acid repeat sequence in the third cytoplasmic loop, which is not found in the isolated rat gene (see below; figure 1). The location of this repeat sequence corresponds to the location of the alternatively spliced 29 amino acid sequence of the D2 receptor.

Through the cloning of several D4 genes and cDNAs it became clear that a region within the third cytoplasmic loop of the human D4 receptor was polymorphic (Van Tol et al. 1992). Subsequent detailed analyses of this region by Southern blot, polymerase chain reaction, and sequence analysis demonstrated that the polymorphic sequence occurs as a 48 bp

tandem repeat of 2 to 10 repeat units (figures 1 and 2). The different polymorphic repeat variants of the D4 are classified according to the number of repeats D4.2 to D4.10. Thus far 18 different repeat units have been identified which display over 90 percent sequence similarity among each other, and which have been identified by different Greek letters (Lichter et al. 1993). Although the first and last repeat units are always the so-called alpha and zeta units, respectively (there is one exception: an allele has been identified in which the last unit codes for a xi unit that is identical in amino acid sequence to the zeta unit), the other units can be found in any position between the alpha and zeta units. This has resulted in the identification of 27 different alleles in over 200 analyzed chromosomes (Asghari et al. 1994; Lichter et al. 1993; Van Tol et al. 1992) (figure 2).

In Northern blot analysis of cells transfected with the human D4 gene, several D4 cDNA variants indicate that the repeat sequence is not spliced out of the D4 gene, but is part of the coding sequence. This is supported by the fact that there are no sequences in or surrounding the repeat, which could be used as splice donor and acceptor sites. Moreover, exon trapping failed to excise this sequence. Thus the 27 variant alleles would actually code for 20 different D4 receptor variants. The putative amino acid sequence for the different repeat regions demonstrate a high Pro content in this region (30 percent to 40 percent). Recently, such Pro-rich segments have been identified as potential SH3-binding domains (Ren et al. 1993; Yu et al. 1994). On the other hand, it might be postulated that the variation in the third cytoplasmic loop sequence might have consequences for the specificity and/or efficacy in signal transduction as seen for the two alternatively spliced forms of the D2 receptor. Thus far, there is only limited evidence to support this speculation (Asghari et al., in press).

PHARMACOLOGY

The most extensive pharmacological characterization of the D4 receptor has been done by transient expression of this receptor in COS-7 cells. The pharmacological characterization of receptors derived from the expression of the entire gene or cDNA in COS-7 does not show any differences, except for the fact that higher levels of expression can be obtained with the cDNA than with the gene cloned into identical expression vectors. Transient expression of the D4 receptor in COS-7

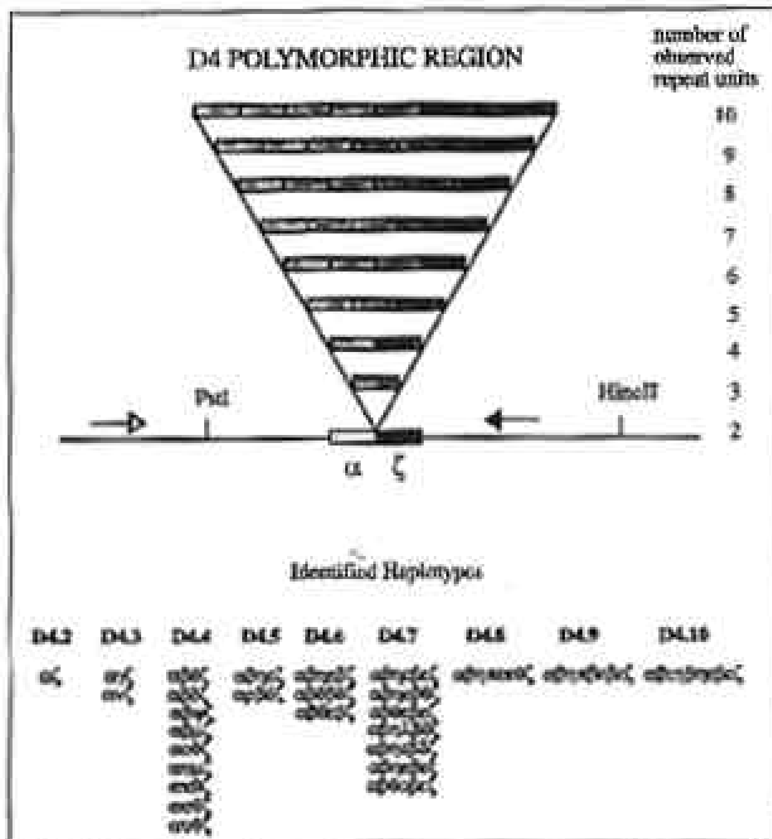

FIGURE 2. *The polymorphic 48 bp repeat region of the human D_4 receptor gene. The polymorphism can be detected by Southern blot analysis using genomic DNA digested with HincII/PstI (Van Tol et al. 1992) or by PCR analysis using specific primers (indicated by arrows) (Lichter et al. 1993). By sequence analysis of this D_4 repeat region of different individuals 18 different 48 bp repeat units have been identified (marked by different Greek letters), which can give rise to 27 different observed haplotypes (Asghari et al. 1994; Lichter et al. 1993).*

cells resulted in the detection of concentration-dependent and saturable [3H]spiperone binding with an affinity dissociation constant of approxi-mately 100 picomolars (pM). The [3H]spiperone binding could be competed with dopamine (inhibition constant (Ki) approximately 27nano-molars (nM)), but less efficiently by norepinephrine and serotonin (Ki>2micromolars (µM). Dopamine competition of [3H]spiperone binding revealed the presence of a high affinity site with a dissociation constant of 500 to 1,000 pM and a low affinity site of 10 to 50 nM. Inclusion of 200µM Gpp[NH]p in the binding buffer resulted in the conversion of the biphasic dopamine competition curves to a single affinity site of approximately 10 to 50 nM (Asghari et al. 1994; Van Tol et al. 1991, 1992). These binding data suggest functional coupling of the D4 receptor in COS-7 cells.

Dopamine D4 receptor binding characteristics have been determined for several D2 agonists and antagonists. This revealed a D4 pharmacological profile that has several similarities with the D2 receptor; however, some remarkable differences have also been detected (figure 3). Probably the most striking is the relatively high affinity of the atypical neuroleptic clozapine for the D4 receptor (Ki 10 to 20 nM) as compared to the D2 receptor (Ki 100 to 200 nM), and the poor affinity of the D2 antagonist raclopride for the D4 receptor (1 to 2 µM) (Asghari et al. 1994; Van Tol et al. 1991, 1992). Furthermore, the D4 receptor demonstrates stereo-selectivity for (+)- and (-)-butaclamol, several (+)- and (-) aporphines, (+)and (-) PHNO, (+) and (-) quinpirole, but not for (+) and (-)-sulpiride (Seeman and Van Tol 1993).

Thus far, no compound has been reported that is selective for the D4 receptor. Although clozapine seems to be tenfold more selective for the D4 receptor as compared to other dopamine receptors, the muscarinic, 5-HT2, and 5-HT7 receptors have similar affinities for clozapine as the D4 receptor. However, by making use of the differential affinities of the benzamides [3H]emonapride and [3H]raclopride for the different D2-like receptors, the density of D4-like sites in brain tissue can be indirectly determined (Seeman et al. 1993). Scatchard analysis with [3H]emonapride will detect and allow determination of the total density of all D2-like receptors (D2, D3, D4), while such analysis with [3H]raclopride will only detect and reveal the density of the D2, D3 receptor pool. Therefore, the difference in maximal density detected by both radioligands in scatchard analysis will reflect the density of a receptor pool that is D4-like.

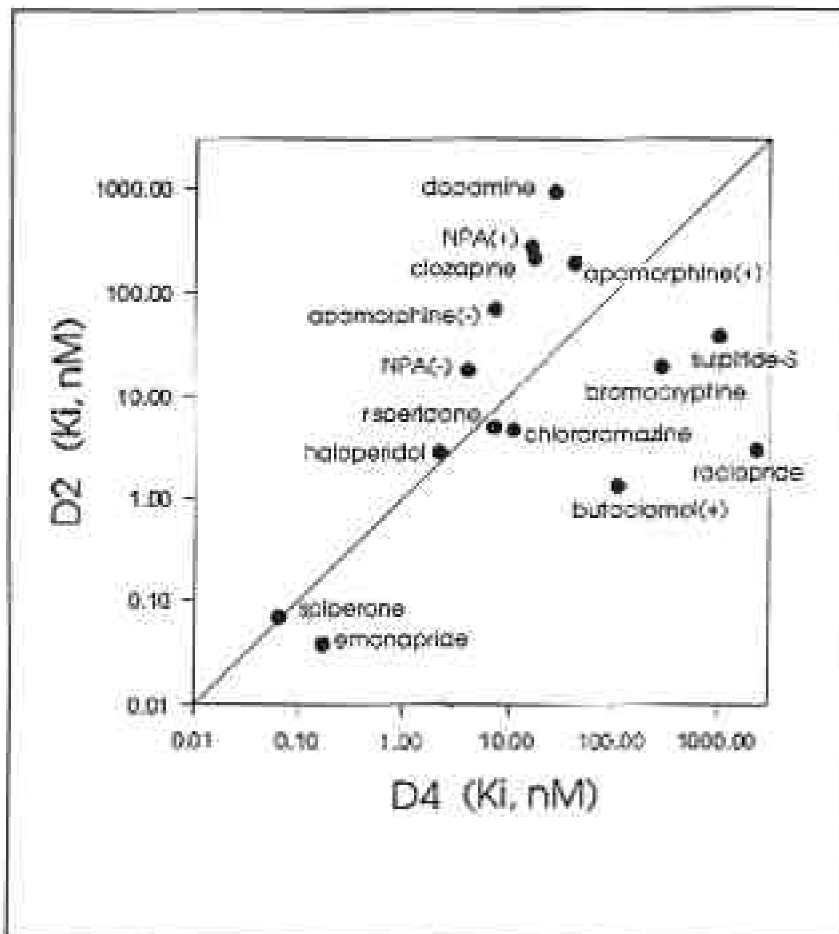

FIGURE 3. *Correlation plot for the affinities of several dopamine agonists and antagonists at the cloned D_2 and D_4 receptor expressed in COS-7 cells. The affinity dissociation constants are determined by competition analysis of [3H]spiperone binding as described previously (Van Tol et al. 1991). For reference a line indicating the position for equal affinities is drawn within the plot.*

Seven different polymorphic repeat variants of the human D_4 receptor have been characterized for their affinities to clozapine, emonapride, haloperidol, raclopride, spiperone, and dopamine (Asghari et al. 1994). Thus far, none of the tested ligands has shown any major differences in

their affinity for several D4 receptor variants. Moreover, for all variants tested, two similar affinity states for dopamine binding were observed that were sensitive to the inclusion of Gpp[NH]p. Under standard DA receptor binding conditions, no major differences have been observed in pharmacological characteristics between variants of the D4 receptor. However, small differences in the sensitivity for sodium chloride on clozapine binding have been observed between D4 variants (Asghari et al. 1994; Van Tol et al. 1992). As yet, it is unclear whether these observed differences are indicative of differences in receptor function. Genetic association studies have thus far not indicated a good correlation between differences in clozapine responsiveness in the patient population and the different D4 isoforms (Shaikh et al. 1993). However, inclusion of four different polymorphic markers for the D4 receptor gene (figure 1) in these analysis gives some ability to predict clozapine responsiveness (Kennedy, personal communication, July 1994).

COUPLING TO ADENYLYL CYCLASE

In order to determine whether the D4 receptor possesses the ability to block adenylyl cyclase activity, the D4.2 variant was cloned by homologous recombination into vaccinia virus. This recombinant vaccinia virus could infect several cell types which would, upon infection, express D4 receptors that display a pharmacological profile identical to that described previously. Approximately 2 to 3 days after infection, cells express D4 receptor levels, as determined by [3H]spiperone binding, of approximately 1 pmol/mg protein. Functional analyses of GH4C1 cells, mouse fibroblast L cells, and Rat-1 cells demonstrated that all these cells, upon infection with recombinant D4 vaccinia virus, could block adenylyl cyclase activity by dopa-mine (Bouvier et al. 1993). Detailed analyses of infected Rat-1 cells demonstrated that dopamine could block forskolin-stimulated adenylyl cyclase activity and increased intracellular cyclic adenosine monophos-phate (cAMP) levels with a 50 percent effective concentration (EC50) of about 10 nM. This activity could be blocked by spiperone and clozapine, but not by raclopride, which was in agreement with the affinities of these drugs for the D4 receptor (Bouvier et al., in press).

As well, several forms of the human D4 receptor were stably transfected into Chinese hamster ovary cells (CHO-K1). These cell lines express the dopamine D4 receptor at concentrations of approximately 200 to 400 fmol/mg protein. Stimulation of these cells

by various concentrations of dopamine did not significantly change the intracellular cAMP levels, compared to nontransfected cells and nonstimulated cells. However, in the D4-expressing cell lines, DA could reduce forskolin-induced intracellular cAMP increases by up to 90 percent (Asghari et al., in press). This reduction was concentration dependent with an EC50 of approxi-mately 15 nM (figure 4). Comparative analysis of various cell lines expressing the D4.2, D4.4, or D4.7 variants failed to show any major differences in either efficacy or EC50 of DA to block adenylyl cyclase activity, although the potency of DA for D4.7 was slightly reduced (Asghari et al., in press). The D2 antagonists emonapride, haloperidol, and clozapine could inhibit the dopamine-induced cAMP changes in forskolin-stimulated cells in a concentration-dependent manner and with a rank order that was in close agreement with the rank order seen for the affinities of these compounds for the D4 receptor. Raclopride was not able to block dopamine's activity at the D4 receptor at concentrations less than 5 μM, which is expected considering the low affinity of this ligand for the D4 receptor. The D4.2, D4.4, and D4.7 all displayed similar functional profiles for these four D2 antagonists.

EXPRESSION OF THE D4 GENE

Dopamine D4 receptor messenger RNA (mRNA) has been detected in various brain regions from humans, monkey, and rat. Northern blot analysis of several dissected brain regions has shown a distribution for this receptor that is dissimilar to the D2 and D3 receptors. A regional distribution study in monkey brain showed relatively high levels for D4 mRNA in frontal cortex, amygdala, midbrain, and medulla (Van Tol et al. 1991). Lower levels were detected in striatum and hippocampus. Although D4 mRNA is detectable by Northern blot analysis in poly A+ enriched RNA preparations, D2 mRNA can be monitored easily by the same methodology in total RNA preparations, suggesting two orders of magnitude difference in the density of both RNAs (Bunzow et al. 1988). This is confirmed by the relatively low signal that is detected by in situ hybridization. In situ hybridization data basically confirm the locali-zation seen by Northern analysis, although cellular localization is more detailed and thus revealed the presence of D4 mRNA at relatively high levels in dentate gyrus (Mansour et al. 1991; Meador-Woodruff et al. 1991, 1994; O'Malley et al. 1992). Interestingly, D4 receptor mRNA has also been detected in relatively high levels in rat heart (O'Malley et al. 1992) and retina (Cohen et al. 1992).

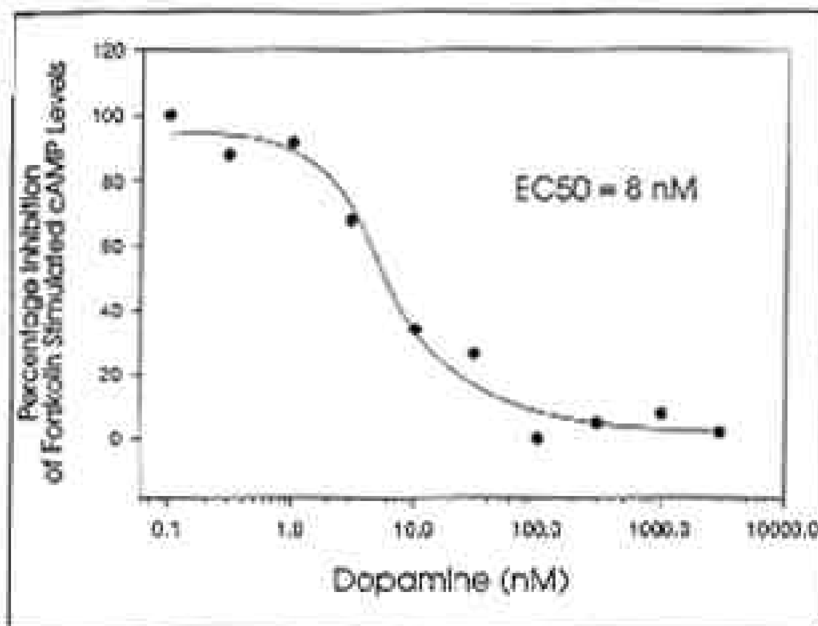

FIGURE 4. *Inhibition of forskolin-stimulated cAMP levels in CHO-K1 cells stably expressing the human D_4 receptor. The cells express approximately 300 fmol/mg protein D_4 receptors as determined by [3H]spiperone binding. Cyclic AMP levels were stimulated with 10 μM forskolin or 10 μM forskolin with varying concentrations of dopamine for 20 min. Cyclic AMP levels were determined by radioimmunoassay essentially as described by Albert and colleagues 1990.*

Alternatively, the author attempted to monitor the location of D4 binding sites. By using the differential binding characteristics of [3H]raclopride and [3H]emonapride for D2, D3, and D4 receptors (see above), D4-like binding sites were detected in caudate putamen of human and rat but not in frontal cortex. This can be interpreted to indicate that a large number of the D4 mRNA containing cortical neurons have their projections with D4 sites in noncortical areas, including caudate putamen. Less likely is that this RNA is not translated into a functional protein. Preliminary data using in vitro autoradiography of [3H]emonapride in the presence of an excess of unlabeled raclopride (> 100 nM) confirms the location of these raclopride "insensitive" [3H]emonapride binding sites seen by scatchard analysis, and also revealed several D4-like binding sites in other rat brain areas, including entorhinal cortex, colliculi, and central

gray area (Nobrega, personal communication, June 1994). Whether these sites are genuine D4 receptors awaits further characterization.

Little is known as yet about whether, and how, D4 receptor density is regulated. Experiments in which rats have been treated for a prolonged period with haloperidol indicate a twofold increase in both D4 mRNA and D4 binding sites, as defined by emonapride and raclopride, in caudate putamen (Schoots et al. 1995). Interestingly, the number of dopamine D4-like binding sites is also considerably increased in postmortem caudate putamen tissue of schizophrenics (Seeman et al. 1993). In the schizophrenic tissues the D4 receptor levels were elevated approximately sixfold over control tissues. Although the majority of the tested tissues originated from schizophrenic individuals who were treated with neuroleptics, similar increases were also seen in the drug-naive individuals, while no significant changes were measured in tissues obtained from patients with Huntington's disease who were treated with neuroleptics. Furthermore, as described above, rats treated with neuro-leptics only showed a twofold change in receptor levels, suggesting that at least a certain proportion of the increased receptor density must be attributed to the disease. On the other hand, the relation of the D4 receptor to schizophrenia is unclear. Genetic linkage and association studies of schizophrenia and bipolar affective disorder with several polymorphic markers for the D4 receptor have not supported this receptor as the primary cause for these disorders (Barr et al. 1993; Macciardi et al. 1994). Similar results have been obtained for the D2 and D3 receptors (Kennedy 1994). This, however, does not rule out any of these receptors as therapeutic targets to control these disorders, since drugs with different D2-like binding profiles and clinical profiles, like raclopride, haloperidol, and clozapine are all effectively used in the treatment of these diseases.

CONCLUDING REMARKS

The role of the dopamine D4 receptor in the DA system with respect to addiction is unclear. Although the DA system has been clearly established as a modifier of self-reward behavior, the recognition of several new DA receptor genes warrants a reevaluation of several components of the system. The absence of a wide variety of agonists and antagonists that are highly selective for the different receptors has prevented the evaluation of the functional role of these receptors in drug addiction. From studies done by Caine and Koob (1993) it is clear that different DA receptors might contribute differently to self-

reward behavior. With respect to the D4 receptor, one published study indicates a higher frequency of the occurence of the alleles D4.3 and D4.6 in alcoholism (George et al. 1993); however, another study failed to demonstrate such an association (Adamson et al. 1995).

To establish unequivocally that the D4 receptor is not a genetic factor contributing to addiction, other paradigms might also have to be tested. As for the roles of the D4 receptor and other newly recognized DA receptors in addiction behavior, researchers are largely ignorant about the different behavioral functions of these receptors. Although the development of highly specific D4 agonists and antagonists might be of great value, alternative approaches should be investigated. As shown for the 5-HT1B receptor (Hen et al., this volume), the use of DA receptor gene "knockout" mice might be of great value for unraveling the functional roles of the dopamine receptors for different behavioral paradigms.

One of the most interesting molecular features of the human D4 receptor is probably the extensive polymorphic repeat sequence in the coding sequence of this receptor. The identification of at least 27 different haplo-types for the repeat encoding 20 putative different D4 receptor proteins has revealed another level of receptor diversity which is thus far unprecedented within the G-protein-coupled receptor superfamily. Until now it was recognized that receptor diversity was generated by the existence of several genes coding for different subtypes or by alternative splicing. Large structural polymorphisms as described for the D4 receptor might account for differences in drug responsiveness and susceptibility to neuropsychiatric disorders, including addiction. It would be of interest to see whether such extensive interindividual differences in coding sequence also exist for other proteins, or whether this is a unique feature of the D4 receptor.

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Click here to go to page 39