Cryptic Initiation at the Human D4 Receptor Reveals a Functional Role for the Amino Terminus†

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ABSTRACT: It was found that deletion of the initiator methionine of the D4 receptor results in the use of a cryptic initiation site in the putative first transmembrane region. We made use of this observation to investigate the role of the amino terminus of the D4 receptor. *In vitro* transcription and translation of D4.4 and a D4.4 deleted for the initiation codon (D4.4ΔNH_2) resulted in the formation of protein products with a molecular mass of about 44 and 40.5 kDa, respectively. The molecular mass of 40.5 kDa suggests initiation in the putative first transmembrane region. Transient expression of various deletion mutants indicated that this receptor form can be expressed at up to 70% of the D4.4 control levels and provided support for the existence for an alternative translation initiation site in the first transmembrane domain, most likely at nucleotide +112 (the initiator methionine codon is designated as +1). The D4.4ΔNH_2 mutant was stably expressed in CHO cells. Pharmacological analysis demonstrated no major differences in antagonist binding with the regular D4.4 receptor, while dopamine and quinpirole binding affinities were about 5-fold decreased. The half-maximal level (EC_{50}) for blocking forskolin-stimulated cAMP levels by dopamine was about 10-fold lower as compared to D4.4. Furthermore, the functional efficacy is decreased by about 40%. These data suggest that the amino-terminal domain is not essential for proper expression, but does interfere with the functional activity of the receptor, possibly through stabilization of the active state. To our knowledge this is the first demonstration that the amino terminus of a dopamine receptor is involved in signal transduction.

The dopamine D4 receptor belongs to the family of D2-like dopamine receptors (D2, D3, and D4) which serve as targets for antipsychotics in the treatment of schizophrenia. The human D4 receptor is of particular interest because it has the highest affinity of all the dopamine receptors cloned to date for the atypical antipsychotic clozapine which is devoid of extrapyramidal side effects (Van Tol et al., 1991).

An interesting feature of the human dopamine D4 receptor is that several variants have been observed in the human population. The most common variation is a polymorphic imperfect direct tandem repeat sequence of 16 amino acids in the putative third cytoplasmic loop region (Van Tol et al., 1992). Thus far, we have observed 27 different haplotypes coding for 20 different receptor forms (Lichter et al., 1993; Asghari et al., 1994). Three of the most common occurring variants did not display large pharmacological or functional differences (Asghari et al., 1994, 1995). Naturally occurring mutant forms of the D4 receptor also include a single nucleotide change in transmembrane domain 5 of the receptor which changes a valine into a glycine residue, rendering this receptor form functionally inactive (Seeman et al., 1994; Liu et al., 1995), a 13 base pair (bp)[1] frameshift deletion in the second transmembrane domain (Nöthen et al., 1994), and a 12 bp deletion in the amino-terminal domain (Catalano et al., 1993). Here we present evidence for the potential existence of an amino-terminal truncated D4 variant. We have made use of this variant to study the role of the amino terminus for D4 receptor's pharmacology and function.

To date there is little information on the role of the amino-terminal region of catecholaminergic receptors. Thus far, it has been recognized that catecholaminergic receptors belong to class IIIb membrane proteins which do not contain a signal sequence (Guan et al., 1992; Singer, 1990). Amino-terminal glycosylation of catecholamine receptors is of little importance for ligand binding and receptor function (Rands et al., 1990; Kobilka, 1990). However, recently it was shown by Green et al. (1994) that naturally occurring mutations in the amino-terminal domain of of the β_2-adrenergic receptor modified agonist-mediated receptor down-regulation. Here we present evidence that the amino-terminal domain of the D4 receptor is involved in signal transduction.

MATERIALS AND METHODS

Materials. COS-7 and CHO-K1 cells were obtained from ATCC. Fetal calf serum, horse serum, Geneticin (G418), and α-MEM were obtained from GIBCO/BRL Ltd. (Grand

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[1] Abbreviations: bp, base pair(s); α-MEM, Dulbecco's α-minimum essential medium; IBMX, 3-isobutyl-1-methylxanthine; EC_{50}, concentration of agonist required to produce 50% maximal response; Gpp[NH]p, guanylyl imidodiphosphate.

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Island, NY) and a central media preparation service (Medical Sciences Building, University of Toronto). The eukaryotic expression vector pRc/RSV was obtained from Invitrogen (San Diego, CA), and the cloning vector pbluescript (SK$^-$) from Stratagene (La Jolla, CA). The expression vector pCD-PS was a kind gift of Dr. T. Storman. Materials required for large-scale plasmid preparation were purchased from QIAGEN (Chatsworth, CA). Materials for in vitro transcription translation were obtained from Promega (Madison,WI; product number L4610). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). *Bal*31 and *T4* DNA ligase were purchased from GIBCO/BRL Ltd. [^{3}H]Spiperone (120 Ci/mmol) was purchased from Amersham (Arlington, IL). [^{3}H]Leucine (145 Ci/mmol) was obtained from Du Pont−New England Nuclear. Dopamine, forskolin, guanylyl imidodiphosphate (Gpp[NH]p), and anti-3′,5′-cyclic adenosine monophosphate antiserum were purchased from Sigma Chemicals Co. (St. Louis, MO); raclopride, Astra Arcus AB (Södertälje, Sweden); haloperidol, Janssen Pharmaceutica (Beerse, Belgium); clozapine, Sandoz Pharma Ltd. (Basel, Switzerland); *S*-sulpiride, Ravizza (Milan, Italy); YM-09151-2 [(n)emonapride], Yamanouchi Pharmaceutical Co. (Tokyo, Japan); 3-isobutyl-1-methylxanthine (IBMX) and dry acetone, Aldrich Chemical Company, Inc. (Milwaukee, WI).

D4 Plasmid Constructs. The D4.2, D4.4, and D4.7 cDNA constructs in the plasmid pbluescript (SK$^-$), and the eukaryotic expression vectors pCD-PS and pRc/RSV have been described previously (Van Tol et al., 1992; Asghari et al., 1994, 1995). The 5′ deletion mutants of the D4.4 cDNA were in essence generated by opening the D4.4 cDNA containing pbluescript plasmids with the restriction enzyme *Eco*RV or *Sma*I (partial digest) followed by *Bal*31 deletions for various lengths of time. The truncated D4 inserts were excised by a *Xba*I digestion and subcloned into the *Eco*RV-*Xba*I sites of pbluescript. Next, the D4 deletion mutants were excised as *Hin*dIII-*Xba*I or *Kpn*I-*Xba*I fragments and subcloned into the *Hin*dIII-*Xba*I sites of pRc/RSV or the *Kpn*I-*Spe*I sites of pCD-PS. The deletions were examined by restriction site analysis and sequence analysis. Sequence analysis was done by the dideoxy chain termination method using Sequenase (version 2.0) and 7-deaza-dGTP (United States Biochemical, Cleveland, OH). Restriction endonuclease digests, ligation reactions, and *Bal*31deletions were done as recommended by the manufacturers of the enzymes.

In Vitro Transcription and Translation. The D4 cDNAs containing pbluescript plasmids (20 ng/μL) were used in the commercially available transcription/translation system from Promega using T7 RNA polymerase and [^{3}H]leucine (0.5 mCi/mL) (TnT T7 coupled reticulocyte lysate system; Promega Corp., Madison, WI) (Van Oostbree et al., 1992). The reactions were done for 90 min at 30 °C as recommended by the manufacturer of the system. The reaction products were analyzed by SDS−polyacrylamide gel electrophoresis. The reaction was taken up in an equal volume of 2× Laemlli buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol, and 5% β-mercaptoethanol). This sample (25 μL) was run on a 10% acrylamide gel (NOVEX, San Diego, CA) in 23.9 mM Tris base, 190 mM glycine, and 0.1% (w/v) SDS. Next, the proteins in the gel were fixated for 1 h in 10% (v/v) glacial acetic acid, 30% (v/v) methanol. For fluorography, the gel was treated with EN^{3}HANCE (NEN Research Products, Boston, MA), dried, and exposed overnight to X-ray film.

Cell Culture. CHO-K1 cells and subclones stably expressing D4 receptors (Asghari et al., 1995) were grown in α-MEM supplemented with 2.5% fetal calf serum and 2.5% horse serum at 37 °C and 5% CO_2. COS-7 cells were grown under identical conditions using α-MEM supplemented with 10% fetal calf serum. CHO-K1 cell lines stably expressing one of each of the D4 variants were created by electroporation with pRc/RSV constructs of the D4 variants. Transfected cells were continuously grown in the presence of G418-[CHO-K1, 400 μg/mL (active)]. After clonal selection and expansion of several G418-resistant cells, stable expression of D4 receptors was determined by [^{3}H]spiperone binding. Transient expression in COS-7 cells was achieved by electroporation of the pCD-PS and pRc/RSV constructs into these cells as described previously (Asghari et al., 1994, 1995).

Cyclic AMP Assays. CHO cells ($\sim$1.2 × 10^6 cells/35 mm plate) stably expressing D4 receptors were incubated in HBBS buffer (118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-Glucose, 20 mM Hepes, and 0.3 mM IBMX, pH 7.2) with and without dopamine (0.0001 μM−3 μM) in the presence or absence of forskolin (10 μM). After a 30 min incubation period at 37 °C, the cells are harvested in permeabilization buffer (2.5% Triton X-100 in HBBS), vortexed, and spun for 5 min at 13 000 rpm in a microcentrufuge. The cAMP content of the supernatant was measured by radioimmunoassay (Asghari et al., 1995) as described in the protocol provided for the use of the cAMP antiserum by Sigma Chemical Co. (Sigma product no. A-0670).

Ligand Binding Studies. Binding analysis was done as described previously (Van Tol et al., 1991, 1992; Asghari et al., 1994). In short, for saturation binding analysis, cell membranes were incubated in duplicate for 2 h at RT with increasing concentrations (10−3000 pM) of [^{3}H]spiperone (120 μCi/mmol). Competition binding analysis was done by coincubation of 200−250 pM [^{3}H]spiperone and increasing concentrations (10−3000 pM) of dopamine, emonapride, haloperidol, clozapine ,and raclopride. Nonspecific binding was determined by coincubation of the [^{3}H]spiperone with 30 μM dopamine. The incubation was terminated by rapid filtration, and [^{3}H]spiperone retained on the filters was detected by liquid scintillation counting.

Data Analysis. The ligand binding data were analyzed by the non-linear least-squares curve-fitting program LIGAND. The EC_{50} values of the functional assays were determined by computer-assisted nonlinear least-squares curve-fitting using the algorithm $f(x) = R^*[X]^n/([X]^n + E^n)$ for which R is the maximum response, [X] is the concentration of drug tested, E is the half-maximal response, and n is the slope factor.

RESULTS

In Vitro Transcription and Translation. In order to determine the fidelity at which the polymorphic repeat region in the D4 is translated, we expressed D4.2, D4.4, and D4.7 pbluescript-containing plasmids in an *in vitro* transcription−translation-coupled T7 RNA polymerase reticulocyte lysate system. Using SDS−PAGE, the radioactive reaction products were analyzed. The measured molecular mass of the three D4 receptor variants were approximately 41, 44, and

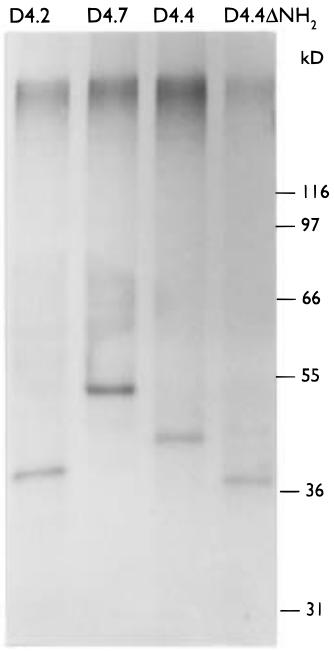

FIGURE 1: In vitro transcription and translation of the D4.2 (lane 1), D4.4 (lane 2), D4.7 (lane 3), and D4.4 (lane 4) deleted for the first 25 nucleotides downstream from the initiator methionine. The samples were labeled by [^{3}H]leucine incorporation and separated by polyacrylamide gelelectrophoresis under denaturing and reducing conditions. Protein size markers (kDa) are indicated on the side.

49 kDa (Figure 1), which is in close agreement with the predicted amino acid sequence as deduced from the cDNA sequence of these proteins. No other dominant translation products could be observed. However, a control vector in which the D4.4 sequence was deleted up to nucleotide +25 (D4ΔNH$_2$+25) resulted in a translation product of 40.5 kDa (Figure 1). The yield of this product was apparently equal to that of the full-length cDNAs.

Expression of 5′ Deletion Mutants. To determine whether D4.4ΔNH$_2$ can be detected by radioligand binding, the D4ΔNH$_2$+25 was cloned into the eukaryotic expression vectors pRc/RSV and pCD-PS. Sequence analysis confirmed that during subcloning no new upstream initiation codons were introduced. Transient expression of the truncated D4.4 forms in these two vectors in COS-7 cells resulted in the detection of [^{3}H]spiperone binding levels that were 20% (pCD-PS) and 50% (pRc/RSV) of nontruncated D4.4 in the control vectors. The affinity constant of the amino-terminal truncated form for [^{3}H]spiperone (approximately 95 pM) is not significantly different from the regular D4.4 receptor.

Progressive deletion of the 5′ coding sequence to nucleotides +83, +86, +100, and +105 resulted in clearly detectable [^{3}H]spiperone binding when transiently expressed in COS-7 cells using the pRc/RSV expression vector. In contrast, deletions up to nucleotides +117, +118, and +128 resulted in inconsistent detection of [^{3}H]spiperone binding which was on average <5% of control levels.

Deletion to nucleotide +89 resulted in the reconstruction of an in-frame initiation codon starting at position +88 in the pRc/RSV expression vector. For this mutant, the first 29 amino acids of the D4 receptor are deleted, and the Leu at amino acid position 30 is replaced by Met. Transient expression of this construct in COS-7 cells resulted in 34% ± 10% higher levels of expression than the control vector.

Table 1: Pharmacological Profile of D4.4ΔNH$_2$[a]

drug	D4.4ΔNH$_2$	D4.4
[^{3}H]spiperone	0.09 ± 0.01 (4)	0.1 ± 0.01 (6)
nemonapride	0.08 ± 0.01 (6)	0.16 ± 0.03 (6)
haloperidol	2 ± 1 (5)	1.5 ± 0.3 (6)
clozapine	31 ± 7 (5)	14 ± 1 (6)
raclopride	1390 ± 110 (2)	1522 ± 111 (4)
S-sulpiride	423 ± 101 (4)	248 ± 9 (2)
dopamine	135 ± 82 (5)	4 ± 1 (6)
dopamine + G	147 ± 75 (5)	22 ± 9 (6)
quinpirole	14 ± 5 (4)	2 ± 1 (2)
quinpirole + G	19 ± 6 (4)	5 ± 0.1 (2)

[a] The affinities for spiperone were determined by Scatchard analysis, while the affinities for the other compounds were obtained by competition of [^{3}H]spiperone. The data are expressed as the average (nM) ± SE The number of independent analyses is indicated in parentheses. G indicates the inclusion of 200 μM Gpp[NH]p in the binding assay. The affinities presented for D4.4 are the average of new data and data from the same cell line published by Asghari et al. (1995). The affinities are determined by LIGAND using single-site analyses. Several of the curves obtained for the agonists dopamine and quinpirole were displayed significantly better using two-site analyses (see Results).

In order to investigate whether the alternative downstream initiation codon might contribute to the production of D4 receptor in the presence of an intact upstream Kozak sequence, we made a 7 bp frame-shift deletion mutant for nucleotides +54 to +60 (D4ΔNH$_2$+54−+60). Transient expression levels, as measured by [^{3}H]spiperone binding, of the pRc/RSV vectors expressing D4.4, D4ΔNH$_2$+25, and D4ΔNH$_2$+54−+60 in COS-7 cells showed that D4ΔNH$_2$+25, and D4ΔNH$_2$+54−+60 were expressed at 53 ± 3% (±SE; n = 8) and 18 ± 2% (±SE; n = 6) of D4.4 levels, respectively.

Pharmacological Characterization of D4.4ΔNH$_2$. For this purpose, the D4ΔNH$_2$+25 in pRc/RSV was stably expressed in Chinese hamster ovary-K1 (CHO-K1) cells. Three different clonally selected cell lines (CHOD4.4ΔNH$_2$-7, -11, and -15) were used to determine the affinities of various drugs by competition of [^{3}H]spiperone binding (Table 1). The levels of [^{3}H]spiperone binding sites (B_{max}) in CHOD4.4ΔNH$_2$-7, -11, and -15 cells are 120, 52, and 103 fmol/mg of protein. None of these cell lines showed significant differences in the pharmacological profile. The affinities of the examined antagonists for D4ΔNH$_2$+25 are similar to those measured for D4.4 (CHO-K1 clonal cell line D4.4-7; Asghari et al., 1995). In contrast, the agonists dopamine and quinpirole displayed 5−30-fold decreased affinities for the D4.4ΔNH$_2$ receptor. Careful examination of the competition curves using LIGAND indicated that for both quinpirole and dopamine the D4.4 receptor displayed high- (K_h) and low-affinity states that could be converted to a single-affinity state by inclusion of 200 μM Gpp[NH]p in the binding assay. However, for the D4.4ΔNH$_2$ receptor, only 1 out of 4 of the quinpirole competition curves (K_h 623 pM) and 3 out of 5 of the dopamine competition curves displayed a high-affinity state (K_h 1.4 nM), while all the D4.4 curves for the agonists dopamine (K_h 360 pM) and quinpirole (K_h 560 pM) displayed high-affinity states.

Functional Characterization of D4.4ΔNH$_2$. The functional activity of the D4.4ΔNH$_2$ receptor was assessed in the cell lines CHOD4.4ΔNH$_2$-7 and -11. Dopamine inhibited forskolin-stimulated (10 μM) cAMP levels in these cells by 53 ± 3% (±SE, n = 8) with an EC$_{50}$ of about 165 ± 31 nM

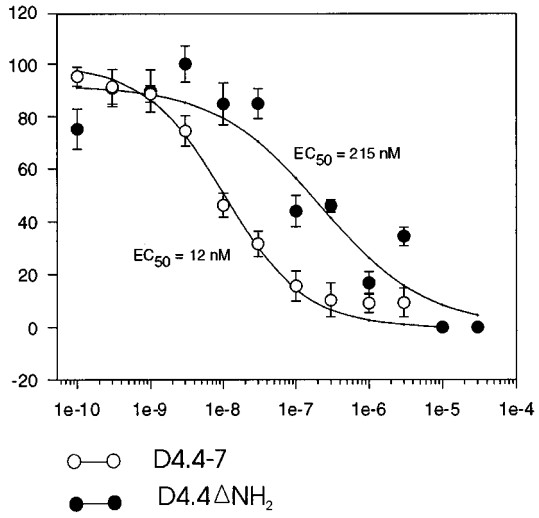

FIGURE 2: Inhibition of forskolin-stimulated cAMP levels by dopamine in Chinese hamster ovary cells (CHO-K1) that express the D4.4 receptor (cell line D4.4-7; ○) and the D4.4 receptor that is deleted for the first 25 nucleotides downstream from the initiator codon (cell line D4.4ΔNH$_2$-11; ●). These curves represent the data from a single experiment in which each point is the average of triplicate measurements. The EC_{50} for the individual curves is indicated in the graph.

(±SE, $n = 8$)(Figure 2). There was no significant difference between the two cell lines. As seen before (Asghari et al., 1995), dopamine inhibited forskolin-stimulated cAMP levels in the D4.4-expressing cells (CHOD4.4-7, B_{max} 341 fmol/mg of protein) by 92 ± 3% (±SE, $n = 8$) at an EC_{50} of about 13 ± 4 nM (±SE, $n = 8$) (Figure 2).

DISCUSSION

The *in vitro* transcription−translation experiments indicated that deletion of the initiation codon resulted in the use of an alternative initiation codon giving rise to a protein of 40.5 kDa. Progressive deletion of the D4 cDNA suggests that sequences in between nucleotides +105 and +117 are of importance for the alternative initiation. Analyses of the D4 cDNA sequence for downstream Kozak sequences (PuNNAUGPu; Pu, purine; N, any nucleotide) (Kozak, 1986, 1987) within the first 300 bp of coding sequence were negative. The use of the non-AUG initiation codons CUG and GUG has been reported [for examples, see Hann (1994)]. Scanning for PuNNC/GUGPu sequences resulted in the identification of several potential downstream initiation sites. The CUG codon at position +112 is in the above-mentioned context and might serve as a potential initiation codon that can explain both the detection of the [^{3}H]spiperone binding site in the progressive 5′ deletion experiments and could also give rise to a protein product with a predicted molecular mass of 40.75 kDa.

The results demonstrate that D4.4ΔNH$_2$ can be expressed as a functional target for various neuroleptics in the plasma membrane. This suggests that the amino terminus is not essential for intracellular transport and functional folding of the D4 receptor, which is also supported by the high levels of expression of an amino-terminal deletion mutant with an initiator methionine reconstructed at amino acid position 30. The observation that the affinities of the antagonists used are not different from that for D4.4 suggests that the regions important for the binding of these antagonists are in the remainder of the receptor. This supports the view that receptor folding is minimally affected by amino-terminal truncation. Agonist binding is significantly decreased, and the detection of high-affinity states is clearly inconsistent as compared to the D4.4.

Although for various G protein-coupled receptors the extracellular amino terminus serves as an agonist binding domain, this is not likely the case for this receptor because: (a) dopamine binding is not obliterated by amino-terminal truncation, (b) the Asp and Ser residues that have been found to be essential for catecholamine binding are conserved in the D4 receptor (Strader et al., 1988, 1989), and (c) dopamine-activated D4.4ΔNH$_2$ can inhibit forskolin-stimulated cAMP increases. However, the amino-terminal truncation caused a 10-fold decrease in the EC_{50} for dopamine to inhibit forskolin-stimulated cAMP increase as compared to the regular D4.4. Furthermore, the efficacy to inhibit this response was reduced by about 40%. These effects are unlikely to be caused by the loss of amino-terminal glycosylation (Kobilka, 1990; Rands et al., 1990).

The experimental data do not allow conclusions as to whether the observed changes are sequence-dependent or caused by overall structural changes. However, it was recently shown that single amino acid changes in the amino terminus of the β_2-adrenergic receptor will affect agonist-mediated receptor turnover, but not agonist binding and functional activity (Green et al., 1994). The reduced functional efficacy of D4.4ΔNH$_2$ is probably unrelated to receptor density since two different clonal cell lines, which have a 2-fold difference in expression levels of this variant, display identical reduced functional efficacies. The inconsistent detection of agonist high-affinity states, decreased functional EC_{50}, and efficacy of D4.4ΔNH$_2$ suggest that the extracellular amino-terminal domain plays a role in the efficiency of D4 signal transduction.

In order to determine whether an amino-terminal truncated receptor form might exist *in vivo*, various factors have to be considered (Kozak, 1991a) such as the length of the leader sequence (Kozak, 1991b), the strength of the Kozak context of the upstream initiator methionine (Kozak, 1986, 1987), and secondary structures downstream from the initiation codon (Kozak, 1990). In an attempt to address this question, we made a 7 bp deletion from nucleotides +54 to +60. In this construct, the 5′ leader sequences of the mRNAs are not affected, and the initiator methionine is in a sufficient distance from the cap-site (71 nucleotides) to avoid bypassing of the ribosome complex. The initiation codon is in a relatively strong Kozak context (CGCGCCATGG) which is unaffected by the deletion. The sequences do not contain a strong hairpin loop structure with the base of its stem between nucleotides +12 and +15. Therefore, the deletion is unlikely to destroy such secondary structures that have been shown to enhance the use of the upstream initiation codon and thus reduce leakiness. The construct did not address the potential use of an internal ribosome entry site (McBratney et al., 1994) which could potentially be destroyed by the deletion and result in underestimation of the use of the downstream initiation codon. Nevertheless, the downstream initiation codon is used at about 18% of the upstream initiation codon, suggesting that *in vivo* some of the total D4 receptor population might exist in this form. The relatively low levels of D4 receptors in brain currently prohibit us from verifying the *in vivo* existence of D4ΔNH$_2$.

The results presented demonstrate that the extracellular amino-terminal domain of the D4 receptor is involved in agonist-mediated signal transduction, but is not essential for proper expression and the binding of various antagonists. The data support a model in which the amino terminus contributes to stabilization of the active state of the receptor.

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