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# Human $D_3$ dopamine receptor in the medulloblastoma TE671 cell line: cross-talk between $D_1$ and $D_3$ receptors

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**Abstract In search of a cell line in which the $D_3$ dopamine receptor is expressed endogenously, we found that the neuron-derived human medulloblastoma cell line TE671 expresses the human $D_3$ ($hD_3$) and $D_1$ ($hD_1$) receptor, but neither the $D_2$ or $D_4$ receptors. Exposure of TE671 cells to the $D_3$ agonist 7-OH-DPAT (DPAT), or to the $D_1$ agonist SKF-38393 (SKF) increased the expression of $hD_3$ or $hD_1$ mRNA, respectively. Moreover, whereas DPAT had no effect on $hD_1$ mRNA levels, stimulating the cells with SKF caused an increase in both $hD_1$ and $hD_3$ transcript levels. These results suggest (i) that following ligand stimulation, $hD_3$ and $hD_1$ receptors are upregulated to enhance their own receptor expression, and (ii) that upregulation of $hD_1$ receptor transcripts leads to a stimulation of the $hD_3$ dopamine receptor transcripts.**



*Key words:* Dopamine receptor; $D_1$; $D_3$; TE671; Human

## 1. Introduction

Dopamine receptors have generally been divided into two broad categories, referred to as D1 and D2 receptors. Both belong to the family of receptors which are coupled to their specific effector functions via guanine nucleotide regulatory (G) proteins [1]. Both D1 and D2 receptors are present predominantly in the central nervous system (CNS) and exhibit similar regional localizations. The D1 receptor family consists of $D_1$ and $D_5$ [2,3] while thus far three D2-like receptors have been cloned including the $D_2$ [4], $D_3$ [5], and $D_4$ [6] subtypes.

The $D_3$ dopamine receptor is thought to be associated with behavioral aspects of dopaminergic function [7], and thus represents a therapeutic target in a number of neurological disorders, notably Parkinson's disease and schizophrenia [8]. The $D_3$ receptor is present at much lower levels in the brain than the $D_2$ subtype, and is expressed predominantly in limbic areas including the olfactory tubercle, nucleus accumbens and hypothalamus [5,9]. The only brain regions in which it has been found to be expressed in the absence of the $D_2$ and/or $D_4$ subtypes are the islands of Calleja [10,11]. Recent work from our laboratory has demonstrated that mice lacking the $D_3$ dopamine receptor are hyperactive and exhibit reduced levels of anxiety [12,13]. In addition, the $D_3$ receptor is expressed in the dopaminergic neurons of the substantia nigra, which, together with its high affinity for the autoreceptor-selective compounds AJ76 and UH232, supports the notion that it may act as an autoreceptor [5].

One of the principal problems in studying the functional properties of the $D_3$ receptor is the difficulty of finding high affinity $D_3$-specific ligands which do not bind to the $D_2$ and $D_4$ subtypes. This, together with the relatively low levels of $D_3$ receptor mRNA expression in the brain, and its colocalization with the $D_2$ and/or $D_4$ receptor subtypes, has hampered studies of the functional properties of the $D_3$ receptor. The availability of the cloned cDNA encoding the $D_3$ receptor provides a useful means of studying its activity in ectopic expression systems, but has the drawback that the receptor is not in its normal cellular environment and may therefore lack some of the necessary elements of its signal transduction pathway. In addition, in transfected cell systems the receptor's expression is not under the control of its own promoter elements, but is under the control of the promoter present in the transfection vector. In order to study the functional properties of the $D_3$ receptor in a more native environment, we have searched for a cell line in which this subtype is expressed endogenously. We report here that the neuron-derived human medulloblastoma cell line TE671 expresses the $D_3$ and $D_1$ receptor subtypes, but neither the $D_2$ nor $D_4$ dopamine receptors. We also show that stimulation of the $D_3$ receptor leads to its own upregulation, and stimulation of the $D_1$ receptor leads to upregulation of both $D_1$ and $D_3$ receptors, providing direct evidence of cross-talk between these two dopamine receptors.

## 2. Materials and methods

### 2.1. Materials

Cell culture media were from Gibco Laboratories (Grand Island, NY, USA). Oligonucleotide probes were synthesized by the laboratory of oligonucleotide synthesis at the Weizmann Institute of Science. Dopamine, 7-OH-DPAT (7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; DPAT), butaclamol, haloperidol, SKF-38393 (1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride; SKF), and SCH-23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SCH) were purchased from RBI (Natick, MA, USA); U99194A maleate (5,6-dimethoxy-2-(di-*n*-propylamino)indan melate; U99) was a gift from Upjohn (Kalamazoo, MI, USA).

### 2.2. Methods

*2.2.1. Cell culture.* TE671 cells were obtained from the ATCC and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (fetal calf serum), 1 mM glutamine, 30 μg/ml gentamicin sulfate, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml fungizone. Cells were maintained in a humidified incubator (5% $CO_2$) at 37°C. Adherent cells were dislodged by treatment with trypsin (0.25%) and were seeded at approximately $2\times10^6$ cells/10-cm diameter dish, one day before the experiment. The cells were stimulated with the drugs in DMEM containing 0.1% BSA for the time periods indicated, while exposure to antagonists was carried out 10 min before agonist treatment.

*2.2.2. Genomic library screening.* An EMBL human genomic library (Clontech) was screened using a randomly primed cDNA fragment encoding the human $D_3$ receptor, or end-labeled oligonucleotides based on the $D_3$ receptor's cDNA sequence [14]. DNA from positive clones was isolated using Lambda maxi-prep columns (Qiagen), and was excised using the enzyme *Xho*I. DNA preparations were

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Table 1
Primer sequences used for RT-PCR

| Receptor | Sequence (5′ to 3′) | Sense/nonsense | Residues (nt) | Size of PCR product (nt) |
|---|---|---|---|---|
| $hD_3$ | tct agATGGCATCTCTGAGTCAGCTG | Sense | 1–21 | 705 |
| | CCTGACACTGTTGCACTGAC | Antisense | 705–686 | |
| $hD_1$ | GGATTGCCACTCAAGCGGTC | Sense | 99–118 | 1254 |
| | CTCTATGGCATTATTCGTCGCAGG | Antisense | 1330–1353 | |
| β-Actin | CCTTCAACACCCCAGCCATGTACG | Sense | Exon 4 | 501 |
| | TGCCACCAGACAGCACTGTGTTGGC | Antisense | Exon 5 | |
| $hD_2$ | ggaattcCCATGCTGTACAATACG | Sense | 775–798 | 994 |
| | gga aTTCCTGCTCACGGTTCG | Antisense | 785–1769 | |
| $hD_4$ | TGGCACGTCGCGCCAAGCTG | Sense | 769–788 | 540 |
| | CATCTCCTTGGTCCCTGAG | Antisense | 3′ non-coding | |

subjected to restriction digestion, Southern analysis, and hybridization with appropriate oligonucleotide probes based on the $D_3$ receptor sequence, in order to allow identification and characterization of the genomic fragments. Suitable fragments were excised and subcloned into pBluescript (Stratagene) for sequence analysis. All molecular biological techniques were performed according to standard procedures [15].

*2.2.3. Northern analysis.* Total RNA was prepared from cultured cells using Tri-Reagent (MRC, OH, USA), and from human or mouse tissues using the guanidium thiocyanate-caesium chloride method [16]. For Northern analysis, 25 μg total RNA was denatured and was then transferred onto and fixed to nylon Genescreen Plus membranes (Boston, MA, USA). Prehybridization, hybridization and washes were performed according to the manufacturer's instructions, prior to autoradiography.

*2.2.4. RT-PCR.* Total RNA from TE671 cells was reverse transcribed and subjected to PCR, essentially as described previously [9]. For PCR, $D_3$-specific primers, based on the sequence of the human $D_3$ dopamine receptor [14] were used, containing *Xba*I adapters (shown in small letters) (5′-ccgtctagATGGCATCTCTGAGTCAGCTG-3′ and 5′-ccgtctagaCCTGGCAGCTAGAAATGG-3′). PCR products were electrophoresed, and fragments were subcloned to the vector pBluescript (Stratagene) by standard procedures [15].

For semiquantitative RT-PCR total RNA was extracted from TE671 cells as described above. The sequences of the oligonucleotide primers that were used for PCR amplification of fragments of the $hD_1$, $hD_2$, $hD_3$, $hD_4$ and β-actin mRNA are shown in Table 1. PCR amplification was performed as described previously [9] in a DNA thermal cycler (Progene, Techne); 23 cycles were carried out for β-actin, 27 cycles for $hD_3$ and $hD_1$, and 36 cycles for $hD_2$ or $hD_4$. The amount of cDNA taken for PCR and the linear range of amplification for each primer pair were established in independent preliminary studies. Oligonucleotides specific for the $hD_3$ receptor (5′-TGGGTGGTATACCTGGAGG-3′, $hD_1$ receptor (5′-ACATTCGACAGGCTTTCCATTACC-3′, or for β-actin (5′-TGCCACCAGACAGCACTGTGTTGGC-3′) were labeled with [$\gamma$-$^{32}$P]dATP using T4 polynucleotide kinase (Boehringer), and were used in hybridization. The membranes were hybridized and washed according to the manufacturer's recommendations and then exposed to a phosphorimager (BAS 1000, Fuji) for 2 h.

## 3. Results

### 3.1. Identification of endogenous $D_3$ receptor expression in TE671 cells

Total RNA was extracted from TE671 cells and subjected to Northern analysis using a 450-bp *Eco*RI-*Sal*I N-terminal fragment of the murine $D_3$ dopamine receptor [9]. A specific signal was obtained in TE671 cells at approximately 4 kb, different in size from the 8.3-kb mRNA for the murine $D_3$ receptor (Fig. 1, left). This 4-kb signal for the human $D_3$ receptor mRNA was also observed using a probe derived from the 3rd cytoplasmic loop of the human $D_3$ receptor and was detected in RNA from both TE671 cells and from human neonatal brain tissue (data not shown), confirming that the $D_3$ receptor expressed in this cell line resembles the human $D_3$ receptor expressed in brain tissue. To determine whether TE671 cells express the $D_2$ subtype, the Northern blot in Fig. 1, left, was deprobed, and was rehybridized using a *Hin*dII-*Sac*I fragment of the $D_2$ dopamine receptor. No $D_2$ receptor signal could be detected in the TE671 cells, whereas a strong signal was seen at approximately 2.9 kb in the mouse olfactory tubercle (Fig. 1, right), as expected for the $D_2$ subtype [4].

To characterize further the $D_3$ receptor expressed in TE671 cells, RT-PCR was performed on TE671 cell RNA, using primers based on the N-terminal and C-terminal regions of the human $D_3$ dopamine receptor, previously isolated from neonatal brain tissue [14]. Ethidium bromide staining revealed a 1.2-kb PCR product, which hybridized with a human $D_3$-specific oligonucleotide. This product was subcloned and sequenced, and found to represent the full length human $D_3$ receptor, confirming that the $D_3$ receptor present in the cell line TE671 is identical to that in brain tissue[1]. The TE671-derived clone contained only two silent changes (at positions 876 and 900) from the published human $D_3$ gene [14] which was isolated from human brain tissue. No specific signals were amplified by PCR using primers specific for the $D_2$, and $D_4$ receptors, signifying that these dopamine receptor subtypes are not expressed in the TE671 cell line (data not shown). However, TE671 cells were found to contain $D_1$ dopamine receptor mRNA using RT-PCR with $D_1$-specific primers, and sequencing of the amplified product showed it to be identical to the previously described human $D_1$ receptor [2].

### 3.2. Comparison of the murine and human $D_3$ receptor genes in the third cytoplasmic domain

The nucleotide sequence of the PCR product of the 1.2-kb full-length $D_3$ receptor encodes a protein shorter than the long isoform of the mouse $D_3$ receptor ($D_{3L}$) by 46 amino acids, located in the receptor's putative third cytoplasmic loop. In addition, this 46 amino acid stretch overlaps with the 21 amino acids lacking in the short isoform of the murine $D_3$ receptor ($D_{3S}$) [9]. We have previously examined the intron-exon organization of the murine $D_3$ receptor and found that the 5′ and 3′ borders of the 46 amino acid stretch coincide with intron 4 and the alternatively spliced intron 5, respectively [17]. This led us to speculate whether there may be other (longer) splice variants of the human $D_3$ receptor. Since this could only occur if an intron was present in this region, we examined this section of the human $D_3$ receptor

[1] GenBank accession number: U32499.

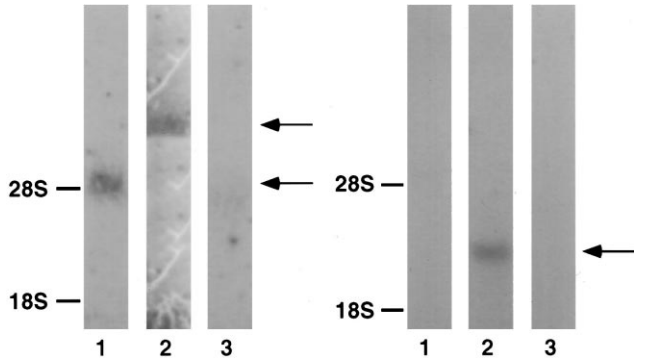


Fig. 1. Identification of $D_3$ dopamine receptor mRNAs in TE671 cells. Northern analysis of mRNA from TE671 cells (1), mouse olfactory tubercle (2) and CHO cells (3), using cDNA probes encoding the $D_3$ receptor (left) and $D_2$ receptor (right).

gene. A human genomic library was screened using a 340-bp *Nco*I-*Eco*RI C-terminal fragment of the $D_3$ receptor, extending from the distal half of the third cytoplasmic loop to the C-terminus. Examination of two genomic clones containing the putative third cytoplasmic loop revealed the presence of an intron exactly at the position where the human and murine $D_3$ receptors diverge, and restriction analysis and Southern blotting demonstrated this intron to be over 7 kb in size (Fig. 2). Thus, in the third cytoplasmic domain of the $D_3$ receptor, the human $D_3$ receptor gene appears to have one 7-kb intron, whereas the murine homologue contains two introns, and has exonic sequence encoding an extra 25 ($D_{3S}$) or 46 ($D_{3L}$) amino acids.

### 3.3. Upregulation of transcripts of $hD_3$ and $hD_1$ dopamine receptors

TE671 cells provide an easy and useful means of investigating the influence of dopaminergic agonists on $D_3$ receptor levels, as they endogenously express $D_3$ receptors in the absence of $D_2$ and $D_4$ dopamine subtypes. We therefore treated TE671 cells with dopamine (100 μM) in the presence or absence of the D2 antagonist butaclamol (10 μM), for increasing periods of time, and examined the effect on the level of the $D_3$ receptor mRNA. Exposures to dopamine of 20–30 min produced elevated $hD_3$ mRNA levels (Fig. 3a,b), while the mRNA levels of β-actin did not change (data not shown). This rise in $hD_3$ mRNA levels started to drop with longer exposures to dopamine, showing this to be a short-lived effect. The increase in $hD_3$ mRNA was sensitive to the dopaminergic antagonist butaclamol confirming the receptor specificity of this effect (Fig. 3a). The specificity of the signal was also confirmed by RT-PCR. Cells were treated for 30 min, and RT-PCR was performed on cellular RNA using specific primers (Table 1) to amplify a 700-bp fragment from the 5′ end of the $hD_3$ receptor. The mRNA expression of the $hD_3$ receptor was increased following dopamine treatment and this was attenuated by the antagonist haloperidol (Fig. 3b). This increase in $hD_3$ receptor mRNA levels was both rapid and short-lived.

As mentioned earlier, RT-PCR on TE671 cell RNA with receptor specific primers demonstrated that while $D_2$ and $D_4$ receptors are not present in these cells, the $hD_1$ subtype is expressed. We therefore used Northern blot analysis to examine the effect of dopaminergic agonists on $hD_1$ receptor mRNA levels. Exposure of the cells to 100 μM dopamine caused a significant increase in mRNA levels of the $hD_1$ receptor after 20 min; this effect persisted up to 40 min stimulation, and was abolished by the presence of butaclamol (Fig. 3c). This was confirmed by RT-PCR using specific $hD_1$ receptor primers. Dopamine significantly increased the level of the $hD_1$ receptor mRNA, and addition of the antagonist haloperidol abolished the effect (Fig. 3d).

### 3.4. Cross-talk between $hD_1$ and $hD_3$ receptors in TE671

The endogenous expression of both $D_1$ and $D_3$ receptors in the same cell line allowed us to analyze whether cross-talk occurs between these two receptor systems. To differentiate between these two systems, $D_1$- and $D_3$-specific ligands were used to treat the cells. TE671 cells incubated with the $D_3$-specific agonist DPAT showed a rapid increase in $hD_3$ mRNA levels, which was abolished in the presence of the $D_3$-specific antagonist U99 (Fig. 4). The $D_3$ agonist DPAT had no effect on $hD_1$ mRNA levels. However, stimulating the cells with the $D_1$ agonist SKF caused an increase in both $hD_1$ and $hD_3$ transcript levels (Fig. 4). The specific $D_3$ antagonist U99, given together with SKF, had no significant

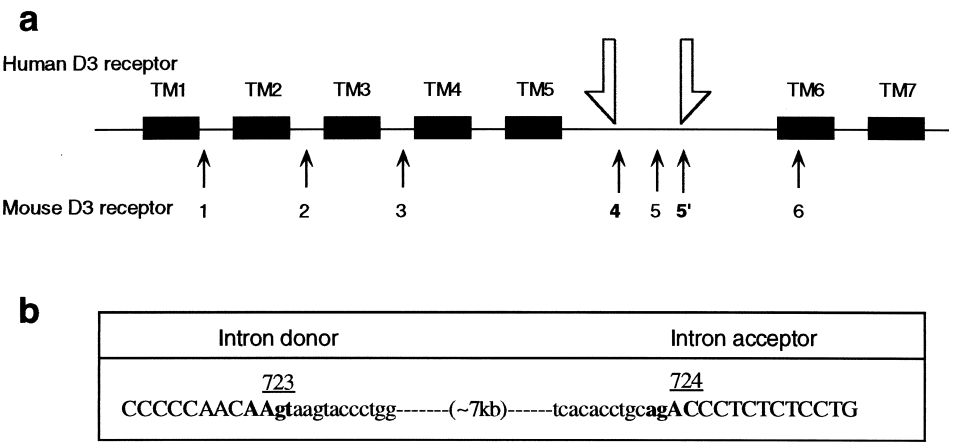


Fig. 2. The intron at the third cytoplasmic loop of the human $D_3$ receptor gene. a: Schematic representation of the position of the newly identified intron in the human $D_3$ receptors. b: DNA sequence of the donor and acceptor sites of the newly identified intron in the human $D_3$ receptor.

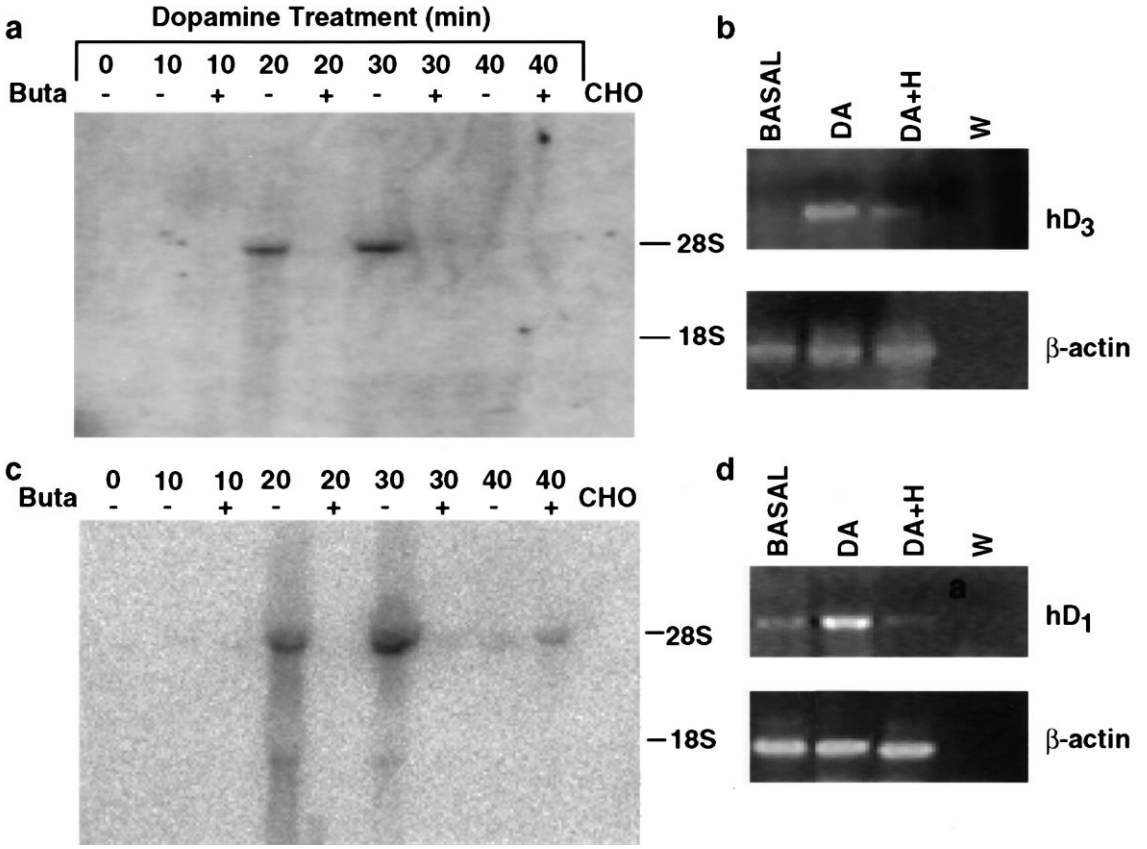


Fig. 3. Up-regulation of $hD_3$ and $hD_1$ dopamine receptor transcripts in TE671. Northern blot analysis of mRNA extracted from TE671 cells stimulated with dopamine (100 μM), for the indicated times, in the absence or presence of butaclamol (buta; 10 μM), and hybridized with a $D_3$ (a) or a $D_1$ (c) specific probe. Ethidium bromide staining of RT-PCR products using $D_3$ (b) or $D_1$ (d) primers on TE671 cells stimulated with dopamine (DA; 100 μM) for 30 min, in the absence or presence of haloperidol (H; 10 μM). W is a water control.

effect on the level of $hD_3$ and $hD_1$ mRNAs. However, the $D_1$ antagonist SCH abolished the effect of SKF on $hD_1$ and markedly decreased its effect on $hD_3$ receptor mRNA level (Fig. 4).

## 4. Discussion

In this paper we report the identification of a cell line (TE671) which endogenously expresses both the $D_3$ and $D_1$ dopamine receptors in the absence of other dopamine receptor subtypes, and demonstrate that this cell line presents a useful and convenient system for examining specific dopamine receptor functions. The TE671 cell line is derived from neuronal origin and has been widely used as a model of human medulloblastoma, having similar properties to cells originated from the CNS [18]. It thus represents an excellent tool for studying regulation of $D_3$ receptor expression and activity.

The cloning and sequencing of the $D_3$ receptor from TE671 cells confirmed that this receptor is identical to that expressed in human brain, and led us to compare it with the murine $D_3$ receptor. We have previously characterized the structural organization of the murine $D_3$ receptor gene [17] and showed it to contain two introns in the region encoding the putative third cytoplasmic loop, one of which contains a variable splice site which gives rise to the murine $D_{3S}$ receptor isoforms [9]. Partial analysis of the human $D_3$ receptor gene was performed by Giros et al. [14] who identified introns corresponding to introns 1 and 6 in the murine $D_3$ receptor gene. The present report presents the first direct evidence of the intron organization in the 3rd cytoplasmic loop of the $hD_3$ receptor gene, and shows that the 7-kb intron present is located at a corresponding position to introns 4 and 5 in the murine $D_3$ recep-

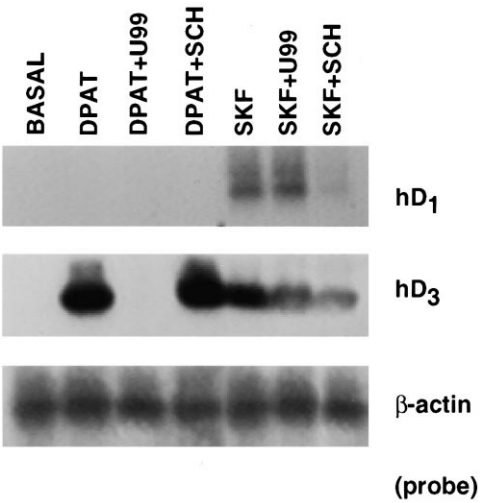


Fig. 4. Cross-talk between $hD_1$ and $hD_3$ receptors in TE671 cells. PCR amplification of RNA-derived cDNA of TE671 cells stimulated with either the $D_3$ agonist DPAT (10 μM) or $D_1$ agonist SKF (10 μM), alone or in combination with the $D_3$ antagonist U99 (1 μM) or the $D_1$ antagonist SCH (1 μM). Southern blots were hybridized with $hD_1$ or $hD_3$ specific probes (see Section 2 for details).

tor gene. In addition, the coincidence of the intron boundaries with the location of the extra 46 amino acids in the murine and rat receptors gives rise to the suggestion that other, longer forms of the human $D_3$ receptor may exist, analogous to the $D_{3S}$ or $D_{3L}$ receptors present in the mouse.

Conceivably, there are at least two mechanisms by which the mRNA levels of the $hD_1$ or $hD_3$ receptors could be elevated in the TE671 cells. First, receptor activation could lead to an increase in the stability of the receptor mRNA. If so, this mRNA should contain sequence elements that interact with cellular factors to regulate its stability [19]. A second possibility is that TE671 cells contain regulatory elements that modulate the transcriptional rate of the dopamine receptors. Presumably, these could be enhancer or silent elements that interact with specific transcription factors and would be either activated or inhibited, respectively, by dopamine receptor activation. It should be emphasized that since the TE671 cells express the dopamine receptors endogenously, they contain all the 3′ and 5′ untranslated regions that may be involved in the transcription and/or stability of the dopamine receptor mRNA.

Perhaps most interesting is the coexpression of $D_3$ and $D_1$ receptors in TE671 cells, and the finding that cross-talk occurs between these two receptor systems. Coexpression of $D_3$ and $D_1$ receptors within the same neurons has been reported in both the shell and core regions of the nucleus accumbens [20], in neostriatal medium spiny neurons [21], and in striatonigral neurons [22]. Moreover, both $D_1$ and $D_3$ receptors are present in granule cells of the islands of Calleja, where the highest $D_3$ receptor density in brain is found [10,11]. Obviously, this colocalization raises the question of interaction between these receptor systems. In TE671 cells, activation of either $hD_3$ or $hD_1$ receptors by dopamine results in activation of signal transduction cascades that are specific to each receptor, leading to the increase in mRNA. We have shown that stimulation of the $hD_1$ receptor by SKF activates a signal transduction cascade that leads to upregulation of both the $hD_1$ and $hD_3$ transcripts, while $hD_3$ receptor activation leads to its own upregulation but has no effect on $hD_1$ mRNA levels. In the brain, however, the endogenous ligand dopamine will presumably not distinguish between receptor subtypes, and will activate both receptor cascades, unless some system operates to sequester or down-regulate one or other of these signaling pathways. Thus dopamine stimulation may lead to upregulation of both $hD_1$ and $hD_3$ receptors in cells where they are coexpressed. It seems paradoxical that $D_1$ stimulation should lead to up-regulation of $D_3$ receptors, since these are classically thought to have opposing effects, particularly with respect to adenylate cyclase stimulation. However, recent data from our laboratory [23] show that the $D_3$ receptor may also activate adenylate cyclase; consequently it may act in concert with the $D_1$ receptor to amplify a signal via cAMP production, while also activating or inhibiting other effector systems which differ from $D_1$ modulated pathways. It will be interesting to determine how receptor activation leads to changes in mRNA levels, and the TE671 cell line should present an excellent system for dissecting the downstream signaling components of the $D_1$ and $D_3$ receptors, and for studying how these two dopaminergic systems 'talk' to one another.

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