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COMMENTARY

THE THIRD DOPAMINE RECEPTOR (D_3) AS A NOVEL TARGET FOR ANTIPSYCHOTICS

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Until recently it was widely accepted that dopamine (DA†) affects its target cells in brain and endocrine tissues via interaction with only two receptor subtypes, termed D_1 and D_2, differing one from the other by their pharmacological specificity and their opposite effect on adenylate cyclase [1, 2]. It was also generally admitted that the therapeutic efficacy of antipsychotics derived from their high affinity binding to D_2 receptors.

However, we have repeatedly raised the idea that antipsychotic agents interact to a variable extent with more than a single DA receptor subtype, i.e. that the dual categorization of DA receptors was incomplete. Our conviction was based mainly upon the observation that a series of "atypical antipsychotics", although inactive at D_1 receptors, were able to distinguish subclasses of D_2 receptors in binding studies (in brain but not pituitary) and in behavioral studies [3]. However, no highly selective agent could be identified. In addition, the hypothesis that DA autoreceptors might differ pharmacologically from postsynaptic D_2 receptors was advanced [4] but failed to gain general acceptance [5, 6]. Hence, the idea that more than a single molecular entity, the D_2 receptor, was responsible for the various actions of antipsychotics remained controversial, in spite of its substantial clinical relevance.

This situation has started to be modified with the advent of molecular biology in this field, which has confirmed the existence of additional DA receptors. Their existence throws a new light onto the modes of action and side-effects of many drugs used in neurology and psychiatry. This is particularly the case for the D_3 receptor that we recently identified in rat [7] and human brain [8] and which appears as a major target for antipsychotics. This Commentary is focused mainly upon this "third receptor" but data are put in perspective by comparison with data describing other members of the already large DA receptor family.

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† Abbreviations: DA, dopamine; TMs, transmembrane domains; PCR, polymerase chain reaction; and RT-PCR, reverse transcription-polymerase chain reaction.

‡ See note added in proof.

Molecular biology reveals multiple dopamine receptor genes

The first cloning of a DA receptor gene, that of the D_2 receptor, was achieved largely by serendipity, i.e. was the result of a search for genes displaying sequence similarity with those of the β-adrenoreceptors [9, 10]. This initial discovery, in turn, paved the way for the cloning of a series of DA receptor genes, based upon the significant sequence homology these receptors display. As expected, the D_1 receptor, which is nearly as abundant as the D_2 receptor in brain, was the first to follow [11–14]. Then came the genes of a series of less abundant and less expected receptors which markedly expand the DA receptor family: the D_3 [7, 8], D_4 [15] and D_5 receptors [16]‡. The amino acid sequence of all these receptors, as deduced from their established nucleotide sequence, reveals that they belong to a larger superfamily, that of receptors with seven transmembrane domains (TMs) and coupled to their intracellular transduction system by a G protein. These features are similar to those of rhodopsin, the "receptor for light", with which they display sequence homology and, presumably, a common phylogenic origin.

The various genes of the DA receptor family can be classified in two groups according to their organization: (a) intronless genes, i.e. those of the D_1 and D_5 receptors, in which the coding nucleotide sequence is continuous; and (b) genes having their coding sequence contained in discontinuous DNA segments (exons) interspersed among sequences (introns) that do not form a part of the mature mRNA. This last organization, found in the rhodopsin gene as well as the D_2, D_3 and D_4 receptor genes (Fig. 1), may lead, via a mechanism of alternative splicing (in which a given exon in the pre-mRNA is either present or absent in the final mRNA), to the biosynthesis of several distinct proteins encoded by a unique gene.

The definition of two subfamilies of DA receptors, the D_1-like and the D_2-like, based upon the gene organization, is consistent with a similar distinction based upon pharmacology and signalling systems (Tables 1 and 2). Remarkably enough, the D_2 and D_3 receptor genes have 4 out of 6 and 5 introns, respectively, located at similar positions, suggesting relatively recent divergence from a common ancestral gene. The human D_3 receptor was assigned to

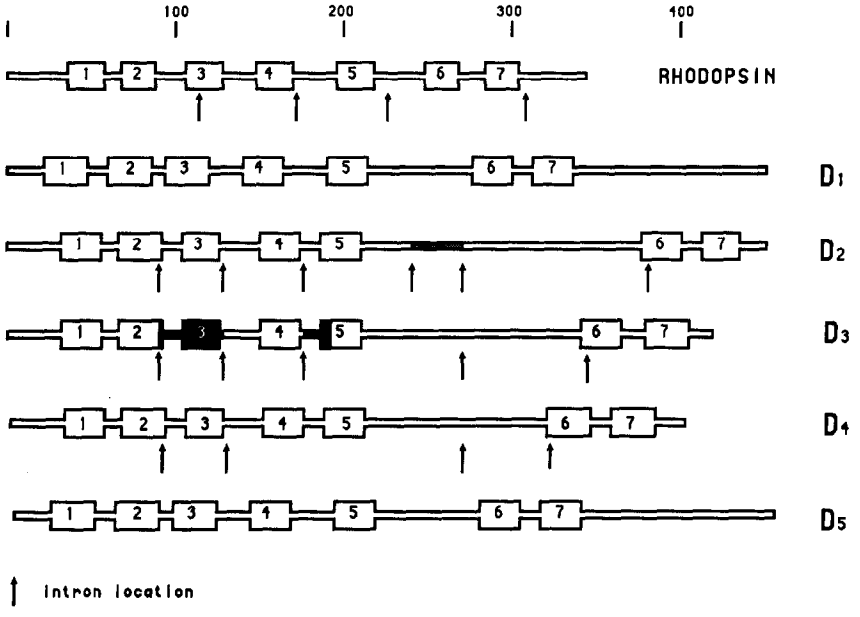

Fig. 1. The family of dopamine receptor genes: Organization and comparison with rhodopsin. The scale indicates the length of the amino acid sequence. Arrows indicate the position of introns; shaded areas correspond to alternative exons.

Table 1. Synopsis of dopamine receptor subtypes

	D_1	D_2	D_3	D_4	D_5
Coding sequence	446 a.a.	D_{2A} = 443 a.a. D_{2B} = 414 a.a.	400 a.a.	387 a.a.	477 a.a.
Chromosome localization	5 q31—q34	11 q22–q23	3 q13.3	11 p	4 p16
Highest brain densities	Neostriatum	Neostriatum	Paleostriatum (isl. Calleja, n. accumbens)	Medulla Fr. cortex	Hippocampus
Pituitary	No	Yes	No	?	No
Dopamine Neurons (A9, A10)	No	Yes	Yes	?	?
Affinity for dopamine	Micromolar	Micromolar	Nanomolar	Submicromolar	Submicromolar
Characteristic antagonist	SCH-23390	Haloperidol	UH 232	Clozapine	SCH-23390
Adenylyl cyclase	Stimulates	Inhibits	?	?	Stimulates
Characteristic agonist	SKF-82526	Bromocriptine	Quinpirole	?	SKF-82526

chromosome 3 [8] and localized to its long arm (3 q13.3) by *in situ* hybridization [17], whereas the other DA receptors are localized to different chromosomes.

Several polymorphisms were identified recently on the human D_3 receptor gene, including a BalI polymorphism in the N-terminal tail which is conveniently studied by polymerase chain reaction (PCR); it is inherited in a codominant way, according to Mendelian laws and can be used in genetic linkage studies [18].

Molecular cloning and structure of the rat and human D_3 receptors

The molecular cloning of the D_3 receptor involved a combination of screenings of cDNA and genomic libraries and reverse transcription-polymerase chain reaction (RT-PCR). A clone isolated from a rat brain cDNA library [19] was used to screen a genomic library, and a positive clone was shown to contain the 5′ part of a gene homologous to the D_2 receptor gene. RT-PCR was performed with a specific primer in the sequence of this clone and a degenerated primer designed in TM7 of the D_2 receptor. The PCR product was subcloned, sequenced and used in a screening of a rat genomic library which provided a clone containing the 3′ end of the coding region. The full-length cDNA was finally obtained by RT-PCR with specific primers flanking the coding region and RNA from olfactory

Table 2. Pharmacology of dopamine receptor subtypes

	K_i (nM)				
	D_1	D_2	D_3	D_4	D_5
Agonists					
Dopamine*	2,300	2,000	30	450	230
Apomorphine	680	70	70	(4)†	360
Bromocriptine	700	5	7	500	500
Pergolide	1,400	20	2		900
Quinpirole	>20,000	1,400	40	50	>20,000
SKF-38393	150	10,000	5,000	10,000	100
Antagonists					
Haloperidol	30	0.6	3	5	40
Pimozide		10	11	40	
(−)Sulpiride	40,000	10	20	50	80,000
UH 232		40	10		
Clozapine	140	70	500	9	250
SCH-23390	0.3	1,000	1,000	3,500	0.3

Values for human D_2 and D_3 receptors transfected in CHO cells are from this laboratory, whereas those for D_1, D_4 and D_5 receptors were taken from Refs. 15 and 16.
* Dopamine values were obtained in the presence of Gpp(NH)p.
† High affinity component.

tubercle as template [7]. A similar approach was used for the cloning of the human D_3 receptor cDNA. Specific sequences flanking the coding region were obtained by screening a human genomic library with corresponding rat probes. The full coding sequence was then obtained in RT-PCR, using primers designed in these sequences and RNA from human mammillary bodies [8].

The open reading frame of the D_3 receptor corresponds to a sequence of 446 amino acid residues in the rat but only 400 residues in humans, the main difference residing at the level of the third putative intracytoplasmic loop (i_3). There is relatively little sequence homology at the level of this loop between D_2 and D_3 receptors, contrasting with the high amino acid sequence homology at the level of the TMs where the dopaminergic ligands are thought to bind: for instance, homology at this level is as high as 78% between the human D_2 and D_3 receptors (Fig. 2).

The structural knowledge derived from physical studies of the opsins [20] and discrete manipulation of the β-adrenergic receptor gene ("site-directed mutagenesis") [21] can be extended to other members of the superfamily, e.g. the DA receptors, particularly the human D_3 receptor. They all comprise a pattern of seven stretches of 20–25 hydrophobic amino acids postulated to form transmembrane α-helices, connected by alternating extracellular and cytoplasmic loops constituted by hydrophilic residues. The N-terminal part constitutes a glycosylated extracellular domain.

The transmembrane helices constitute the ligand binding domain. particularly three amino acid residues thought to interact with catecholamines: an aspartic residue (Asp^{110} in the human D_3 receptor) in TM3, which forms an ion pair with the protonated amine group of DA, and two serine residues (Ser^{193} and Ser^{196}) in TM5, which presumably form a hydrogen bonding interaction with the two hydroxyl groups of DA. This last interaction, specific for DA and agonists, could cause a conformational change in the helix, transmitted to the i_3 loop.

Among the various intracytoplasmic domains, the i_3 loop appears as the main area for interaction with G proteins. It is clear that the length of this loop allows us to extend the classification of DA receptors: the intronless D_1 and D_5 receptors are characterized by a short i_3 loop (and a long c-terminal tail) and are coupled to G_s proteins which activate adenylate cyclase; on the other hand, the D_3 as well as the D_2 and D_4 receptors, which display a long i_3 loop and a short C-terminal tail (Fig. 1), might be coupled to G_i (or G_o) proteins inhibiting adenylate cyclase. In fact, these domains are those exhibiting the largest sequence dissimilarity among the various DA receptor subtypes, a feature which may reflect selective interaction of each member with one member of the large family of G proteins, leading to distinct intracellular signals.

Splice variants of D_3 receptor mRNA

Alternative splicing was shown to occur in the case of the D_2 receptor, potentially leading to two distinct receptors differing by a stretch of 29 amino acids at the level of the third intracytoplasmic loop which were called $D_{2(444)}$ (or D_{2L} for D_2 long and D_{2A}) and $D_{2(415)}$ (or D_{2S} for D_2 short and D_{2B}). These two isoforms of the D_2 receptor display identical pharmacology but are expressed differently among cerebral areas, and may interact differently with various G proteins [19, 22, 23]; in addition, their relative abundance is affected by neuroleptic treatments [24].

In the case of the D_3 receptor, alternative splicing gives rise potentially, in addition to the 446 amino acid receptor, to two truncated proteins of 109 and 428 amino acids [25]. Thus, PCR amplification, using primers flanking the entire coding sequence of the

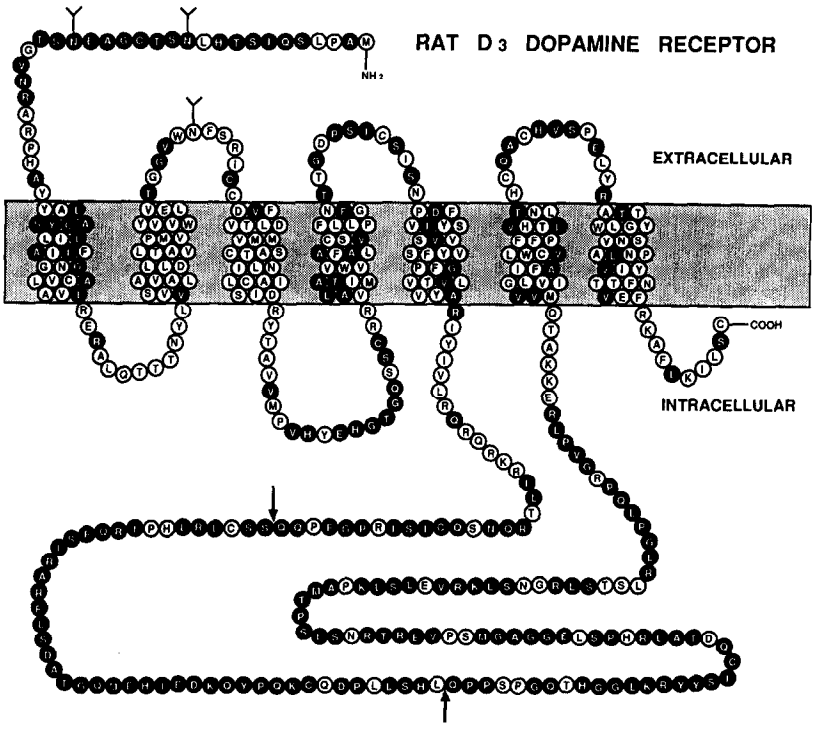

Fig. 2. Proposed membrane topography of the rat D_3 dopamine receptor and its relationship with the D_2 receptor. Darkened circles represent residues which differ between rat D_2 and D_3 receptors. The portion of the third intracytoplasmic loop limited by arrows is absent in the human D_3 receptor; otherwise its structure is highly homologous to that of the rat D_3 receptor.

D_3 receptor and mRNAs of various rat brain areas in which it is expressed, gave rise, in addition to the typical cDNA, to two other products with sizeable deletions of 113 bp in TM3 and 54 bp in 02, respectively: hence the designation of the proteins potentially encoded by these two transcripts as D_3(TM3-del) and D_3(02-del), respectively.

Two distinct alternative splicing mechanisms underlie the production of these two mRNAs. In the case of D_3(TM3-del), the process involves combinatorial exons, the "cassette" exon being the second exon (Fig. 1). Since the latter does not comprise nx3 nucleotides, this introduces a frameshift in the sequence and the splice product encodes a 109 amino acid protein. By contrast, in D_3(02-del) mRNA, the in-frame 54 bp deletion does not correspond to a full exon: alternative splicing occurs within the fourth exon where an internal acceptor site can be used by the splicing machinery, thereby giving rise to a mRNA encoding a 428 amino acid protein.

Whereas the structure of D_3(TM3-del) makes it unlikely that the protein may function as a receptor, this is not so clear in the case of D_3(02-del), whose structure may still be compatible with the occurrence of seven TMs, as revealed by the hydropathy profile. However, CHO clones stably expressing D_3(02-del) mRNA failed to show any dopaminergic binding activity, as assessed with various radioactive ligands.

What could be the function, if any, of these truncated products of the D_3 receptor gene? Indeed, both encode potential integral membrane proteins, possibly involved in cell signalling. Nevertheless, the idea that these truncated forms lack any direct biological activity in signal transduction cannot be discarded. They could be formed at random during biosynthesis of the functionally active D_3 receptor. Althernatively, this may represent a mechanism controlling the abundance of the active D_3 receptor. Finally, since multiple D_3 receptor gene transcripts are also found in human brain [8], it cannot be excluded that defects in the alternative splicing mechanisms, leading to the formation of inactive receptors, may occur during psychiatric diseases.

Anatomical distribution of D_3 receptor mRNA in rat brain

The distribution of D_3 receptor gene transcripts in rat brain areas, as established using Northern or PCR analysis or visualized by *in situ* hybridization histochemistry [7, 26], markedly differs from those of the D_1 [27] or D_2 receptor [28] gene transcripts. For instance, only a weak D_3 receptor hybridization signal was detected in restricted parts of the striatum, whereas the whole striatum contains the highest densities of DA axons and D_2 receptor mRNA (Fig. 3). By contrast, the D_3 receptor mRNA is highly expressed in the olfactory tubercle–island of Calleja complex, the bed nucleus of stria terminalis and nucleus accumbens. These areas constitute, with the

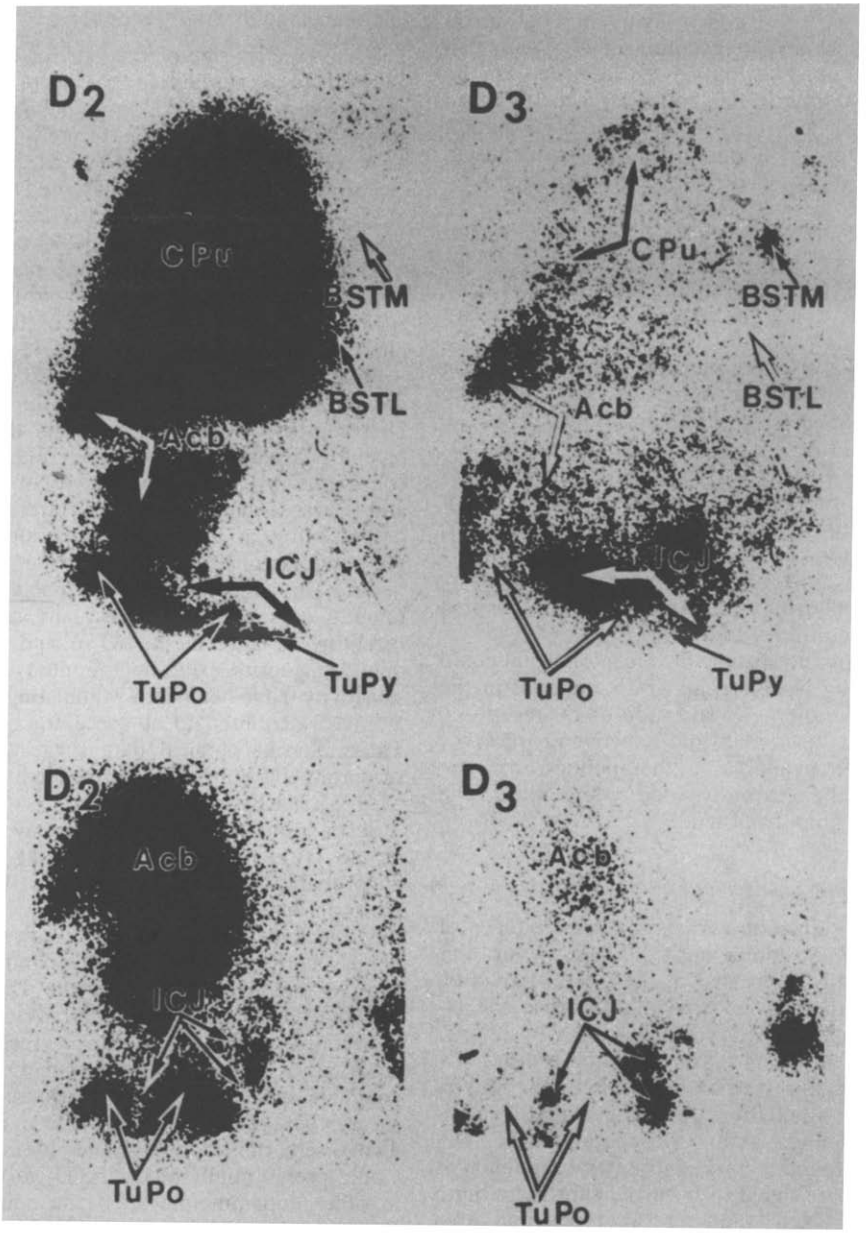

Fig. 3. Comparison of the distributions of D_2 and D_3 receptor mRNAs established by *in situ* hybridization in sagittal (top) and frontal (bottom) sections of rat telencephalon. Note the non-overlapping complementary distributions of the two transcripts in the ventral striatum, particularly at the level of olfactory tubercle–islands of Calleja and basal nucleus of the stria terminalis. Abbreviations: Acb, accumbens nucleus; BSTL and BSTM bed nucleus of the stria terminalis, lateral or medial part; ICJ, islands of Calleja; CPu caudate putamen; and TuPo and TuPy polymorph and pyramidal layers of the olfactory tubercle.

ventral and ventomedial parts of the caudate putamen, the "ventral striatum", a territory receiving afferents from the prefrontal or allocortex and amygdala and its major DA inputs from the A10 cell group in the ventral tegmental area. It projects to the ventral pallidum and the latter to the mediodorsal thalamic nucleus which selectively innervates the prefrontal contex [29]. This connectivity has led to the designation of this territory as the "limbic" part of the striatal complex, in which D_3 receptors may, therefore, mediate a large part of DA signals. The remainder of the striatal complex, which is innervated mainly by DA projections from the substantia nigra, receives its cortical inputs from

the somatic neocortex and is highly enriched in D_1 and D_2 receptors. D_3 receptor signals were also detected in other "limbic" areas such as the hippocampus, septum or mammillary nuclei in the hypothalamus. This suggests a major participation of D_3 receptors in dopaminergic transmissions in limbic areas known to be associated with cognitive, emotional and endocrine functions.

D_2 receptor mRNA is also highly expressed in these areas but there is *no strict overlap* with D_3 receptor mRNA distribution. For instance, the highest levels of D_3 receptor mRNA in brain are detected in the islands of Calleja, in which the D_2 receptor signal is weak, whereas a reverse situation is found in the olfactory tubercles (Fig. 3). In the bed nucleus of the stria terminalis, only cells of the medial division strongly and selectively express D_3 receptor mRNAs (Fig. 3). The two receptor subtypes differ by the much higher affinity of dopamine for the D_3 receptor and, possibly, by their intracellular signalling systems (see below). Hence, it seems likely that different kinds of signals may be generated by DA in neighboring but topographically distinct cerebral structures.

Interestingly, no specific D_3 receptor signal could be detected by Northern and PCR analyses in the pituitary, a prototype localization of D_2 receptors. This allows us to predict that selective D_3 receptor ligands, when available in therapeutics, will not affect, like the currently used neuroleptics, the activity of mammotrophs.

The D_3 receptor as a second autoreceptor

In situ hybridization reveals a weak D_3 receptor signal in the substantia nigra [26]. However, this signal is mainly expressed in the lateral part and, here again, there is no true overlap with the D_2 receptor signal, which is strongly expressed all over the whole compacta. The hypothesis that D_3 receptors are expressed by DA neurons themselves was verified after their lesioning using local 6-hydroxydopamine. After degeneration of DA neurons, we found a marked ipsilateral reduction of the D_3 receptor signal in both the substantia nigra ($-65 \pm 10\%$) and the ventral tegmental area ($-69 \pm 14\%$). In the same tissue extracts, the D_2 receptor mRNA levels were similarly affected, i.e. by -88 and -65%, respectively [7].

This establishes that both D_2 and D_3 receptors are not only located postsynaptically but are also expressed by DA neurons belonging to the A_9 and A_{10} cell groups. This suggests that both play the role of autoreceptors. Such a role for the D_3 receptor is consistent with its pharmacological profile (see below).

Many distinct functions were previously attributed to DA autoreceptors, i.e. inhibitions of impulse flow, DA synthesis and release at either nerve terminals or dendrites, and co-transmitter release. D_2 and D_3 autoreceptors might variously participate in all of these actions and in various brain areas. Finally, the question as to whether a single cell expresses both D_2 and D_3 receptors remains to be answered, namely by *in situ* hybridization studies at the cellular level.

Pharmacology of the D_3 receptor

The pharmacology of the rat [7], or human D_3 receptor* was studied in transfected CHO cells expressing a high level of sites labelled with high affinity by [^{125}I]iodosulpride, formerly considered to be a D_2 receptor-selective ligand [30]. The D_3 receptor can be considered, like the D_4 receptor, as a "D_2-like" receptor: it poorly recognizes "D_1-specific" ligands such as SKF-38393 or SCH-23390, whereas it binds "D_2-specific" agonists, e.g. quinpirole, or antagonists, e.g. sulpiride (Table 2). However, several salient features of the D_3 receptor pharmacology should be underlined.

First, dopamine, as well as agonists such as TL99, quinpirole or quinerolane, display high affinities at D_3 receptors. This may account for the role of D_3 receptors as autoreceptors since (i) DA in very low concentrations reduces the activity of DA neurons, and (ii) these agonists seem to act preferentially at autoreceptors as judged in animal models, such as the butyrolactone-induced increase of DA synthesis [5, 31]. This suggests that some functions attributed to autoreceptor stimulation actually involves the D_3 receptor. In agreement, AJ 76 and UH 232, the only antagonists exhibiting (limited) D_3 receptor-selectivity, have behavioral stimulating properties in animals, attributed to autoreceptor blockade [32]. These pharmacological data suggest that the D_3 receptor plays a major role in the feedback inhibition of DA transmission.

Second, most antipsychotics display high affinities at the D_3 receptor, indicating that this receptor is probably blocked during the treatment of schizophrenia and related disorders. The degree of this blockade would depend, however, on the antipsychotics used since their recognition by the D_3 receptor relative to that of the D_2 receptor is variable. The compounds for which the ratios between K_i values for D_2 and D_3 receptors (K_iD_2/K_iD_3 ratios) are the highest would exert a more complete blockade of DA transmission in limbic areas, where the D_3 receptor is selectively expressed. Conversely, those for which the ratios are the lowest would preferentially block the D_2 receptor present in other dopaminergic areas, including the extra-pyramidal system, mainly implicated in the control of motor function. This could be one of the molecular basis of the distinction of "atypical" neuroleptics. Consistent with this hypothesis is the observation of a high K_iD_2/K_iD_3 ratio measured with atypical neuroleptics such as sulpiride or amisulpride. Nevertheless, the peculiar clinical properties of clozapine are more likely to derive from its higher affinity for D_4 than any other receptor subtype [15].

Interestingly, among antipsychotics having the highest K_iD_2/K_iD_3 ratios are amisulpride, carpipramine, pipothiazine and pimozide, which all exhibit definite desinhibitory actions sought in the treatment of the negative symptoms in schizophrenia. Conceivably, the more efficient blockade of D_3 autoreceptors by these compounds could facilitate

* Sokoloff P, Andrieux M, Besançon R, Pilon C, Martres M-P, Giros B and Schwartz J-C, manuscript submitted for publication.

DA transmission in some brain areas, which might lead to the alleviation of negative symptoms [4]. To address these questions, further studies will be necessary, however, using more selective compounds, the design of which should be facilitated by the use of clonal cell lines expressing a single receptor subtype.

It should be underlined, however, that all these pharmacological data were derived from studies performed with transfected CHO cells in which the D_3 receptor does not appear to be coupled with a G protein [7]. Since coupling affects the recognition of ligands, particularly agonists, the exact pharmacology of the D_3 receptor will be definitely established by studies of the native receptor in cerebral membranes.

Signalling pathway of the D_3 receptor

Via interaction with a G_i protein, the D_2 receptor seems linked to numerous signalling pathways including inhibition of adenylate cyclase or phospholipase C and activation of K^+ channels [33]. More recently, the D_2 receptor expressed in transfected CHO cells was also shown to mediate an enhancement of arachidonic acid release, provided that such release has been initiated by increasing intracellular Ca^{2+} [34, 35]. The potential importance of this novel eicosanoid pathway lies in the fact that it may account for the synergistic interaction between D_1 and D_2 receptors [34].

In CHO cells transfected with D_3 receptors no or variable inhibition of either adenylate cyclase or phospholipase C was evidenced [7] and the arachidonate response was weak [34]. A series of observations suggest that this reflects the coupling of the D_3 receptor to a G protein which is distinct from the G_i involved in D_2 receptor signalization and absent from the recipient CHO cell.

Thus, guanylnucleotides, which rightwardly shift and steepen the competition curves generated with agonists at D_2 receptors, have no such effect at rat D_3 receptors expressed by transfected CHO cells [7]. However, in CHO cells transfected with the human D_3 receptor (which markedly differs from its rat counterpart at the level of the i_3 loop), a modest but replicable effect of guanylnucleotides is observed. Furthermore, this modulatory effect is enhanced in CHO cells co-transfected with the α_o subunit of a G protein, although adenylate cyclase inhibition still cannot be evidenced.*

From these observations it appears that D_3 receptor signalization may involve pathways different from those activated by stimulation of D_2 receptors, via interaction with a distinct G protein(s). In view of the multiplicity of G proteins, it seems important to establish the signalling pathway(s) of native D_3 receptors in brain.

Conclusions

The recent and rapid enlargement of the DA receptor family illustrates again how the diversity of receptor families was severely underestimated by most pharmacologists in the pre-molecular-biology days. The existence of the three pharmacologically distinct "D_2-like" subtypes, i.e. D_2, D_3 and D_4, instead of a single D_2 receptor, which was formerly recognized as *the* target for antipsychotic agents, raises important issues: among the three, which is (are) responsible for the beneficial therapeutic effects as well as for each unwanted side-effect? At this early stage of our knowledge much caution is needed but two clues point to the D_3 as a key receptor in schizophrenia: its selective expression in a phylogenetically old part of the brain known as the limbic system and its relatively preferential binding of several atypical antipsychotics. The last criterion, on the other hand, points to the D_4 as a key target for clozapine, a compound with a spectrum characterized by its activity in a subpopulation of patients resisting to other antipsychotics.

The enlargement of the DA receptor family has revealed that most drugs now currently used in the field seriously lack selectivity. Whether this ensures therapeutic activity or is responsible for side-effects remains to be established, using more selective agents.

After decades of traditional pharmacology during which drugs were used to define receptors and thereafter their genes, we are entering a new era, that of "reverse pharmacology", in which receptor genes are first identified and drugs ultimately derive from this identification. It is expected that this novel process will lead soon to the selective agents which are needed to answer the questions raised and, possibly, to cure some of the more serious human diseases. Furthermore, the probable roles of D_3 and D_4 receptors as targets for antipsychotics raise the possibility that their corresponding genes may be affected in various psychiatric diseases, an hypothesis which is being actively explored in several laboratories.

Note added in proof. The rat homolog of the human D_5 receptor was recently cloned and termed "D_{1B}" [36].

* Andrieux M, Martres M-P, Pilon C and Sokoloff P, unpublished observations.

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