Early Emergence of Three Dopamine D₁ Receptor Subtypes in Vertebrates

MOLECULAR PHYLOGENETIC, PHARMACOLOGICAL, AND FUNCTIONAL CRITERIA DEFINING D₁A, D₁B, AND D₁C RECEPTORS IN EUROPEAN EEL ANGUILLA ANGUILLA

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The existence of dopamine D₁C and D₁D receptors in Xenopus and chicken, respectively, challenged the established duality (D₁A and D₁B) of the dopamine D₁ receptor class in vertebrates. To ascertain the molecular diversity of this gene family in early diverging vertebrates, we isolated four receptor-encoding sequences from the European eel Anguilla anguilla. Molecular phylogeny assigned two receptor sequences (D₁A₁ and D₁A₂) to the D₁A subtype, and a third receptor to the D₁B subtype. Additional sequence was orthologous to the Xenopus D₁C receptor and to several other previously unclassified fish D₁-like receptors. When expressed in COS-7 cells, eel D₁A and D₁B receptors display affinity profiles for dopaminergic ligands similar to those of other known vertebrate homologues. The D₁C receptor exhibits pharmacological characteristics virtually identical to its Xenopus homologue. Functionally, while all eel D₁ receptors stimulate adenylyl cyclase, the eel D₁B receptor exhibits greater constitutive activity than either D₁A or D₁C receptors. Semiquantitative reverse transcription-polymerase chain reaction reveals the differential distribution of D₁A₁, D₁A₂, D₁B, and D₁C receptor mRNA within the hypothalamic-pituitary axis of the eel brain. Taken together, these data suggest that the D₁A, D₁B, and D₁C receptors arose prior to the evolutionary divergence of fish and tetrapods and exhibit molecular, pharmacological, and functional attributes that unambiguously allow for their classification as distinct D₁ receptor subtypes in the vertebrate phylum.

Dopamine is a widespread modulatory neurotransmitter in the central and peripheral nervous system of vertebrates. The physiological roles of dopamine range from the sensorimotor control, thermoregulation, and modulation of appetite to the regulation of reproductive and maternal behavior. At the cellular level, the pleiotropic effects of dopamine are mediated by two specific classes of dopamine receptors, termed D₁ and D₂, distinguishable by their ability to activate (D₁ class) or to inhibit (D₂ class) the enzyme adenylyl cyclase (1). More recently, molecular studies have revealed that D₁ and D₂ receptors are composed of several membrane proteins, each belonging to the G protein-coupled superfamily of receptors defined by their shared overall topology and common signal transduction mechanism, which triggers GDP/GTP exchange on heterotrimeric G proteins (2). Two D₁-like receptors (D₁A and D₁B, more precisely named D₁A and D₁B, respectively) and three D₂-like receptor subtypes (D₂, D₂₁, and D₂₂), each encoded by distinct genes, have been isolated in mammals (3). Based on sequence analysis and gene organization, D₁ and D₂ dopamine receptor classes appear not to be more closely related to each other than to other catecholamine receptor families (4, 5). As with other members of the monoamine receptor family, D₁-like and D₂-like receptors are probably of independent origin and have acquired separately and convergently the ability to bind their endogenous ligand (5).

In contrast with dopamine D₂-like receptor genes, which have only been extensively characterized in mammals, D₁-like receptor gene diversity has been recently examined in a small set of other vertebrate species. Besides D₁A and D₁B receptors, additional D₁-like receptor genes have been isolated from amphibians (Xenopus D₁C receptor (6)) and birds (chicken D₁D receptor (7)). Similarly, D₁-like receptor sequences not classified as either D₁A or D₁B have been described in fish (Ref. 8 and GenBank™ sequence X81969). Although Xenopus D₁C and chicken D₁D receptors significantly differ from both vertebrate and mammalian D₁A and D₁B receptors on the basis of their amino acid sequence and distinct pharmacological profiles, it is unclear whether they are truly reflective of distinct D₁ receptor subtypes or if their presence is merely associated with restricted to specific species.

As with other members of the catecholamine receptor gene family, the molecular diversity of D₁-like receptors appears to be the product of gene duplication events occurring during the evolutionary history of a particular species (9). Since the multiplicity of D₁-like receptors is a common characteristic of amphibian, avian, and mammalian genomes, the gene duplication events that underlie the origin of these receptor subtypes must have occurred significantly before or close to the emergence of tetrapods. In order to gain insights into the nature and temporal occurrence of these important genetic events, it is necessary to ascertain the genetic diversity of the D₁ receptor family in a species belonging to a phylum that diverged before the emergence of tetrapods. Ray-finned fish (actinopterygians) diverged ~420 million years ago from flesh-finned fish (sarcopterygians)
from which the tetrapod ancestor descended. Therefore, we searched for the full complement of D1-like receptor diversity in a modern representative of actinopterygians, the European eel Anguilla anguilla (a teleost). We report here that (a) eel dopamine D1 receptors are comprised of three distinct D1 receptor subtypes, which express molecular, pharmacological, and functional signatures that define unequivocally the characteristics of vertebrate D1A, D1B, and D1C receptors; (b) the diversity of the vertebrate D1 receptor gene family occurred and was clearly established before the divergence of actinopterygians from sarcopterygians.

EXPERIMENTAL PROCEDURES

Dopamine D1-like Receptor Diversity—An unidirectional cDNA library was constructed in part with the cDNA synthesis kit and UniZAP XR cloning kit (Stratagene). Briefly, 10 μg of poly(A)+ RNA from eel brain and pituitary were reverse-transcribed with avian myeloblastosis virus reverse transcriptase using a hybrid oligo(dt) linker-primer, which contains an Xho I site, in the presence of cyto-dCTP. Second strand synthesis was performed with RNase H and DNA polymerase I, and the cDNAs were blunt-ended with Klenow DNA polymerase, ligated to an EcoRI adapter, and finally digested with XhoI and EcoRI. Following size fractionation by gel filtration on Sephacryl S400, cDNAs over 1 kilobase pair were ligated into the Uni-ZAP XR vector arms and in vivo packaged with Gigapack II Gold extract. The library contained ∼6 × 10⁹ independent clones.

Approximately 1 × 10⁶ recombinant phages were plated and transferred in duplicate onto Hybond N filters (Amersham Corp.). The DNA fragment encoding the eel dopamine Dₐ receptor gene was labeled with [α-³²P]dCTP by random priming to a specific activity higher than 1.10⁶ cpm/μg. Filters were hybridized at 60°C in the hybridization medium as described above, containing 1.10⁶ cpm/μl of [³²P]-labeled Dₐ fragment. The filters were washed twice for 30 min in 0.5 × SSPE, 0.1% SDS at 55°C and autoradiographed. Positive clones were isolated by three rounds of purification and excised in vivo according to the manufacturer’s protocol.

Sequence Analysis and Molecular Phylogeny—All cDNA clones were sequenced in both strands with internal sequence-specific or universal M13 primers using Sequenase 2.0 (Amersham), either by hand or on an ABI373 sequencer (Genome Express, Grenoble, France). The deduced amino acid sequences of the cloned eel D₁ receptor genes were aligned with all D₁-like sequences available by February 1996: human D₁A (X58967), rat D₁A (M35077), opossum D₁A (S67258), goldfish D₁A (L08602), Xenopus D₁A (U07863), chicken D₁A (L36877), Fugu D₁a-like sequence (X80174), human D₁B (X58545), rat D₁B (H68118), Xenopus D₁B (U07864), chicken D₁B (L36878), Tilapia D₁A (X81869), Fugu D₁-like sequence (X80177), and Drosophila D₁ (X77234). The alignments of sequences, deletions of the invariant or noninformative positions, distance calculation, tree constructions, and bootstrap analysis were carried out on a PC with the MEGA software package (15). Cos-7 Cell Expression and Ligand Binding—DNA fragments were subcloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen). COS-7 cells grown in 150-mm plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C, 5% CO₂ were transfected with recombinant plasmid (10 μg/10⁶ cells) by either the DEAE-dextran procedure or by electroporation as described previously (16), cells were collected 24 h after transfection, and membranes were prepared in buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 1.5 mM CaCl₂, 5 mM KCl, 5 mM MgCl₂, 120 mM NaCl). For saturation experiments, membranes (30–50 μg/mg) were assayed for D₁ receptor activity using increasing concentrations (0.02–20 nM) of [³H]SCH-23390 (DuPont NEN; 81.4–85 Ci/mmol; 1 Ci = 37 GBq) in a final volume of 1.5 ml and incubated for 90 min at 37°C or room temperature. For competition binding experiments, [³H]SCH-23390 (0.5 nM final concentration) was incubated with increasing concentrations of dopaminergic compounds (10⁻¹¹ to 10⁻⁴ M) and assayed as above. Incubations were terminated by rapid filtration over Skatron filter mats and monitored for tritium in a Pharmacia or Beckman liquid scintillation counter. For all experiments, nonspecific binding was defined in the presence of 1 μM (±)-butaclamol. Kᵢ values were calculated from the estimated IC₅₀ as described by Munson and Rodbard (38) by using the nonlinear least-square curve fitting program, Kaleidagraph (Abelbeck Software). All experiments were conducted in triplicate with all clones tested concurrently.

cAMP Accumulation—In cAMP accumulation experiments, COS-7 cells transiently transfected with the receptor clones were grown for 48–72 h in 6- or 24-well dishes and assayed for cAMP accumulation in the presence or absence of various dopaminergic compounds as indicated in Dulbecco’s modified Eagle’s medium containing 0.5 mM 3-isobutylmethylxanthine and 1 μM propranolol as described (6). For experiments in which the constitutive activity of multiple D₁ receptors were assessed, cells were assayed simultaneously for D₁ receptor activity using 15–20 nM [³H]SCH-23390 to ensure the equivalence of D₁A, D₁B, and D₁C receptor expression, cAMP accumulation as determined by immunodetection as described by the manufacturer (Amersham).

Reverse Transcription-PCR Analysis of the Multiple D₁ Receptor mRNA Distribution—10 μg of total RNA extracted from different sections of eel brain were treated with 5 units of RQ1 DNase (Promega) for 30 min at 37°C, extracted twice with phenol/chloroform, and precipitated with ethanol. 5 μg of RNA were then primed with oligo(dt) and

1 The abbreviations used are: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; 6-ATDIN, (+)-2-amino-6,7-dihydroxytetralin; NPA, (+)-N-propylapomorphine.

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reverse-transcribed with 200 units of Superscript II (Life Technologies, Inc.) at 45 °C for 60 min. \( \frac{1}{50} \) of the reaction was used as substrate in subsequent PCR experiments. We firstly checked that all four D₁ receptor transcripts were linearly and similarly enough amplified (never more than a 4-fold difference) by the receptor-specific primers after 15–30 cycles of PCR amplifications. Then, for each receptor, we performed 25 cycles of PCR with receptor-specific primers (see legend to Fig. 6) with cDNAs prepared from different parts of the brain. The quantities of cDNA used as substrate were estimated by co-amplification of the eel cytochrome \( b \) cDNA (GenBankTM sequence D84302).

**RESULTS**

Cloning and Sequencing of Four Distinct D₁-like Receptor Sequences in the European Eel—Amplification of eel genomic DNA by PCR with D₁ specific primers generated a fragment that upon sequence analysis displayed 74% identity with transmembrane segments III–VII of the rat D₁A receptor gene. The full-length sequence of this gene was then obtained using a modification of the 5'- and 3'-RACE technique (see "Experimental Procedures"). Three clones were obtained from independent PCR experiments with the same sequence. This D₁ receptor fragment was secondarily used as a probe to screen at medium stringency a cDNA library prepared from eel brain and pituitary mRNAs. Six independent clones were isolated. Further hybridization analysis and DNA digestion with restriction enzymes revealed that they corresponded to two distinct cDNA sequences. Sequence determination of the longest cDNAs of each of the two classes (clones Eel 441 and 446) revealed that they most likely represented D₁A and D₁B receptor orthologues. Surprisingly, the D₁A cDNA sequence isolated from the library differed slightly from that of the PCR products previously obtained from genomic DNA. Therefore, we named these two D₁A sequences D₁A₁ and D₁A₂ in accord with the standard nomenclature of recently duplicated loci (15).

Taking into account these new sequences and those from *Xenopus* D₁C and chicken D₁D (6, 7), we designed other degenerate primers suitable for PCR amplification in order to detect a larger set of D₁-like receptors from genomic DNA. Using this approach, an amplified DNA fragment sharing 73% sequence identity with the *Xenopus* D₁C gene was isolated. The full-length gene sequence was subsequently obtained using a combination of 5'- and 3'-RACE procedures as described under "Experimental Procedures." Finally, PCR analysis revealed that none of the four eel D₁-like receptor genomic sequences contain introns interrupting their coding regions, as is characteristic for all cloned vertebrate/mammalian D₁ receptor sequences to date (data not shown).

Fig. 1 depicts the deduced amino acid sequence of all four cloned eel D₁-like receptors. Sequence comparisons with other cloned members of the D₁ receptor family clearly indicate that these four sequences encode fish homologues of mammalian D₁
receptors, which can be subdivided into D_{1A}, D_{1B}, and D_{1C} receptor subtypes. This contention is further supported by phylogenetic analysis of multiple receptor sequences (see below). A putative initiation methionine with the predicted Kozak sequence (16) was followed by long open reading frames for all four clones. Two eel D_{1A}-like receptors were encoded by 1335 nucleotides (D_{1A1} and D_{1A2}), the D_{1B} of 1374 nucleotides (eel D_{1B}) and eel D_{1C} was composed of 1344 nucleotides encoding proteins with estimated molecular masses of 49,441, 49,310, 51,940, and 50,084 Da, respectively. The highest observed number of identities shared by all of the known members of the D_{1} receptor class are found in putative transmembrane regions of the deduced amino acid sequence. Within these regions, the two D_{1A} receptors (D_{1A1} and D_{1A2}), the D_{1B} receptor, and the D_{1C} receptor exhibit homologies of ~95, 93, and 97%, respectively, with their corresponding Xenopus, chicken, and mammalian receptors. As with all D_{1} receptors cloned to date, regions of significant sequence divergence between D_{1} receptor subtypes are particularly evident in the amino termini, the third intracellular and extracellular loops, the fourth extracellular domain, and the carboxyl-terminal tails.

Consensus sequences for putative post-translational modifications as well as amino acid residues known to be critical for dopamine binding have been remarkably conserved in the eel D_{1} receptor family and are found at the expected positions in the sequence. For instance, the aspartic acid residue in TM2 thought to mediate the sodium ion effect on ligand binding (17) as well as the aspartic acid in TM3 and the three sequential serine residues in TM5, which are believed to be the key determinant of dopamine binding in the rat D_{1A} receptor (18), are conserved in the eel sequences. Similarly, six cysteine residues are found at homologous positions in all D_{1} receptor sequences. Two cysteines (positions 94 and 185 of the eel D_{1A1} Receptor) are believed to form a disulfide bridge linking the first and second putative extracellular loops. Interestingly, two other cysteines (positions 292 and 304) are also conserved in the third putative extracellular loop and could possibly serve to form a second disulfide bridge constraining receptor structure. Two additional conserved cysteines are found in the cytoplasmic tail of these receptors (positions 344 and 348). The residues homologous to cysteine 337 or 347 of the rat and human D_{1A} sequences, respectively, appear to be myristoylated so as to anchor the C terminus to the membrane and to allow agonist-selective conformational changes to be transmitted at the internal side of their plasma membrane (19, 20). Several sites that are putative substrates for protein kinase A and protein kinase C are conserved in the third intracellular loop of all the vertebrate D_{1} receptors. The serine residues homologous to the serine 380 of the rat D_{1A} sequence shown in vitro to be a substrate for phosphorylation by protein kinase A (21) is conserved in all of the D_{1A} sequences, being replaced, interestingly enough, by a conservative substitution (threonine) in the eel D_{1A1} receptor. Conserved consensus sequences for N-linked glycosylation are found within the amino terminus and the third extracellular loop for all four cloned eel D_{1} receptors. The eel D_{1C} receptor, however, contains one additional consensus site in the amino terminus, as does the Xenopus D_{1C} receptor. Unlike the Xenopus D_{1C} receptor, which contains two additional protein kinase C consensus sites within the carboxyl tail, the eel D_{1C} homologue does not, the functional significance of which, if any, is presently unknown. Finally, as highlighted in Fig. 1, the Xenopus and eel D_{1C} receptor sequences exhibit several specific residues shared with the avian D_{1D} sequence but not with either D_{1A} or D_{1B} receptor sequences. While clearly not definitive, these specific amino acid residues may constitute the “molecular signature” of D_{1C} receptors and, as such, possibly suggest that the chicken D_{1D} receptor is indeed more closely related to the D_{1C} than to the other D_{1} receptor subtypes.

Molecular Phylogeny Analysis of the D_{1}-like Receptor Diversity—The amino acid sequences deduced from the four eel D_{1}-like receptor DNA clones were aligned with all cloned D_{1}-like receptor sequences available so far. As depicted in Fig. 2, we calculated a matrix of pairwise distances (i.e. the number of amino acid substitutions) separating the receptor sequences and constructed a phylogenetic tree with the neighbor-joining method (22), from which evolutionary relationships could be hypothesized. Maximum parsimony analysis of the data gave essentially the same branching order (not shown).

Each of the D_{1} eel receptor sequences is clearly assigned to a well defined receptor subtype. The bootstrap resampling method applied to the tree distance estimated as very robust the branching delineating the D_{1A} (100%), D_{1B} (99%), and D_{1C} (98%) sequences (Fig. 2). Although the D_{1A} sequences appear to have diverged more slowly than D_{1B} or D_{1C} sequences in the same group of species, these divergences remain modest and do not hinder the significance of the analysis. The European eel possesses, therefore, two distinct D_{1A} (D_{1A1} and D_{1A2}), one D_{1B}, and one D_{1C} receptor genes. Other D_{1}-like receptors have been recently isolated from other fish, such as a D_{1A} receptor from goldfish retina (23), and several other D_{1}-like receptor sequences have been isolated from Tilapia (24) and Fugu (8), allowing for the opportunity to assess their evolutionary relationships. Our phylogenetic analysis strongly suggests that eel D_{1C}, Tilapia D_{1C}, Fugu D_{1C}-like, and Xenopus D_{1C} are orthologues (bootstrap value ~ 98) and that they constitute a new subtype of vertebrate D_{1} receptor. As such, the Fugu sequence should be renamed as D_{1C}. The situation for the chicken D_{1D} is less clear, since molecular distance analysis could not unambiguously determine whether this receptor belonged to the D_{1C} subtype, but with a high rate of sequence divergence, or if it represented the first member of another subtype of D_{1}-like receptors specifically duplicated in the avian lineage. Parsimony methods as well as the presence of some specific residues shared by D_{1C} and D_{1D} sequences (see Fig. 1) appear to support
the existence of a D1C/D1D clade (data not shown). This issue could be resolved with the availability of D1D receptor sequences from nonavian species. In any event, the existence of the same paralogous D1-like receptor subtypes in teleost fish and tetrapoda demonstrates that the D1C receptor subtype is common to most vertebrates and that the gene duplication events at the origin of the D1-like receptor diversity arose prior to the separation of actinopterygian fishes from the other vertebrates, ~420 million years ago.

**Eel D1A1 and D1A2 Are Encoded by Two Distinct Genes**—The existence of two D1A receptors with an overall amino acid homology of 94% (within the transmembrane segments) suggests that the genetic diversity of D1 receptors in eel is in fact greater than that observed in other vertebrates. Alternatively, the existence of two highly homologous receptor sequences suggested the possibility that D1A1 and D1A2 receptors could represent alleles of a single gene, even if sequence differences between the two receptors were much higher than usually expected for allelic variants. This possibility was initially strengthened by the fact that the genomic DNA from which the D1A1 receptor was amplified and the mRNAs used to construct the library from which D1A2 had been cloned did not originate from the same animals and were obtained from wild populations, in which large genetic diversity is expected. To distinguish between these two hypothesis, the presence of D1A1 and D1A2 sequences was detected by PCR experiments in 12 individual genomes from eels obtained from different suppliers and very distant geographical areas. Fig. 3 depicts the results obtained following PCR amplification of eel genomic DNA with D1A1- and D1A2-specific primers. As can be seen in Fig. 3, the two receptor sequences, corresponding to D1A1 and D1A2, coexist in a single eel genome. Identical results were obtained with 11 other eel samples (data not shown). The extremely low probability of 12 animals coming from different parts of France to be heterozygous for the same alleles prompted us to conclude that D1A1 and D1A2 sequences indeed corresponded to two different genes (also see below).

**Pharmacological Characterization of the Eel D1-Like Receptors**—In order to further justify our proposed receptor classification scheme based on molecular phylogenic analysis, we characterized D1-like receptors found in the European eel in terms of their pharmacological profiles following transient expression in COS-7 cells. In particular, a comparative analysis of the pharmacological properties obtained for each of the proposed D1 receptor subtypes in the eel with those observed for well defined mammalian or other vertebrate D1-like receptor subtypes (7, 25) would strongly support the differentiation of multiple D1-like receptors into three distinct subclasses. Following expression in COS-7 cells, all four receptors bound the D1 receptor antagonist, [3H]SCH-23390, in a saturable manner to a single class of binding site with high affinity and with estimated dissociation constants (Kd) of 0.140, 0.90, 1.30, and 0.065 pM for eel D1A1, D1A2, D1B, and D1C receptors, respectively. Saturation analysis revealed receptor densities (Bmax) that were on average 0.6, 0.8, 1.0, and 0.8 pmol/mg protein for D1A1, D1A2, D1B, and D1C, respectively. The expression levels of the four eel receptors were essentially similar, facilitating the comparison of their respective pharmacological characteristics. [3H]SCH-23390 binding to membranes of COS-7 cells expressing D1A1, D1B, or D1C receptors was inhibited by various dopaminergic agonists and antagonists, in a stereoselective, concentration-dependent, and uniphasic manner (as indexed by Hill coefficients close to unity) with a pharmacological profile clearly indicative of a D1 receptor. Estimated Kd values for these agents are listed in Table I.

One unique distinguishing pharmacological feature between the mammalian dopamine D1-like receptors is the inherent ability of the D1B receptor to display higher affinity for the endogenous neurotransmitter dopamine than D1A (26). As listed in Table I, and consistent with our proposed classification of these receptors based on molecular phylogeny, the eel D1A2 receptor displayed an affinity for dopamine (~3 μM) ~3-4 fold less than the eel D1B receptor (~880 nM), paralleling their vertebrate and mammalian, particularly rat, counterparts. Moreover, similar to the vertebrate/mammalian D1B receptor, 6,7-ADTN exhibited higher affinity for the Xen D1B receptor than for the D1A receptor, while most antagonists exhibited lower affinities for the eel D1B receptor similar to that seen with the human D1B/D1D receptor. Estimated Kd values for the inhibition of [3H]SCH-23390 binding by a series of compounds to eel D1A2 or D1B receptors are highly correlated to Kd values obtained on the vertebrate or human D1A or D1B receptors, respectively, with essentially 1:1 correspondence in drug affinities (see correlation with *Xenopus* receptors in Fig. 4, B and C). As such, the major differences in drug affinities discriminating between the D1A and D1B receptor subtypes in either vertebrate or mammalian species appear to be conserved and also found for eel sequences. Although not as extensively characterized, the D1A1 receptor appears in this respect to behave as a bona fide D1A1 receptor, displaying affinities for dopaminergic agonists and antagonists similar to that observed for D1A2.

As listed in Table I, the D1C receptor, however, displayed pharmacological characteristics consistent with both types of receptors with an observed affinity for dopamine (~1380 nM), somewhat intermediate to that of D1A2 and D1B receptors. Fig. 4A illustrates a similar pattern for NPA, displaying an affinity for the D1C receptor intermediate to that of D1A2 or D1B. Similar results were obtained for 6,7-ADTN. All other agonists displayed somewhat higher affinity for the eel D1C receptor than either the D1A or D1B receptors, similar to that seen with the *Xenopus* D1C receptor. Most D1 receptor antagonists exhibited affinities at the D1C receptor that were either intermediate to those of D1A2 and D1B receptors or identical to that of D1A2 (see Table I). As illustrated in Fig. 4D, the estimated Kd values of various agonists and antagonists at the eel D1C receptor are highly correlated with those observed on the cloned *Xenopus* D1C with a virtual 1:1 correspondence in drug affinities as indexed by the line of equimolarity. Since none of the receptors displayed guaninenucleotide sensitivity (data not shown), consistent with previous observations (6, 7), it is possible to directly compare affinities of these receptors with their vertebrate/mammalian counterparts expressed in the same cells and

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assayed under similar conditions. While the eel D1C receptor subtype can be pharmacologically differentiated from either D1A or D1B, none of the compounds tested selectively identified or exhibited preferential affinity for the D1C receptor subtype. Future work will need to identify ligands, possibly not of the benzazepine class, that can pharmacologically differentiate the D1C receptor.

Despite the rather unique pharmacological profiles exhibited by eel D1 receptor subtypes, all four receptors were found to couple to the same second messenger system when expressed in COS-7 cells. As illustrated in Fig. 5A, dopamine (10 μM) stimulated the eel D1A2 receptor-mediated production of cAMP ~10-fold over basal levels, an effect that is consistently blocked by pretreatment with the D1 receptor antagonist SCH-23390 (1 μM). The D1A1 receptor exhibits properties identical to those of the D1A2 receptor, and corresponding data are therefore not presented. Cells transfected by the nonrecombinant vector remained insensitive to dopamine. D1B receptor activation stimulated adenylate cyclase activity ~6-fold over basal levels, an effect blocked by SCH-23390 and similar to that seen with the D1C receptor. Given that receptor expression levels in any given experiment were similar (0.7–0.9 pmol/mg protein), it

**TABLE I**

Estimated $K_i$ values for the inhibition of $[^3H]$SCH-23390 binding to COS-7 cells expressing eel dopamine D1 receptor subtypes

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
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<th>$K_i$ (nM)</th>
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<td>D1A1</td>
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<td>D1B</td>
<td>D1C</td>
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<tr>
<td>Clozapine</td>
<td>260</td>
<td>670</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td>Siperone</td>
<td>920</td>
<td>1750</td>
<td>10,800</td>
<td>2930</td>
</tr>
</tbody>
</table>
Cyclase activity. The concentration-dependent cAMP accumulation was essentially similar, with values of 0.9, 1.2, and 0.8 pmol/mg protein, respectively (data not shown). Moreover, these data confirm the notion that SCH-23390 is a pure D1 antagonist, while butaclamol and flupentixol are inverse agonists at the D1B receptor and that the sequence specific motifs regulating this event are absolutely conserved within the D1/D5 receptor family. Interestingly, the D1C receptor also exhibited a significant intrinsic activity promoting cAMP production (~1.5–2-fold) in transfected COS cells, albeit less pronounced than that of D1B and consistent with its binding profile, which is somewhat intermediate to those of the D1A and D1B receptors. As with the D1B receptor, the eel D1C receptor appears to recognize flupentixol and butaclamol as inverse agonists, inhibiting constitutive D1C adenylate cyclase activity to below basal levels.

In summary, pharmacological and functional data obtained from the four eel D1 receptors transiently expressed in a single cell type clearly support the conclusion drawn from the sequence analysis that they belong to three different D1 receptor subtypes, namely D1A, D1B, and D1C, which have undisputable homologues in the other vertebrate species.

Tissue Distribution of the Four D1-like Receptor Transcripts in the Eel Brain—The low abundance of most of the D1-like receptor transcripts in the eel brain prevented the use of Northern blot analysis to study their regional distribution. The D1A2 receptor mRNA was a notable exception that seems to be the most abundant in the eel brain (data not shown). We therefore used reverse transcription-PCR analysis to obtain a relative quantification measured in D1B-transfected cells were significantly higher (~2–3-fold) than in cells expressing D1A2 receptors. As illustrated in the figure, D1B constitutive activity could be antagonized by both butaclamol and flupentixol but not by SCH-23390. This is virtually identical to the profile exhibited by these ligands at mammalian D5/D1B receptor subtypes. Moreover, these data confirm the notion that SCH-23390 is a pure D1 antagonist, while butaclamol and flupentixol are inverse agonists at the D1B receptor and that the sequence specific motifs regulating this event are absolutely conserved within the D1/D5 receptor family. Interestingly, the D1C receptor also exhibited a significant intrinsic activity promoting cAMP production (~1.5–2-fold) in transfected COS cells, albeit less pronounced than that of D1B and consistent with its binding profile, which is somewhat intermediate to those of the D1A and D1B receptors. As with the D1B receptor, the eel D1C receptor appears to recognize flupentixol and butaclamol as inverse agonists, inhibiting constitutive D1C adenylate cyclase activity to below basal levels.

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estimate of the amount of the four mRNA transcripts in the dissected telencephalon, midbrain, pituitary, cerebellum, and brain stem. After 25 cycles of PCR amplifications with gene-specific primers, the abundance of each of the D1-like transcript was measured by quantifying the corresponding hybridization signals relative to cytochrome b mRNA used as an internal standard. As illustrated in Fig. 6 each of the D1-like receptors exhibited a differential distribution pattern. D1A1 mRNAs were found in all the brain regions, being the most abundant in the brainstem. In contrast, D1A2 was mainly expressed in the midbrain, as was also observed for D1B and D1C receptor mRNAs. D1C receptor transcripts were almost absent from the cerebellum. Surprisingly, the pituitary, where D1 receptor-mediated effects are best documented in teleost fish (27), is not a prominent area for D1-like transcription. In the telencephalon, where dopamine projections are highly variable from one species to another (28), the four D1 mRNA subtypes were present at a high level. Quantification of the hybridization signals obtained for the D1A1 and D1A2 transcripts highlighted further their differential distribution and is documented in Table II. In summary, the data obtained from this crude distribution of the D1-like receptor mRNA transcripts provide evidence for the tissue-specific expression of the four corresponding genes in eel, as is also observed for the D1A and D1B Receptors in mammals (29) or for D1A, D1B, and D1C/D1D in Xenopus and chicken (6, 7).

**DISCUSSION**

Four D1 dopamine receptors have been isolated by molecular cloning in the teleost fish A. anguilla (European eel). All these receptors are expressed in the brain and have been pharmacologically and functionally characterized.

The assignment of each of the eel sequences to a particular subtype of D1 receptor was important in order to analyze, through an evolutionary perspective, the functional significance of the dopamine D1 receptor genetic diversity observed in modern vertebrates. The major interest of a comparative approach is to point out the parameters that are really conserved and specific of each of the receptor subtypes and therefore relevant to the physiological role of each of these subtypes. In principle, several different criteria may be used to classify the various molecular forms of G protein-coupled receptors, which include the relative affinity and activity of ligands, the modulation of particular intracellular signaling pathways, the specific tissue distribution, and finally the sequence similarities analyzed by molecular phylogeny methods (5, 30). The convergence of these criteria is probably the best way to provide a robust definition and classification of each of the receptor subtypes isolated from different animal species.

One major criterion of receptor classification into distinct receptor subtypes depends on sequence identities. In this respect, we have recently emphasized that molecular phylogeny methods are very useful to unravel the relationships of the various monoamine receptors and, in particular, to identify orthologous versus paralogous receptors (5). Indeed, the “characters” represented by nucleotides or amino acids at each position in the sequence alignments constitute objective information that can be used for the classification of these molecules on the basis of their shared similarities and differences. Despite the limitations represented by high divergence rates, saturation of sequence similarities, or gene homogenization, the phylogenetic trees constructed from sequence alignments also provide valuable information regarding evolutionary relationships among corresponding molecules and, therefore, on gene duplication events giving rise to the molecular diversity currently observed. We document here one such example for members of the dopamine D1-like receptor family, which is so far the receptor class most largely analyzed in a wide range of vertebrate species.

As illustrated in Fig. 2, molecular phylogeny unambiguously assigns the four eel D1-like receptors to three D1 receptor subtypes named D1A, D1B, and D1C. As such, it provides strong support for the view that the D1C receptor reflects a true receptor subtype, common to most of the vertebrate species. Indeed, Macrae and Brenner (8) recently isolated two D1-like receptors from another teleost fish, the puffer fish Fugu rubripes. The phylogenetic tree clearly shows that these two receptor sequences are, respectively, orthologous to the D1A and D1C subtypes. We can now conclude that D1A and D1B orthologous receptors are found in all the vertebrate species analyzed to date, whereas D1C orthologues are up to now found only in fish and amphibians, challenging the presence of this subtype in mammals.

The presence of two distinct D1A receptors in the eel is also an intriguing feature brought to light by this study. We have accumulated evidence to show that D1A1 and D1A2 receptors are not simply different polymorphic alleles; their sequences are too different to correspond to polymorphic variations, since they are found in 12 different individuals fished from different parts of France and their mRNAs are differentially transcribed in the eel brain. These data imply that an ancestor D1A gene duplicated either recently in the eel lineage or more precociously in an ancestor of modern teleosts, although this assumption awaits the demonstration of the presence of two D1A receptors in other fishes. The duplication of the ancestor of the eel D1A receptor genes is probably not the consequence of genome tetraploidization, as is the case for the toad Xenopus laevis or salmons, since European eels as well as their Japanese relatives are not tetraploids (31).

Finally, the chicken D1D sequence (7), although too divergent from the other vertebrate D1-like sequences to be unambiguously assigned to one of the three D1 subtypes, shares impressive synapomorphies with the D1C receptor subtype. As such, it is still unclear whether the chicken D1D receptor represents a fast diverging D1C sequence or if it corresponds to a new paralogous subtype of the D1 receptor appearing late in the vertebrate phylum, perhaps specifically in the Thecodontia (birds and crocodiles) lineage.

The most commonly used criterion of receptor classification is based on the rank order of potency and relative binding affinity of various dopaminergic agonists and antagonists in vitro. Since this pharmacological parameter basically results from the ligand interactions with a small number of amino acid residues in the binding pocket of the receptors, the classification obtained by this criterion should closely resemble that obtained on the basis of sequence comparison. It is indeed the case for the eel D1 receptors that display pharmacological profiles that closely conform to those previously observed for each of the three D1 receptor subtypes (6, 7). In particular, the two
eel D1A receptors (D1A1 and D1A2) have very similar binding abilities. Comparison of the binding properties of the eel D1 receptors with those of the other vertebrate D1 receptors delineates salient conserved characteristics for each of the D1 receptor subtypes. Most importantly, a higher affinity of the D1B subtype for the endogenous ligand dopamine, as compared with D1A and D1C, is a distinguishing property of the D1B receptor subtype in all of the vertebrate receptors analyzed so far. In this respect, the D1C receptor displays an intermediate affinity for dopamine in both *Xenopus* and eel. Most antagonists bind the eel D1C receptor with estimated affinity values very close to that of D1A2. One interesting conserved discriminating feature, however, is the rank order of affinity for NPA (D1A > D1C > D1B), 6,7-ADTN (D1B > D1C > D1A), haloperidol (D1A > D1C > D1B), and spiperone (D1A > D1C > D1B). In contrast, the benzazepine SCH-23390, the canonical D1 receptor ligand, does not discriminate between the various receptor subtypes in all of the vertebrate species tested and should be considered indeed as a generic marker of the D1 receptor family in vertebrates. Whether the newly described D1C receptor subtype can be further discriminated from D1A or D1B receptors to display exquisite sensitivity for other dopaminergic compounds or “second generation” D1-like agonists and antagonists is currently under investigation.

It is worth mentioning that drug-based discrimination of the various subtypes of vertebrate dopamine D1 receptors is better described by a “profile” of binding affinities for several drugs that can be readily compared in different species than by the particular properties of single “specific” ligands. Indeed, confusion in receptor classification may occur when minor sequence differences between orthologous receptors (species homologues) are recognized by specific ligands or, on the contrary, when paralogous receptors (true subtypes) are not distinguished by different ligands (32). Examples of such heterodox behavior are also observed in the D1 receptor family, where flupentixol displayed 7-fold higher affinity for the chicken than for the *Xenopus* D1B receptor (7).

The third classification criterion is provided by the differential coupling of the receptors to intracellular signaling molecules via direct interactions with α and βγ subunits of heterotrimeric G proteins. This criterion is difficult to fully apply in the case of cloned receptors, since their activity is evaluated by introducing them in cell lines where the full range of “natural” intracellular pathway activation may not always be obtained (33). Nevertheless, the four eel D1-like receptors can be significantly activated adenylyl cyclase in COS-7 cells. This property, which historically led to the definition of the D1 receptor class, remains the key parameter of D1 receptor characterization. From an evolutionary point of view, this plesiomorphic property was acquired by the common ancestor of all of the D1 receptors and should be found even in early diverging species. This statement is supported by the fact that the *Drosophila* dopamine D1 receptor mediates dopamine activation of adenylyl cyclase, although this molecule retains little of the pharmacological profile that defines vertebrate D1 receptors (25).

Interestingly, cells transfected with the eel D1B receptors consistently exhibit a higher basal cAMP level than control, D1A-transfected, or D1C-transfected cells, as found in other species. It suggests that one functional differentiating characteristic of the D1B receptor is its constitutive activity, a property inherent in the mammalian D1/D1B receptor (34, 35) and which appears to have been absolutely conserved throughout the evolutionary course of the D1B receptor subfamily. As such, constitutive activation of adenylyl cyclase by the D1B receptor system appears fundamental to this receptor subtype and functionally relevant to the physiology of dopamine in the vertebrate nervous system. At present, it is difficult to ascertain whether all vertebrate D1C receptors share, at a somewhat attenuated level, the ability to display constitutive activity (see Fig. 5) and whether this property is common throughout the evolutionary history of the D1C receptor subtype. In this regard, the *Xenopus* D1C receptor does not appear to be constitutively active (6), although direct comparison with the eel D1C receptor is difficult due to the widely disparate levels of receptor expression. The precise determination of D1C subtype characteristics regarding adenylyl cyclase modulation will require analysis in a larger set of animal species in order to determine whether its relatively high intrinsic activity is indeed conserved.

The fourth criterion that may identify receptor subtypes expressed in a single animal species is its tissue specific distribution profile. Although, as of yet, we were not able to provide a precise and complete description of the tissue distribution of the four eel D1 receptor mRNAs, the semiquantitative PCR analysis suggested that the various eel D1 receptor subtypes are differentially transcribed in various segments of the eel brain. Differential distribution of receptor mRNAs also characterizes mammalian D1A and D1B receptors. D1A receptors are present at very high levels in the striatal and olfactory regions, whereas D1B receptors are expressed mainly in the hippocampus and cortex in the human and also in the parafascicular nucleus of the thalamus in rat (29, 36, 37). The localization of the D1A and D1B receptors seems to be essentially nonoverlapping in mammals. This characteristic could be extended to the other vertebrate species and may render the multiplicity of D1 receptors essentially nonredundant. The physiological consequence for the expression of four dopamine D1 receptors in a teleost fish can be only hypothetical at present, but it probably relates to dopamine functions (not only cellular effects) selected for eel adaptation to a changing milieu. European eels, like their American and Asian relatives, have a very complex physiology in which dopamine is thought to contribute significantly to the sensorimotor, feeding, and reproductive behavior during its whole life cycle. The role of dopamine in such a life cycle could have driven the conservation of more dopamine receptor subtypes than found in other vertebrates living in more constant environments. Be that as it may, the acquisition of differential expression territories by the various duplicated genes during evolution would be a mechanism of utmost importance for the conservation of paralogous genes.

In summary, the comparison of the the eel D1 receptor sequences as well as of some of their pharmacological and functional characteristics with those of the other D1 vertebrate subtypes, although still incomplete, pinpoints defining characteristics and features specific for each of the D1A, D1B, and D1C receptor subtypes found in vertebrates. Understanding the physiological relevance of these defined functional homologies will now require an appreciation of the relationship between the differential localization of the various D1 receptor subtypes within the brains of the main groups of vertebrates and the synaptic organization of dopaminergic pathways in these species.

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Early Emergence of Three Dopamine D₁ Receptor Subtypes in Vertebrates: MOLECULAR PHYLOGENETIC, PHARMACOLOGICAL, AND FUNCTIONAL CRITERIA DEFINING D₁A, D₁B, AND D₁C RECEPTORS IN EUROPEAN EEL ANGUILLA ANGUILLA

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