Cloning and sequence analysis of a hypothalamic cDNA encoding a D<sub>1c</sub> dopamine receptor in tilapia

Anne E. Lamers a, Diet Gröneweld a, Dominique P.V. de Kleijn a, Felix C.G. Geeraedts a, Jack A.M. Leunissen b,*, Gert Flik a, Sjoerd E. Wendelaar Bonga a, Gerard J.M. Martens a

a Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands
b CAOS / CAMM centre, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands

Received 3 January 1995; revised 9 April 1996; accepted 11 April 1996

Abstract

Physiological and pharmacological studies have indicated that during acid stress a D<sub>1</sub>-like dopamine receptor becomes functional on intermediate pituitary melanocyte-stimulating hormone cells of tilapia (Oreochromis mossambicus). As a first step towards physiological expression studies we isolated a D<sub>1</sub>-like dopamine receptor from a tilapia hypothalamus cDNA library. Construction of a phylogenetic tree of most of the D<sub>1</sub>-like receptors known in human, rat, Xenopus, goldfish and Drosophila revealed that the here presented clone is most likely the tilapia equivalent of the Xenopus D<sub>1</sub> dopamine receptor.

Keywords: Cloning; Sequence analysis; Dopamine receptor D<sub>1c</sub>; (Hypothalamus); (O. mossambicus)

Dopamine signals are transduced via two types of dopamine receptors, D<sub>1</sub>-like and D<sub>2</sub>-like [1]. D<sub>1</sub>-like receptors are coupled to a stimulatory G-protein to effect a stimulation of a second messenger system in the cell (see review Ref. [2]). D<sub>2</sub>-like dopamine receptors are generally coupled to an inhibitory G-protein enabling the inhibition of a second messenger. The D<sub>1</sub>-like and the D<sub>2</sub>-like receptors can be distinguished pharmacologically using specific D<sub>1</sub> and D<sub>2</sub> agonists and antagonists. Receptor subclasses of the D<sub>1</sub>-like and D<sub>2</sub>-like receptors have been identified at the molecular level: D<sub>2</sub> like receptors have been classified into D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>; D<sub>1</sub>-like into the subtypes D<sub>1a</sub> and D<sub>1b</sub> [2,3]. Recently an additional D<sub>1</sub>-like receptor subtype was found in the Xenopus, termed D<sub>1c</sub> [4].

The regulation of release of α-melanocyte-stimulating hormone (α-MSH) from the pituitary neurointermediate lobe (NIL) of tilapia (Oreochromis mossambicus) during adaptation to acidified water has been described previously [5,6]. Pharmacological studies revealed that a D<sub>1</sub>-like dopamine receptor activity was induced in the α-MSH-producing cells of the tilapia NIL when the fish were exposed for 7 days to acid water (pH 4.5) [7]. This receptor has a higher affinity for dopamine than the D<sub>2</sub>-like receptor present in the NIL. The activation of these D<sub>1</sub>-like and D<sub>2</sub>-like receptors appears to result in stimulation of α-MSH release at picomolar concentrations of dopamine but in inhibition at nano- to micromolar dopamine concentrations. As a first step towards a study of dopamine receptor expression in the tilapia pituitary gland, we here describe the isolation and sequencing of a hypothalamic cDNA clone encoding a tilapia D<sub>1c</sub>-like dopamine receptor.

Construction of the tilapia hypothalamic cDNA library. A tilapia cDNA library was constructed from about 4 µg hypothalamic poly(A) RNA of fish raised in fresh water (pH 7.8), using the AZAP-cDNA synthesis kit (Stratagene, see also Ref. [8]). RNA was isolated by the acid guanidinium-thiocyanate/phenol-chloroform procedure [9] and subsequently poly(A)<sup>+</sup> RNA was purified with an oligo (dT) cellulose column (Stratagene) according to the manufacturer’s instructions. cDNA was synthesized using an oligonucleotide containing a poly(dT) sequence and an XhoI restriction site. EcoRI adaptors were ligated and the cDNA was directionally cloned into EcoRI-XhoI sites of...
Fig. 1. Nucleotide sequence and deduced amino acid sequence of hypthalamic cDNA clone D11 encoding the tilapia D₁₅ dopamine receptor. Numbering starts at the putative initiation methionine and ends at the termination codon. The positions of the transmembrane (TM) regions are overlined. Arrowheads indicate putative glycosylation sites, the termination codon is indicated with asterisks and the polyadenylation signal is underlined.
Screening of the cDNA library. Approx. 100 000 recombinants of the tilapia hypothalamic cDNA library were screened using a human D₁ dopamine receptor gene probe (hD₅,3z) [11]. The probe was ³²P-labeled by random priming according to standard procedures [10]. After pre-hybridization in hybridization buffer containing 40 mM sodium phosphate-buffered (pH 7.4), 25% formamide, 6 × SSC [1 × SSC = 150 mM NaCl and 15 mM sodium citrate], 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml denatured herring sperm DNA, 0.1% polyvinylpyrrolidone, 1 mM EDTA and 2 × Denhardt’s solution [1 × Denhardt’s solution is 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin and 0.1% Ficoll 400], filters were incubated with the probe in hybridization buffer at 42°C. After 18 h, filters were washed twice for 30 min with 2 × SSC at room temperature, 0.1% SDS and twice for 30 min with 2 × SSC, 0.1% SDS, at 56°C. Hybridization-positive clones were purified, and Bluescript DNA was prepared by in vivo excision according to the Stratagene protocol.

DNA sequence analysis. DNA sequencing of both strands was performed with T₇ DNA polymerase and the dideoxy chain termination method [12], using subclones and synthetic oligonucleotides. Sequence alignments were performed according to Needleman and Wunsch [13] using the GAP program of the GCG program package [14].

Construction of the phylogenetic tree. The amino-acid sequence of the tilapia D₁-like receptor was aligned with the following dopamine receptors obtained from the databases SWISSPROT and EMBL: goldfish D₁ (gD₁; accession No. P35406), human D₁ (hD₁; P21728), human D₂ (hD₅; P21918), rat D₁a (rD₁a; P18901), rat D₁b (rD₁b; P25115), the Xenopus D₁a (XD₁a; X107863), Xenopus D₁b (XD₁b; X107864), Xenopus D₁c (XD₁c; X107865), and the translated Drosophila melanogaster D₁/5 (dmD₁/5; X77234). Invariant positions were deleted from the alignment, 1000 bootstrap samples were created using the SEQBOOT [15] program, and phylogenetic trees were obtained with the programs NEIGHBOR [16] and PROTPARS [17,18].

Isolation and sequence analysis of hypothalamic cDNA encoding a tilapia D₁-like dopamine receptor. Screening of approx. 100 000 recombinants of the amplified tilapia hypothalamic cDNA library resulted in the isolation of three hybridization-positive phage plaques. Restriction analysis revealed that the three clones contained the same 4.7 kb insert. One clone (pTDAl) was used for further analysis. Analysis of the nucleotide sequence of the pTDAl clone revealed an open reading frame (ORF) coding for a protein of 368 amino acids (Fig. 1). Seven putative transmembrane regions, characteristic for G-protein coupled receptors, may be assigned to the protein. The clone contained an extremely long 5′ non-coding region of approx. 2.2 kb and a 3′ non-coding region of about 1.3 kb.

The deduced amino-acid sequence of pTDAl showed high similarity to the D₁-like dopamine receptors (Table 1). The degree of amino-acid sequence identity between the putative G-protein coupled receptor and the human/rat D₁/D₁a, the human/rat D₁/D₁b, and the Xenopus D₁c receptors is about 66%, 65% and 74%, respectively. The amino-acid sequences of the two human stimulatory dopamine receptors (D₁ and D₁) show about 65% identity, whereas the stimulatory human D₁ and the inhibitory human D₁ receptor show only 29% identity [19]. From this we conclude that the tilapia clone encodes a stimulatory (D₁-like) dopamine receptor, most likely of the D₁c subtype. We further term the here presented clone tilapia D₁c (tD₁c).

The 5′-untranslated region of the tD₁c receptor contains several small ORFs of which the four most downstream are shown (Fig. 1). The first three ORFs consist of 66 bases and the fourth of 24 bases. Although small ORFs are generally rare in vertebrate mRNA, they are not uncommon in mRNAs of receptor genes, proto-oncogenes and growth-control genes [20,21]. The function of the small ORFs is yet unknown, but a role in the regulation of translational initiation of the main ORF has been suggested [22,23]. It is not clear whether the small upstream ORFs

### Table 1

<table>
<thead>
<tr>
<th>Amino-acid sequence identities in percentages, between the human D₁ (hD₁), rat D₁a (rD₁a), Xenopus D₁a, human D₁ (hD₁), rat D₁b (rD₁b), Xenopus D₁b (xD₁b), goldfish D₁ (gD₁), Xenopus D₁c (xD₁c), tilapia D₁c (tD₁c) and Drosophila D₁c (dmD₁c)</th>
<th>hD₁</th>
<th>rD₁a</th>
<th>xD₁b</th>
<th>hD₁</th>
<th>rD₁a</th>
<th>xD₁b</th>
<th>gD₁</th>
<th>xD₁c</th>
<th>tD₁c</th>
<th>dmD₁c</th>
</tr>
</thead>
<tbody>
<tr>
<td>hD₁</td>
<td>100</td>
<td>87.3</td>
<td>75.5</td>
<td>63.4</td>
<td>62.8</td>
<td>64.5</td>
<td>65.7</td>
<td>68.3</td>
<td>65.0</td>
<td>38.2</td>
</tr>
<tr>
<td>rD₁b</td>
<td>100</td>
<td>100</td>
<td>75.7</td>
<td>64.5</td>
<td>62.6</td>
<td>65.4</td>
<td>65.4</td>
<td>68.9</td>
<td>65.1</td>
<td>38.1</td>
</tr>
<tr>
<td>xD₁b</td>
<td>100</td>
<td>100</td>
<td>65.7</td>
<td>64.9</td>
<td>67.9</td>
<td>68.3</td>
<td>71.3</td>
<td>64.6</td>
<td>39.3</td>
<td>37.2</td>
</tr>
<tr>
<td>hD₁</td>
<td>100</td>
<td>100</td>
<td>90.4</td>
<td>83.2</td>
<td>76.3</td>
<td>68.6</td>
<td>65.9</td>
<td>36.4</td>
<td>38.0</td>
<td>40.3</td>
</tr>
<tr>
<td>rD₁a</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>82.7</td>
<td>76.0</td>
<td>67.4</td>
<td>65.8</td>
<td>40.8</td>
<td>37.1</td>
<td>100</td>
</tr>
<tr>
<td>gD₁</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>71.8</td>
<td>69.4</td>
<td>67.6</td>
<td>67.6</td>
<td>40.3</td>
<td>37.1</td>
<td>100</td>
</tr>
<tr>
<td>xD₁c</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>74.2</td>
<td>72.4</td>
<td>67.6</td>
<td>67.6</td>
<td>40.3</td>
<td>37.1</td>
<td>100</td>
</tr>
<tr>
<td>tD₁c</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>74.2</td>
<td>72.4</td>
<td>67.6</td>
<td>67.6</td>
<td>40.3</td>
<td>37.1</td>
<td>100</td>
</tr>
<tr>
<td>dmD₁/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>
Numbers in branches indicate the bootstrap values calculated with the PROTPARS program.

are translated, because none of the AUG triplets of the small ORFs are in a favourable context for translational initiation (A/GNNATGG is considered the ideal context for translational initiation [24]).

Comparison of the tD$_{lc}$ receptor with D$_1$-like receptors of other species. Fig. 2 shows a alignment of the amino-acid sequence of the tD$_{lc}$ receptor with those of human D$_5$, rat D$_{lb}$, Xenopus D$_{lb}$, human D$_1$, rat D$_{la}$, Xenopus D$_{la}$, goldfish D$_1$, Xenopus D$_{lc}$, and Drosophila melanogaster D$_{1/5}$. The highest degree of identity is located in the transmembrane regions, the first and second intracellular loops, the first extracellular loop and in the regions adjacent to the transmembrane regions VI and VII of the third intracellular loop and the cytoplasmic tail. The length of the cytoplasmic tail of the tD$_{1/5}$ receptor is intermediate compared to that of the goldfish D$_1$ [25] and those of the mammalian D$_{la}$ and D$_{lb}$ receptors and the amphibian D$_{lc}$ receptor [4,19,26]. Presumably, this has no consequence for binding to the G-protein, as recent studies have revealed that only the region of the cytoplasmic loop nearest to the transmembrane VII is involved in G-protein coupling [27,28].

The percentages of overall sequence identity between the tD$_{lc}$ dopamine receptors and hD$_1$, hD$_5$, rD$_{la}$, rD$_{lb}$, xD$_{la}$, XD$_{lb}$, xD$_{lc}$, gfD$_1$, and dmD$_{1/5}$ receptors are shown in Table 1. The tD$_{lc}$ receptor is related similarly the mammalian D$_{1/la}$ and D$_{5/1b}$ receptors, and showed the highest identity (74.2%) with the recently discovered third D$_1$-like receptor of the Xenopus (XD$_{lc}$) [4]. Phylogenetic trees of members of the D$_1$-like dopamine receptor family were constructed to verify the identity of the tD$_{lc}$ receptor. The topologies obtained by the NEIGHBOR and PROTPARS programs were essentially identical. The consensus tree of 1000 bootstrap samples of the PROTPARS program is shown in Fig. 3. The consensus tree indicates that the Drosophila D$_{1/5}$ receptor, the human D$_1$/rat D$_{la}$/Xenopus D$_{la}$/goldfish D$_1$ receptors, the human D$_5$/rat D$_{lb}$/Xenopus D$_{lb}$ receptors and the Xenopus D$_{lc}$/tilapia D$_{lc}$ are four significantly divergent groups within the family of D$_1$-like dopamine receptors ($P = 1.00$). The sequence of emergence of the three subtypes from the ancestral D$_1$ gene cannot be concluded from this tree.

References


Fig. 3. Phylogenetic tree of the D$_1$-like dopamine receptor family. Numbers in branches indicate the bootstrap values calculated with the PROTPARS program.

Fig. 2. Alignment of the amino-acid sequences of human D$_5$ (hD$_5$), rat D$_{lb}$ (rD$_{lb}$), Xenopus D$_{lb}$ (xD$_{lb}$), human D$_1$ (hD$_1$), rat D$_{la}$ (rD$_{la}$), Xenopus D$_{la}$ (xD$_{la}$), goldfish D$_1$ (gfD$_1$), Xenopus D$_{lc}$ (xD$_{lc}$), tilapia D$_{lc}$ (tD$_{lc}$), and Drosophila D$_{1/5}$ (dmD$_{1/5}$) dopamine receptors. The one-letter amino-acid code is used. Black boxes indicate identical amino acids in all receptors, hatched boxes indicate conservative substitutions and gaps ( - ) are introduced to achieve maximum similarity.