0026-895X/01/6003-462–473$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:462–473, 2001

Vol. 60, No. 3
923/925604
Printed in U.S.A.

A Na^+/Cl^--Dependent Transporter for Catecholamines, Identified as a Norepinephrine Transporter, Is Expressed in the Brain of the Teleost Fish Medaka (*Oryzias latipes*)

CHRISTINE ROUBERT,[1] CORINNE SAGNÉ, MARIKA KAPSIMALI, PHILIPPE VERNIER, FRANK BOURRAT, and BRUNO GIROS

Institut National de la Santé et de la Recherche Médicale (INSERM) U-513, Faculté de Médecine de Créteil, Créteil, France (C.R., C.S., B.G.); INSERM U-288, Paris, France (C.R., C.S., B.G.); and Centre National de la Recherche Scientifique Unité Propre de Recherche 2197, Institut de Neurobiologie Alfred Fessard, Gif-sur-Yvette, France (M.K., P.V., F.B.)

Received March 2, 2001; accepted May 25, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

We report the isolation, functional characterization, and localization of a Na^+/Cl^--dependent catecholamine transporter (meNET) present in the brain of the teleost fish medaka. This carrier is very similar to the human neuronal norepinephrine transporter (NET) and the human neuronal dopamine transporter (DAT), showing 70 and 64% amino acid identity, respectively. When expressed in COS-7 cells, this transporter mediates the high-affinity uptake of dopamine (K_M = 290 nM) and norepinephrine (K_M = 640 nM). Its pharmacological profile reveals more similarities with NET, including a high affinity for the tricyclic antidepressants desipramine (IC_{50} = 0.92 nM) and nortriptyline (IC_{50} = 16 nM). In situ hybridization on the medaka brain shows that meNET mRNA is present only in a subset of tyrosine hydroxylase-positive neurons found in the noradrenergic areas of the hindbrain, such as the locus ceruleus and area postrema. None of the dopaminergic areas anterior to the isthmus contains any labeled neurons. Neither reverse transcriptase-polymerase chain reaction with degenerate primers specific for γ-aminobutyric acid transporter/NET nor autoradiographic experiments with [^{125}I]3b-(4-iodophenyl)-tropane-2b-carboxylic acid methyl ester revealed an additional catecholamine transporter in the medaka brain. Uptake experiments with medaka brain synaptosomes show an endogenous transport with a pharmacological profile identical to that of the recombinant meNET. Thus, meNET is probably the predominant—if not the only—catecholamine transporter in the medaka fish brain. In view of the highly conserved primary structures and pharmacological properties of meNET, it is tempting to speculate that a specific dopamine transport developed later in vertebrate evolution and probably accompanied the tremendous enlargement of the meso-telencephalic dopaminergic pathways in amniotes.

The catecholamines dopamine (DA) and norepinephrine (NE) are essential modulatory neurotransmitters in the nervous system of vertebrates. DA and NE neurotransmission have many common features. DA and NE derive from the aromatic amino acid tyrosine by the same metabolic pathway (Nagatsu and Stjarne, 1998). All their receptors belong to the same group of G-protein-coupled receptors (Valdenaire and Vernier, 1997; Bockaert and Pin, 1999). Termination of the catecholaminergic transmission occurs mainly through a fast and active reuptake by membrane transporters into the presynaptic terminals. In mammals, the neuronal dopamine transporter (DAT) and the norepinephrine transporter (NET) are both closely related members of the Na^+/Cl^--coupled neurotransmitter transporter family (Amara and Kuhar, 1993; Giros and Caron, 1993). They are intrinsic membrane proteins containing 12 putative transmembrane domains (TMD), with both the N and C termini residing within the cytoplasm (Giros and Caron, 1993; Brüss et al., 1995).

DAT and NET share many pharmacological features, including the high-efficiency transport of both DA and NE. The uptake specificity of these carriers is not solely a consequence

This research was supported by grants from Institut National de la Santé et de la Recherche Médicale (to B.G.) and from Centre National de la Recherche Scientifique and University Paris XI (to P.V.). C.R. was supported by Sanofi Recherche and Fondation pour la Recherche Medicale. M.K. was supported by Lilly and Fondation pour la Recherche Medicale. C.S. was supported by Rhône-Poulenc Rorer and Association France Parkinson.

[1]Present address: Sanofi-Synthelabo, Département SNC, 34184 Montpellier Cedex 04, France.

ABBREVIATIONS: DA, dopamine; NE, norepinephrine; DAT, dopamine transporter; NET, norepinephrine transporter; TMD, transmembrane domain(s); fET, *Rana catesbiana* peripheral epinephrine transporter; ceDAT, *Caenorhabditis elegans* dopamine transporter; RTI-55, 3b-(4-iodophenyl)-tropane-2b-carboxylic acid methyl ester; meNET, medaka norepinephrine transporter; PCR, polymerase chain reaction; SSC, standard saline citrate; RT, reverse transcriptase; PBS, phosphate-buffered saline; SERT, serotonin transporter; hNET, human norepinephrine transporter; GAT, γ-aminobutyric acid transporter; GABA, γ-aminobutyric acid; rDAT, rat dopamine transporter; TH, tyrosine hydroxylase; PKC, protein kinase C.

of their pharmacological characteristics. The contribution of each transporter to the synaptic regulation of DA and NE essentially depends on the transporter localization in the corresponding nerve terminals. NET transcript is present only in noradrenergic cells of the nervous system, which are found posterior to isthmus, such as the locus ceruleus, and in peripheral sympathetic nerves (Ordway et al., 1997; Comer et al., 1998; Nishimura et al., 1999). In contrast, DAT mRNA is found anterior to the isthmus, mainly in the mesencephalic dopaminergic nuclei substantia nigra and ventral tegmental area. The corresponding protein is transported to the terminals of these neurons, mostly to the dorsal and ventral striatum (Freed et al., 1995; Nirenberg et al., 1996). The catecholaminergic nuclei found on both sides of the midhindbrain junction are well conserved in all vertebrates but are specified by different developmental mechanisms (Smeets and Reiner, 1994; Ye et al., 1998; Goridis and Brunet, 1999).

DAT and NET have been isolated from several mammalian species, including human (Pacholczyk et al., 1990; Giros et al., 1992), rat (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991; Brüss et al., 1997), mouse (Donovan et al., 1995; Fritz et al., 1998), and bovine (Usdin et al., 1991; Lingen et al., 1994). Recently, a peripheral epinephrine transporter (fET) was characterized in the bullfrog *Rana catesbiana* (Apparsundaram et al., 1997), and a single catecholamine transporter (ceDAT) was found in the genome of the nematode *Cænorhabditis elegans* (Jayanthi et al., 1998) and the arthropod *Drosophila melanogaster* (Pörzgen et al., 2001). Thus, it is possible that the simultaneous presence of DAT and NET is not a conserved character in all Bilateria (including vertebrates). In particular, DAT and NET expression may depend on specific gene duplications and the differentiating mechanisms of the corresponding neurons. The presence or absence of one of these transporters may have strong influence on the effects of catecholamines in the nervous system of a particular species.

To examine in more detail the role of catecholamine transporters in the vertebrate brain, we chose to search and characterize them in the teleost fish medaka (*Oryzias latipes*). The medaka is becoming a popular vertebrate model (Ishikawa, 2000), in addition to the widely used zebrafish (*Danio rerio*). It is an easy-to-breed, small, aquarium fish with transparent embryos. It is suitable for large-scale mutagenesis, and its genome is certainly less redundant than that of zebrafish. An important reason to study a ray-finned fish is that this vertebrate group has evolved independently from the sacropterygians, the lineage that led to tetrapods and includes mammals. Thus, the characteristics shared by ray-finned fishes and tetrapods are likely to be ancestral and will serve as a basis for analyzing changes in the roles of catecholamine transporters in vertebrates.

The brain anatomy and catecholaminergic systems of several teleost species have been studied extensively (Ekström et al., 1986; Roberts et al., 1989; Ekström et al., 1990; Sas et al., 1990; Corio et al., 1991; Meek, 1994), including medaka (Kapsimali et al., 2001). These studies show that despite the everted telencephalic development of the teleost brain, the anatomy of catecholaminergic nuclei and pathways are highly conserved compared with mammals (Smeets and Reiner, 1994; Reiner et al., 1998). Consequently, it seems very attractive to examine, in a comparative perspective, the nature, characteristics, and localization of the catecholamine transporters in fishes.

Materials and Methods

Chemicals. [^{3}H]Dopamine (42 Ci/mmol) and [^{3}H]norepinephrine (39 Ci/mmol) were purchased from Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [^{125}I]RTI-55 (2200 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA). Dopamine, norepinephrine, nortriptyline, nomifensine, desipramine, fluoxetine, and citalopram were obtained from Sigma/RBI (Natick, MA). Cocaine was kindly provided by Dr. M.-H. Thiebot (Paris, France), and *d*- and *l*-amphetamine were kindly provided by Dr. C. Pifl (Vienna, Austria).

Cloning of meNET. A cDNA probe from the rat DAT coding sequence was obtained by PCR amplification of the plasmid TS3-pCMV5 (Giros et al., 1991) with the primers 5′-CCTGCTATCAGTCATCGGCTTTGC and 5′- AGCAGAACAATGACCAGCACCAGG, which correspond to nucleotides 207 to 230 and 749 to 726 of the rat DAT sequence and flank the large extracellular loop. The PCR amplification was run at 94°, 54°, and 72°C for 30 cycles (1 min each). Plaques (700,000) from a cDNA λZAPII library prepared from total brain of medaka (Joly et al., 1997) were transferred onto nitrocellulose filters (BAS85; Schleicher & Schuell, Dassel, Germany) and prehybridized at 42°C for 3 h in 30% formamide, 1× Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 0.1% ficoll), 0.01% SDS, 20 mg/ml salmon sperm DNA, 20 mg/ml yeast tRNA, 20 mM Tris-HCl, pH 7.4, and 4× SSC (60 mM, Na_3 citrate, 0.6 M NaCl, pH 7.0). Hybridization was carried out overnight at 42°C in the same buffer containing also 10% dextran sulfate and 5×10^5 cpm/ml of the nick-translated PCR probe (labeled with [^{32}P]dATP and [^{32}P]dCTP at a specific activity of 1 to 2×10^9 cpm/mg of template). The filters were washed twice for 20 min in 2× SSC, 0.1% SDS at 42°C, and twice for 20 min in 0.2× SSC, 0.2% SDS at 42°C. Positive clones were plaque-purified after a second round of enrichment, and the pBluescript-containing fragments were excised from the phages and sequenced in both orientations by the dideoxynucleotide chain-termination method using an automated sequencer (ABI Prism 377; Applied Biosystems, Foster City, CA).

Transfection of COS-7 Cells and Uptake Experiments. A full-length *Eco*RI fragment from a positive clone was subcloned in the *Eco*RI site of the pRc/CMV expression vector (Invitrogen, Carlsbad, CA) and sequenced to confirm the proper orientation. COS-7 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal calf serum (Valbiotech, Paris, France), and 100 Units/ml penicillin-streptomycin (Invitrogen) at 37°C and 7% CO_2. The cells were transfected by the phosphate-calcium method using 5 to 10 μg of a cesium chloride-prepared plasmid. One day after transfection, cells were plated in 24-well dishes, and uptake experiments were performed 72 h after transfection, as described previously (Giros et al., 1992). For the determination of IC_{50} values, uptake assays were performed for 10 min in the uptake buffer (5 mM Tris base, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, and 5 mM D-glucose, final pH, 7.4) at 37°C using 20 nM [^{3}H]DA with competitors added 5 min before. For determination of K_M and V_{max} values, uptake assays were performed with 20 nM [^{3}H]DA diluted with increasing concentrations of unlabeled DA (20–30 mM). Nonspecific [^{3}H]DA uptake was determined in the presence of 10 mM nomifensine (Giros et al., 1992). Assays were terminated by rapid removal of the supernatant followed by two successive washes with ice-cold uptake buffer. Cells were lysed in 0.5 ml of 0.1 M NaOH, and the radioactivity was quantified by direct liquid-scintillation counting. Calculations of V_{max}, K_M, and IC_{50} values were performed as described previously (Giros et al., 1992). All experiments were carried out in triplicate.

In Situ Hybridization cDNA Probes. A meNET fragment of 680 base pairs was amplified by PCR (HiTaq; Bioprobe, Montreuil sous Bois, France) on meNET cDNA using two specific primers

(sense 5′-AAAGGTGTGGGCTACGCTGT and antisense 5′-TTTTGAGCCATGTATCCCAG) and subcloned into the pCRII vector (Invitrogen). A medaka tyrosine hydroxylase fragment of 388 base pairs was amplified by PCR (Promega, Charbonnières, France) on medaka adult brain cDNA by using two oligonucleotides (antisense 5′-CGTGCCTTCCGYGTGTTCCAGTG and sense 5′-CTGGTAGKTCTGGTCYTGGTAGGGCT) and subcloned in the pCRII vector. In both cases, plasmid DNA were digested by appropriate restriction enzymes and transcribed with T7 RNA polymerase from the corresponding promoter. Probes were labeled by adding digoxigenin-UTP to the RNA synthesis reaction medium, and all other nucleotides were unlabeled and present in excess, according to the manufacturer's protocol (Roche Molecular Biochemicals, Meylan, France). Sense probes transcribed from the SP6 promoter were used as negative controls.

Tissue Preparation and In Situ Hybridization. Thirty medaka fish were killed by immersion in ice-cold water and fixed overnight in 4% paraformaldehyde in phosphate buffer. The brains were dissected, postfixed for 1 h in the same fixative, and kept in methanol at −20°C at least for 2 h before the in situ hybridization experiments. Whole medaka brains were processed for in situ hybridization according to the methods used by Joly et al. (1997), except that the duration of the proteinase K treatment was extended from 30 to 45 min to ensure efficient permeabilization of the tissue. After the final step (alkaline phosphatase reaction), the brains were postfixed for 15 min in PAF 4%, washed in PBS, dehydrated, and wax-embedded. Serial sections (8 μm) were prepared in the transverse, sagittal, and horizontal planes. They were counterstained with nuclear-fast red and photographed using a DMRD microscope (Leica, Wetzlar, Germany).

Reverse Transcriptase-Polymerase Chain Reaction Experiments. Total RNA was extracted from the medaka telencephalon and rat brain using the acid/guanidium method (Chomczynski and Sacchi, 1987) and treated with DNase, recovered by phenol-chloroform extraction, and ethanol. Reverse transcriptase (RT)-PCR was performed according to the manufacturer's instructions using the Access RT-PCR system (Promega). The primers used (sense 5′-TGCTACAARAAYGGHGGHGGTGCC and antisense 5′-CCYTTCCAKAGGCTRAARTA) flanked the large second extra-cytoplasmic loop. The RT-PCR products were inserted into PCRII.1 vector (Invitrogen). Competent cells were transformed according to the manufacturer's instructions. The clones were screened by restriction analysis, and those bearing the estimated 530-base-pair product were sequenced.

In Vitro Autoradiographic Binding of the Cocaine Analog [^{125}I]RTI-55. Medaka brains were extracted, fixed overnight with 0.1 M phosphate buffer, pH 7.4 (1× PBS) containing 4% paraformaldehyde, rinsed twice with 1× PBS, immersed in 30% sucrose for 48 h, frozen in cold isopentane (−40°C), and kept at −80°C until used. Slide-mounted tissue sections (15 μm) were thawed and brought to equilibrium in sucrose buffer (320 mM sucrose, 10 mM sodium phosphate, 10 mM sodium iodide, pH 7.4) for 10 min at room temperature. The sections were then incubated in the absence or presence of cocaine (50 μM), desipramine (100 nM), citalopram (100 nM), or fluoxetine (10 μM) for 20 min before the addition of [^{125}I]RTI-55 (50 pM) for 60 min at room temperature. Free and nonspecifically bound [^{125}I]RTI-55 was removed by washing the sections twice for 20 min in ice-cold sucrose buffer, twice in water for 5 s, and once in 20% ethanol for 10 s. The sections were dried under a cool stream of air and exposed to a β-max film (Amersham Pharmacia Biotech).

Synaptosome Preparation and Uptake Experiments. Synaptosomes were prepared according to the method described by Javitch et al. (1985) with minor modifications. Ten medaka fish brains were dissected at 4°C and homogenized in 1 ml of ice-cold sucrose buffer (sucrose 0.32 M, 1 mM EDTA, and 10 mM Tris-HCl) in a tapered grinder with Teflon pestle (Kontes Glass, Vineland, NJ). The homogenate was diluted (1:3) in sucrose buffer and centrifuged at 1000*g* for 5 min. The supernatant was collected and the pellet was resuspended in 3 ml of sucrose buffer and centrifuged again at 1000*g* for 5 min. The supernatants were pooled and centrifuged at 12,000*g* for 25 min. This pellet was resuspended in 1 ml of sucrose buffer and used for uptake experiments. The uptake buffer was the same as the one used for uptake into cells (except when 120 mM LiCl was used to substitute NaCl). The experiments were performed in 500 μl, comprising 25 μl of the synaptosome suspension, 4 nM [^{3}H]DA (or alternatively 5 nM [^{3}H]5-hydroxytryptamine when specified) (Amersham Pharmacia Biotech), and various uptake inhibitors as indicated and then preincubated for 5 min before the radiolabeled substrates. The dose-response curve for DA uptake was obtained by isotopic dilution with unlabeled DA mixed with [^{3}H]DA. After an incubation of 10 min at 37°C, the uptake was stopped by the addition of 3 ml of ice-cold buffer and immediate filtration through GF/B glass-fiber filters presoaked in 0.05% polyethylenimine (Whatman, Clifton, NJ). The filters were washed twice with 3 ml of ice-cold buffer, and the radioactivity was quantified by direct liquid-scintillation counting. Nonspecific [^{3}H]DA binding on filters (5–6% total) was systematically subtracted. Total uptake (100%) in the presence of Na^+Cl^- buffer was 3153 ± 258 cpm of [^{3}H]DA for 10 min.

Results

Low stringency screening of 700,000 plaque-forming units of a medaka brain cDNA library with a rat DAT cDNA probe led to the isolation of 19 clones that were purified and sequenced. Among them, six overlapping clones corresponded to the same partial or complete coding region that strongly resembles the mammalian DAT and NET. The other isolated clones all shared similarities with various subtypes of the GABA transporter family; 5, 2, 3, and 3 clones appeared orthologous to the rat GAT1, GAT2, GAT3, and BGT, respectively.

The longest DAT- and NET-related sequence was 3.3 kilobase pairs long and contained an open reading frame of 1875 base pairs encoding a 625-amino-acid protein, thereafter called meNET (Fig. 1). The meNET sequence displays 12 hydrophobic segments that correspond to the putative TMD characterizing the members of the Na^+/Cl^--dependent transporter family. The sequence of meNET bears five putative N-linked glycosylation sites (N42, N192, N200, N207, and N304). The consensus site of the N-terminal tail (N42) is unlikely to be used, because it is anticipated to be located in the cytoplasmic compartment. Among the three consensus glycosylation sites located in the large extracellular loop between TMD3 and TMD4, two sites (N192 and N200) are highly conserved in other vertebrate catecholamine transporters (Melikian et al., 1996), suggesting that they will also be used in meNET (Fig. 1). In addition, an N-glycosylation site in EL3 is unique to meNET. Three possible phosphorylation motives for protein kinase C (S267, S587, and T593) are found in the meNET sequence as well as one consensus site for phosphorylation by cAMP-dependent protein kinase A (S13). Like the already known NETs, meNET also possesses a leucine zipper motif within TMD2 (L103-L122; Fig. 1). This leucine zipper also exists in DATs (Giros and Caron, 1993), in which the second leucine of the motif is substituted with another hydrophobic residue (methionine). This motif has been implicated in protein-protein interactions, but no experimental evidence is currently available concerning its role in the function of Na^+/Cl^--dependent neurotransmitter transporters. The regions encompassing TMD5 to TMD8 correspond to the tricyclic antidepressant binding domain of catecholamine transporters (Giros et al., 1994; Buck and

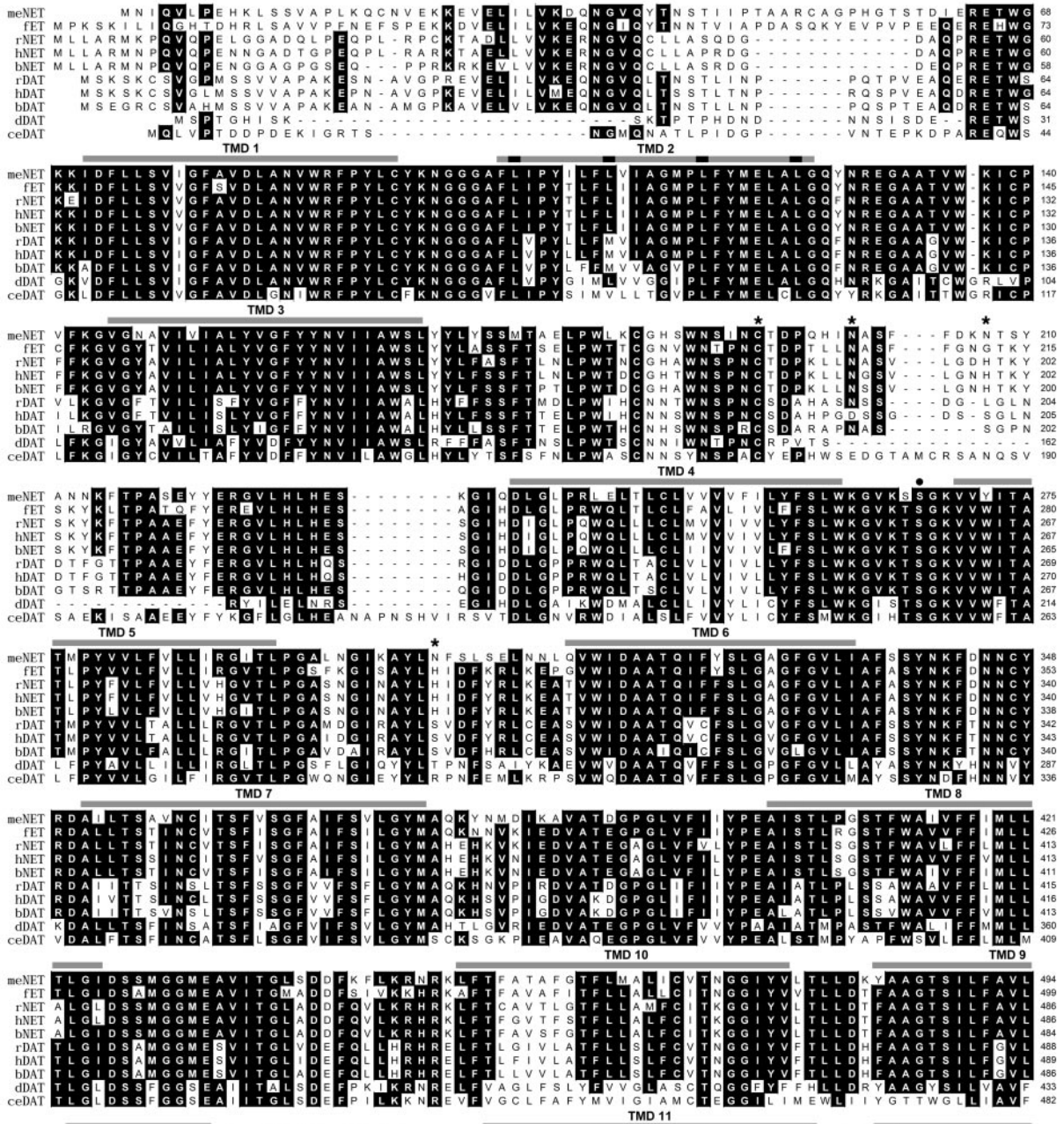

Fig. 1. Alignment of deduced amino acid sequence of meNET with other members of the catecholamine transporter gene family. Alignment was performed by using amino acid sequences of the cloned meNET (this study), frog ET (fET; Apparsundaram et al., 1997), human NET (hNET; Pacholczyk et al., 1990), rat NET (rNET; Brüss et al., 1997), bovine NET (bNET; Lingen et al., 1994), bovine DAT (bDAT; Usdin et al., 1991), rat DAT (rDAT; Giros et al., 1991), human DAT (hDAT; Giros et al., 1992), *D. melanogaster* DAT (dDAT; Pörzgen et al., 2001), and *C. elegans* DAT (ceDAT; Jayanthi et al., 1998). Consensus sequence for PKC (●), leucine zipper motif (■) and N-linked glycosylation sites (*) are shown. A continuous bar (▨) is located on top of the putative transmembrane domains. White-lettered residues on a black background are strictly conserved for at least 6 of 10 sequences. The PKC site in IL2 (S267) as well as two glycosylation sites in EL3 (N192 and N200) are highly conserved among all the cloned catecholamine transporters.

Amara, 1995). Three positions, F331, S355, and I369, are conserved among all tricyclic-sensitive transporters (meNET, NETs, fET, SERTs, dDAT, and ceDAT) but differ only in mammalian DATs and are substituted by Cys, Thr, and Val, respectively. The mutation from Phe to Cys in this position of the hNET has been shown recently to decrease the affinity of tricyclic antidepressants (Roubert et al., 2001).

Alignment of the meNET amino acid sequence with those of the other known monoamine transporters (Fig. 2A) indicates that meNET mostly resembles the fET sequence (72% identity) cloned from the frog *R. catesbiana* (Apparsundaram et al., 1997). In fact, meNET is related more closely to the NET subfamily than to the mammalian DAT, as illustrated in the phylogenetic tree shown in Fig. 2B. Bootstrap values also support this contention (100 for the branching separating DAT subfamily from the NET group, which contains the medaka NET). Although the number of species studied is still small, it is possible that DAT sequences exhibit a larger apparent sequence divergence than NET sequences (note the difference in phylogenetic distances between human and bovine for the NET and the DAT sequences). This accounts for the deeper branching of DAT, indicating that vertebrate DAT sequences did not diverge before NET in vertebrate evolution; instead, they diverged faster. Incidentally, the three monoamine transporter subfamilies (DAT, NET, and SERT) are clearly individualized from each other and from the GABA transporters. In this respect, it should be noted that the so-called DAT from *C. elegans* and *D. melanogaster* branched at the basis of the catecholamine transporter group and cannot be assigned either to the NET or the DAT groups.

To assess the functional characteristics of the cloned meNET cDNA, we directed its expression into COS-7 cells. Cells transfected with meNET cDNA were able to mediate the uptake of [^{3}H]DA and [^{3}H]NE with high affinity in a saturable manner and with a first-order kinetics, whereas in mock transfected cells, no specific accumulation of catecholamines could be detected. These experiments indicate that meNET was able to transport DA (K_M = 290 nM) with a higher affinity than NE (K_M = 640 nM). The meNET displayed similar capacities for DA (V_{max} = 1354 ± 308 fmol/min/10^{5}cells) and NE (V_{max} = 860 ± 150 fmol/min/10^5 cells; Fig. 3). These properties are comparable with those displayed by human or rat NETs, which share a higher affinity for DA but a lower capacity than DAT itself (Table 1).

The ability of various inhibitors of catecholamine transport to block the uptake of [^{3}H]DA was assessed in COS-7 cells transfected with either rDAT, hNET, or meNET (Table 2). We found that meNET exhibited a pharmacological pattern similar to that displayed by DAT and NET for the drugs that block the two catecholamine transporters: cocaine (IC_{50} = 169 ± 42 nM), *d*-amphetamine (IC_{50} = 150 ± 18 nM), benztropine (IC_{50} = 85 ± 23 nM), and nomifensine (IC_{50} = 54 ± 18 nM). In contrast to DAT, meNET was inhibited by nanomolar concentrations of the tricyclic antidepressants desipramine (IC_{50} = 0.92 ± 0.36 nM), nortriptyline (IC_{50} = 16 ± 4 nM), and by the NET-specific compound nisoxetine (IC_{50} = 2.6 ± 1.25 nM). Furthermore, we found no significant differences for meNET inhibition by the *d*- and *l*-stereoisomers of amphetamine as observed with hNET, whereas they displayed stereospecificity toward rDAT. In addition, both the serotonin transporter inhibitor fluoxetine (IC_{50} = 1070 ± 90 nM) and serotonin (IC_{50} > 12,000 nM) showed weak inhibition of meNET-mediated DA uptake (Table 2). Therefore, these pharmacological experiments undoubtedly classified the medaka catecholamine transporter-like sequence as a NET and not a DAT, further justifying its naming.

In mammals, the cellular localization of the transporter transcripts is certainly a better criterion than pharmacology to assign a sequence to the DAT or NET family. The distribution of meNET mRNA was analyzed by in situ hybridization in the medaka brain and compared with the localization of the medaka tyrosine hydroxylase (TH) transcripts. The localization of meNET mRNA was strikingly restricted to a few distinct nuclei of the isthmus and hindbrain (Fig. 4). The midbrain and forebrain were completely devoid of meNET labeling, whereas TH transcripts were detected in numerous nuclei all along the anteroposterior axis of the brain (Fig. 4, A–C). Labeling of the meNET transcripts was observed exclusively in a limited subset of TH-positive cells (Fig. 4). The more anterior meNET-positive nucleus was the locus ceruleus, where all the cells seemed to be labeled by both the meNET and the TH cRNA probes (Fig. 4, D and E). More posteriorly, meNET labeling was observed in a few cells of the nucleus of the solitary tract and in the nucleus of the vagus nerve (Fig. 4F). A strong labeling by both probes was also present in a group of dispersed cells (Fig. 4, F and G) found among the medial longitudinal fascicle, the more lateral descending trigeminal root, and the lateral longitudinal fascicle. Ma (1997) has referred to this group in zebrafish as the interfascicular catecholaminergic neurons. A strong labeling was also observed in the area postrema, but in this area, the number of cells labeled with the meNET probe was approximately half the number of cells labeled with the TH probe (Fig. 4, H and I). Finally, more ventrally, the meNET mRNA was detected in a few cells of the reticular formation.

The striking discrepancy between the distribution of the TH and meNET mRNAs could reflect the presence of an additional catecholamine transporter, which would be present at least in some of the TH-positive and meNET-negative neurons. In particular, the mammalian DAT has been found in the mesencephalic dopaminergic areas, but no labeling with the meNET probe was detected in the midbrain and forebrain in medaka fish. Therefore, to determine whether a DAT-like transporter exists in the medaka brain, we performed a series of additional experiments to specifically address this issue.

We first carried out RT-PCR with degenerate primers designed to recognize all the vertebrate DAT or NET sequences, including meNET. These primers were located in the most conserved regions of DAT and NET (in TMD3 and TMD5), but the resulting PCR product can be easily characterized by the nonconserved second extracellular loop between TMD3 and TMD4. These primers were able to amplify sequences corresponding to rDAT and GAT3 using total rat telencephalon mRNA. They were used to amplify related sequences from mRNA extracted from the whole medaka brain, but also from dissected areas of the forebrain, midbrain, and hindbrain. We reasoned that if a DAT-related transporter exists in the medaka brain, it would be present in the dopaminergic areas anterior to the isthmus. Forty-seven clones were isolated from these experiments. Nine of them were identical to meNET, but no other sequence resembling an additional catecholamine transporter was detected (data not shown).

A

Percent Identity

Divergences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		72.8	71.0	69.9	71.5	65.0	65.1	62.4	51.6	44.1	48.9	45.8	45.9	38.1	39.5	37.0	40.5	40.1	35.7	meNET
2	33.6		74.7	73.4	75.4	65.3	62.8	62.1	52.1	43.6	48.7	46.0	45.2	37.7	39.9	36.8	40.8	40.7	36.0	fET
3	34.7	28.2		93.2	93.0	65.2	65.0	64.3	54.0	44.2	49.7	46.0	45.1	38.7	40.0	37.4	41.8	41.7	37.4	hNET
4	36.5	30.4	7.1		90.7	64.3	64.0	63.2	54.0	44.4	49.9	45.1	44.2	37.3	39.0	36.6	41.0	41.2	37.8	rNET
5	33.8	27.6	7.0	9.4		64.2	63.9	63.3	53.6	42.9	50.1	45.5	44.7	38.5	39.9	36.7	41.3	42.1	37.2	bNET
6	44.8	44.0	43.3	45.6	45.6		92.4	86.4	50.5	42.1	48.0	44.4	43.7	38.4	38.7	35.6	40.8	41.7	36.3	hDAT
7	44.9	44.7	44.2	46.6	46.3	7.5		85.1	51.6	42.6	48.0	43.8	43.1	38.6	39.5	35.9	41.3	41.6	36.7	rDAT
8	47.5	47.5	44.7	46.5	47.1	15.1	16.3		49.1	40.7	47.0	43.9	43.6	38.4	39.2	36.0	40.3	41.4	36.6	bDAT
9	68.3	69.4	64.2	63.1	64.2	70.8	68.3	73.6		53.5	48.6	48.1	47.6	41.3	43.1	41.7	42.0	41.1	41.0	dDAT
10	87.7	89.1	85.7	84.8	89.7	93.1	91.3	96.1	64.3		42.5	40.3	39.5	35.0	36.4	34.3	35.8	35.7	34.1	ceDAT
11	74.5	73.9	71.0	70.5	71.0	74.7	76.1	77.7	72.2	90.9		52.0	50.8	38.6	38.7	40.1	41.5	42.2	39.4	dSERT
12	81.9	80.7	80.5	82.4	81.0	85.1	88.0	85.9	78.2	97.9	63.9		89.5	37.7	39.0	37.6	37.0	38.1	35.6	hSERT
13	81.2	81.8	83.0	84.8	84.4	87.6	88.6	87.9	81.5	99.3	67.4	10.6		37.6	38.5	36.4	36.1	36.1	34.8	rSERT
14	99.9	101.1	100.9	104.9	101.5	102.1	102.7	101.9	96.5	115.0	101.1	102.3	107.5		66.3	62.8	46.5	47.9	37.6	rBET/GAT
15	97.3	97.8	93.8	97.6	93.8	102.3	101.1	98.0	92.1	114.4	98.7	98.0	103.9	41.4		66.3	47.2	47.9	39.2	rGAT2
16	106.3	106.1	105.8	107.0	106.5	113.4	112.8	111.3	99.0	122.0	97.2	102.8	112.1	48.1	42.1		49.0	49.7	36.5	rGAT3
17	98.9	96.0	95.2	96.8	94.2	99.2	98.6	100.4	96.7	115.4	91.0	109.3	111.9	81.2	76.0	78.5		83.4	40.3	torGAT
18	100.7	97.7	93.0	94.6	92.0	95.8	96.4	94.8	97.3	118.2	89.1	110.8	118.2	79.6	75.6	77.0	18.1		40.4	rGAT1
19	109.5	108.9	102.0	99.7	104.5	105.8	106.2	105.1	97.0	120.2	107.9	112.0	115.4	102.4	99.5	104.6	98.3	96.2		rGLYT1a
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	

B

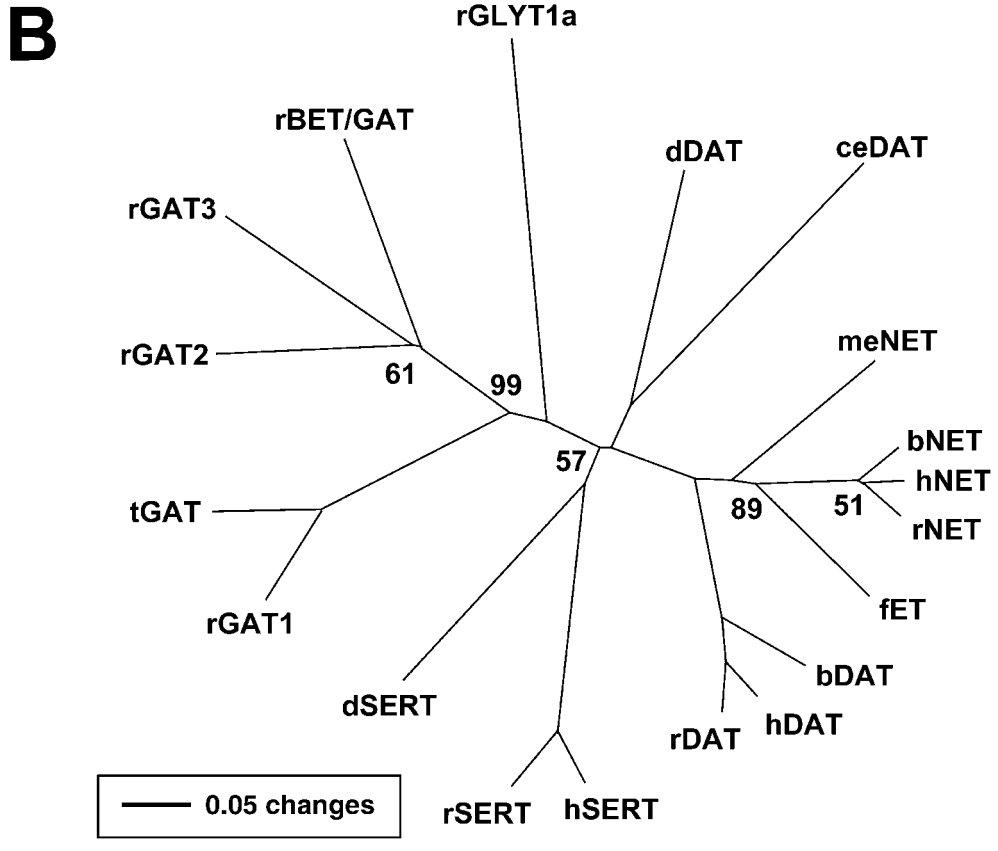

Fig. 2. Phylogenetic relationships of meNET with other members of the catecholamine transporter family. b, bovine; h, human; r, rat; ce, *C. elegans*; f, bullfrog *R. Catesbiana*; d, arthropod *D. melanogaster*; and t, *Torpedo marmorata*. A, identities shared among catecholamine transporters cloned in different species. The values are expressed as the percentage of identical amino acids. B, tree of the phylogenetic distances calculated by the Neighbor Joining Method from an optimized alignment of the amino acid sequences of representative Na^+/Cl^--dependent neurotransmitter transporters. Numbers correspond to the bootstrap values (occurrence of the presented branching after 100 iterations). Unless indicated, these values are equal to 100. Sequences for DAT and NET are those described in the legend to Fig. 1. Other sequences are from cloned rat betaine/GABA transporter (rBET/GAT; Burnham et al., 1996), rat GAT-1 (rGAT1; Guastella et al., 1990), rat GAT-2 (rGAT2; Borden et al., 1992), rat GAT-3 (rGAT3; Clark et al., 1992), *T. marmorata* GAT (tGAT; Guimbal and Kilimann, 1994), and *C. elegans* GAT (CAA98519).

Furthermore, we looked for specific ligand binding on tissue sections. We used as radioligand [^{125}I]RTI-55, an iodinated cocaine analog that exhibits similar affinity for DAT and SERT but has a 10-fold lower affinity for NET in mammals (Eshleman et al., 1999). In coronal sections of the medaka brain, a strong binding of [^{125}I]RTI-55 was observed in preoptic, thalamic regions, and other diencephalic areas, as well as in the dorsal midbrain and more posterior regions of the medaka brain (Fig. 5). However, no labeling was detected in the telencephalon. The observed labeling was totally displaced by 10 μM cocaine, an amine transporter inhibitor (data not shown). Fluoxetine 10 μM (Fig. 5, C and G) and desipramine 100 nM (data not shown) completely displaced [^{125}I]RTI-55 binding, and no remaining labeling could be observed, even after a long exposure time. These high doses of desipramine and fluoxetine should have been able to compete with SERT and NET but not with DAT. After incubation with 100 nM citalopram, a dose that should compete only with SERT but not with meNET or a putative DAT, a significant labeling remained only in subcortical structures (Fig. 5, B and F), which should represent specific meNET labeling. However, we had to use an overnight incubation of the medaka brain with 4% paraformaldehyde to preserve the tissue structures. Thus, we cannot totally exclude that such a treatment may differentially affect DAT binding compared with NET and SERT binding. The results of this experiment showed that apart from SERT and NET, no other cocaine-sensitive binding site could be found in the medaka brain, and thus, no DAT-like binding site was detected.

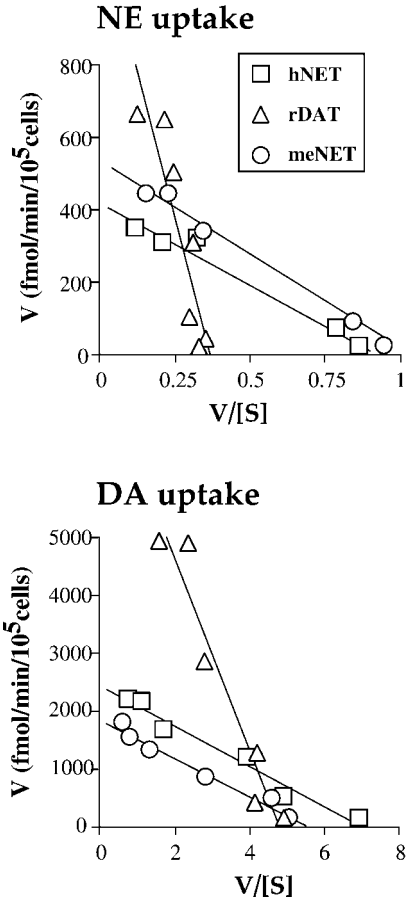

Fig. 3. Functional characteristics of meNET. Eadie-Hofstee representation of concentration dependence of NE and DA transport into COS-7 cells transiently transfected with meNET, hNET, or rDAT cDNA. One representative experiment is shown here. V is expressed in fmol/min/10^5 cells^{-1}.

TABLE 1.

Comparison of catecholamine transport kinetics for meNET, hNET, and rDAT

Transport of [^{3}H]DA and [^{3}H]NE were determined in COS-7 cells transfected with an expression vector containing either meNET, hNET, or rDAT cDNA, as described under *Materials and Methods*. Data are presented as mean ± S.E.M. of three to four independent experiments, each conducted in triplicate.

	Uptake of [^{3}H]NE		Uptake of [^{3}H]DA	
	K_M	V_{max}	K_M	V_{max}
	nM	*fmol/min/10^5 cells*	*nM*	*fmol/min/10^5 cells*
hNET	515 ± 75	530 ± 160	450 ± 65	1245 ± 620
rDAT	2370 ± 1330	1300 ± 80	1500 ± 290	7704 ± 5080
meNET	640 ± 90	860 ± 150	290 ± 32	1354 ± 300

To determine the pharmacological profile of the endogenous uptake of catecholamine in the medaka brain, synaptosomes were prepared to analyze [^{3}H]DA transport. The dose-response for DA indicates a saturable uptake (Fig. 6A). Eadie-Hofstee transformation (Fig. 6A, inset) gives a K_M value of 355 ± 80 nM. The [^{3}H]DA was mostly Na^+-dependent (80% of total uptake in the presence of LiCl) and inhibited by classic NET blockers (Fig. 6B). In fact, nisoxetine (1 μM), benztropine (10 μM), and desipramine (10 nM) were equally potent (80% inhibition), and cocaine (1 mM) was less potent (70% inhibition) in blocking the uptake of [^{3}H]DA detected in medaka synaptosome preparations (Fig. 6B). The dose-response inhibition with cocaine and desipramine gave IC_{50} values of 350 nM and 1.6 nM, respectively (data not shown). Citalopram (1 μM), a specific SERT inhibitor, could not displace the DA uptake by meNET. A high concentration of DA (1 mM) was able to decrease the [^{3}H]DA uptake by up to 90%, which was significantly lower than that observed in the absence of Na^+ (LiCl). The uptake of [^{3}H]5-HT was blocked (79%) by the addition of citalopram (data not shown), therefore confirming the presence of a SERT-like transporter as seen with [^{125}I]RTI-55 labeling. These data show that 80% of

TABLE 2.

IC_{50} values for inhibition of [^{3}H]DA uptake by meNET, hNET, or rDAT

IC_{50} values for inhibition of [^{3}H]DA uptake for various amine transporter inhibitor and substrates in COS-7 cells transiently transfected with meNET, hNET, or rDAT cDNA, as described under *Materials and Methods*. Data represent mean ± S.E.M. of three independent experiments, each conducted in triplicate.

Compound	IC_{50}		
	meNET	hNET	rDAT
		nM	
Cocaine	160 ± 42	91 ± 6	260 ± 22
Nomifensine	54 ± 18	N.D. (8)[a]	N.D. (60)[b]
Benztropine	85 ± 23	N.D.	N.D. (109)[b]
Fluoxetine	1,070 ± 97	255 ± 130	N.D.
Nisoxetine	2.6 ± 1.2	N.D. (6.2)[c]	N.D. (1945)[c]
Nortriptyline	16 ± 4	21 ± 2	12,300 ± 3,900
Desipramine	0.9 ± 0.4	1.2 ± 0.2	4,800 ± 1,100
d-Amphetamine	150 ± 20	66 ± 9	180 ± 70
l-Amphetamine	230 ± 100	35 ± 15	760 ± 180
Serotonin	12,800 ± 300	N.D. (>10,000)[a]	N.D. (>10,000)[b]

N.D., nondetermined.
[a] Pacholczyk et al. (1990).
[b] Giros et al. (1991).
[c] Buck and Amara (1995).

the total DA uptake was achieved in an Na^+-dependent and desipramine-sensitive manner, whereas the remaining 20% uptake did not fulfill the specific criteria of uptake by an Na^+-dependent transporter of the NET/GAT family.

Discussion

Molecular and Pharmacological Characteristics of meNET. A cDNA encoding a unique putative catecholamine

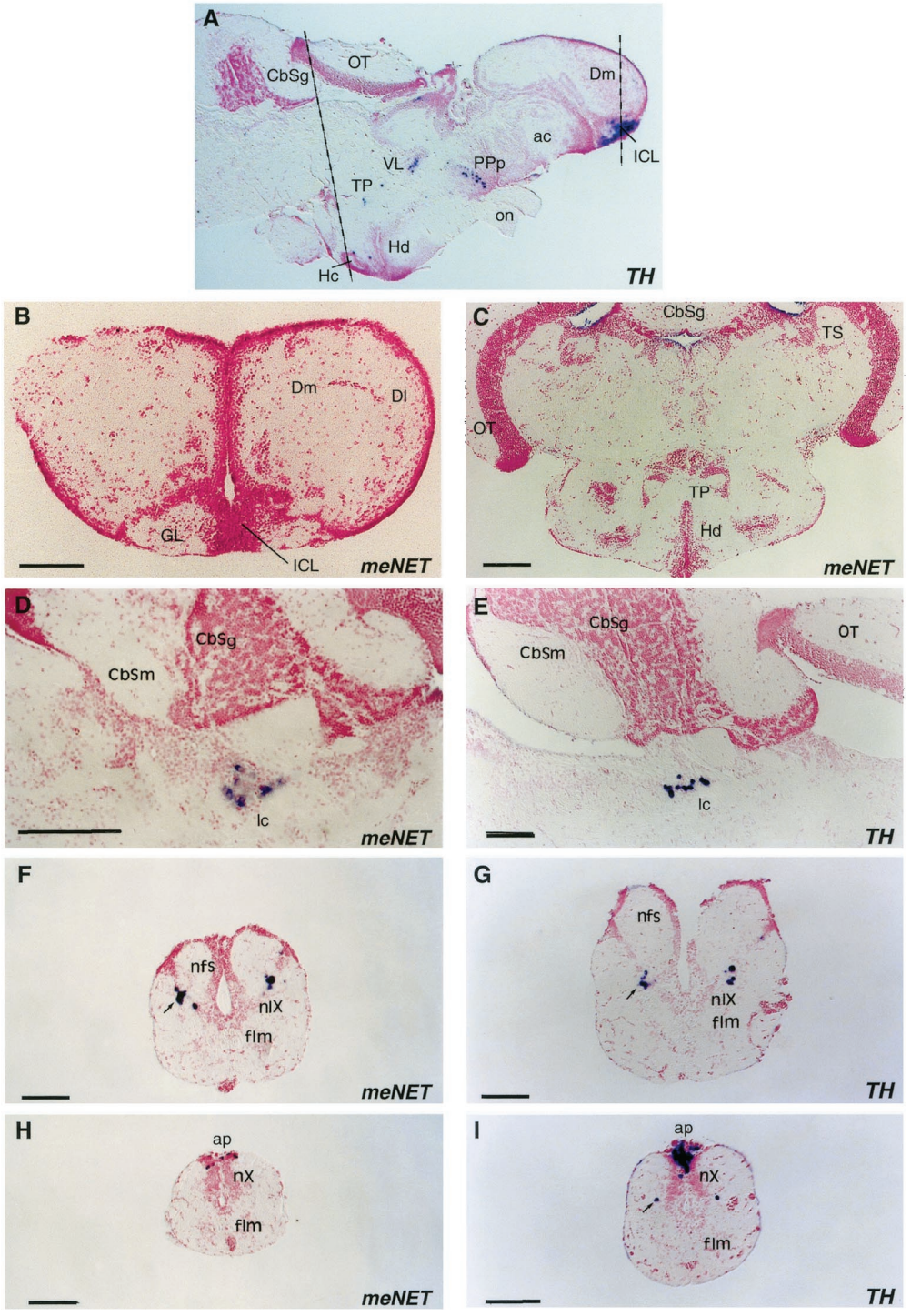

Fig. 4. Distribution of meNET mRNA in the medaka brain and comparison with TH transcript detected with digoxigenin-labeled probes. A, sagittal section (anterior on the right) showing TH transcripts concentrated in discrete areas along the anteroposterior axis of the brain, such as the internal cellular layer of the olfactory bulb (ICL), posterior preoptic parvocellular nucleus (PPp), ventrolateral thalamic nucleus (VL), nucleus of the periventricular posterior tuberculum (TP), dorsal (Hd), and ventral periventricular hypothalamus (Hv). Black lines correspond to the sections presented in B (anterior) and C (posterior), in which no meNET transcripts are detected, as opposed to TH mRNA. meNET (D) and TH (E) transcripts are detected in all the cells of the locus ceruleus (lc). More posteriorly, cells of the interfascicular catecholamine group (arrows) are labeled with meNET (F) and TH (G) probes, respectively. In the area postrema (ap), the number of cells labeled with the meNET probe (H) represents approximately 50% of those labeled with the TH probe (I). I, TH-positive cells (arrow) are located in the posterior part of the interfascicular catecholamine group.

transporter was isolated from a medaka brain cDNA library. The corresponding protein structure, its pharmacological and functional properties, and the transcript distribution suggest it to be a transporter for NE. The protein was thus named meNET, according to the accepted rules of transporter naming.

The cDNA screening performed at moderate stringency with a rDAT probe allowed the additional isolation of only GABA transporters as members of the Na^+/Cl^--dependent transporters; this raises the question of whether meNET was the only catecholamine transporter present in the fish brain. Although no firm conclusion has been reached, a large amount of converging evidence suggests that this would indeed be the case.

First, the primary amino acid structure of meNET is highly typical of the NET/GAT subfamily of Na^+/Cl^--dependent transporters. Indeed, meNET exhibits very high similarities with NET and DAT cloned in various species and a significant resemblance with the DAT cloned in *D. melanogaster* and *C. elegans*. Furthermore, meNET shares many characteristics with all members of the Na^+/Cl^--dependent neurotransmitter transporter family, such as a predicted 12 TMD structure, a large putative extracellular loop bearing multiple N-linked glycosylation sites (Pacholczyk et al., 1990), and a canonical aspartate residue (D83) in the first putative TMD (Kitayama et al., 1992). As in the other known NET and DAT sequences, consensus sites for phosphorylation by PKC and protein kinase A are conserved in the sequence encoded by the meNET cDNA, suggesting that it might be regulated by post-translational modifications. Treatments with phorbol esters and protein kinase C activators have been shown to affect transporter capacity via an internalization of the transporters (Kitayama et al., 1994; Huff et al., 1997; Zhang et al., 1997; Apparsundaram et al., 1998; Bönisch et al., 1998; Daniels and Amara, 1999). However, site-directed mutagenesis of PKC phosphorylation sites in NET (Bönisch et al., 1998) and DAT (C. Pifl, B. G., M. G. Caron, unpublished observations) has demonstrated that these sites are not directly responsible for the observed V_{max} decrease. Further experiments are therefore needed to demonstrate the strategic role of phosphorylation sites for transporter functions, such as membrane-addressing and translocation properties.

Pharmacological experiments clearly characterized this medaka catecholamine transporter as a vertebrate NET. These investigations indicated that like other NETs, meNET has a higher affinity (K_M) for DA than DAT itself (Buck and Amara, 1994). In addition, amphetamine shows stereoselective inhibition of DATs, whereas both stereoisomers have comparable affinity for all NETs (Giros and Caron, 1993; Pifl et al., 1996) and meNET (this study). Like mammalian NETs, meNET is sensitive to tricyclic antidepressants, and this characteristic is also shared by the catecholamine transporters cloned in the invertebrates *C. elegans* (Jayanthi et al., 1998) and *D. melanogaster* (Pörzgen et al., 2001), and the tetrapod *R. catesbiana* (Apparsundaram et al., 1997). Tricyclic antidepressants are also good blockers of the serotonin transporters cloned in mammals (Blakely et al., 1991; Ramamoorthy et al., 1993) and in *D. melanogaster* (Corey et al., 1994; Demchyshyn et al., 1994), but they are poor blockers of mammalian DATs (Giros et al., 1994; Buck and Amara, 1995). Thus, it is likely that tricyclic antidepressant binding is an ancestral property of the monoamine transporters that has been lost in DATs during the course of evolution (see Fig. 2B). All together, the structural and pharmacological features of meNET indicate that it belongs to the catecholamine transporter family, presenting shared properties with either DAT or NET proteins. However, despite some secondary characteristics shared with DATs, meNET is much more closely related to the NET family of transporters isolated in mammalian and nonmammalian species.

Brain Distribution of meNET. The localization of meNET mRNA in the medaka brain also strongly suggests that it is mainly a noradrenaline carrier. The in situ hybridization demonstrated that meNET mRNA is restricted to well-known catecholaminergic nuclei of the isthmus and hindbrain. The meNET mRNA is beyond detection levels in the numerous well-characterized dopaminergic nuclei of the olfactory bulb, hypothalamus, and in the nucleus of the pos-

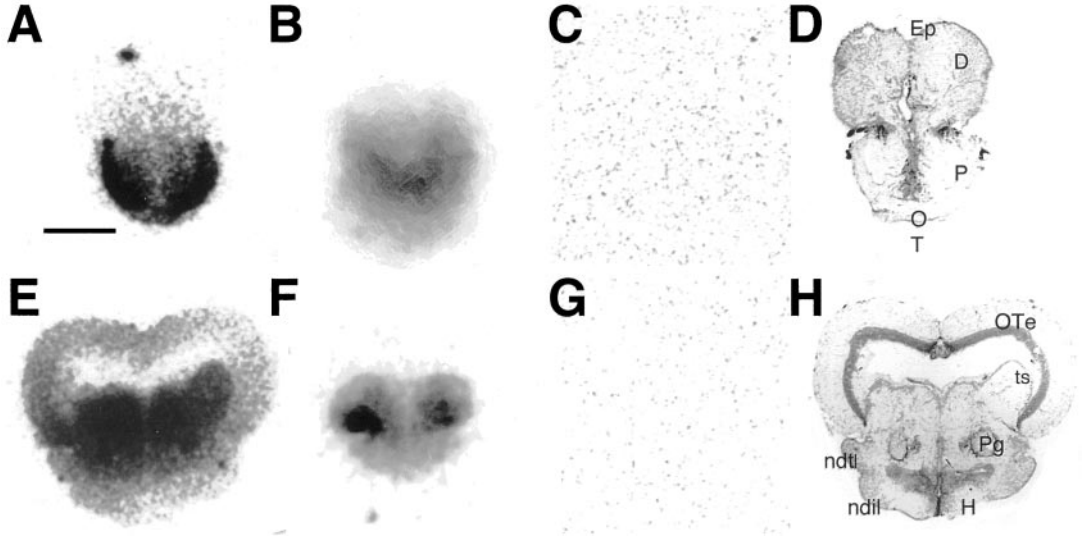

Fig. 5. Detection of [^{125}I]RTI-55 binding sites on medaka brain slices. A and E, [^{125}I]RTI-55; B and F, [^{125}I]RTI-55 + 100 nM citalopram; C and G, [^{125}I]RTI-55 + 10 μM fluoxetine; D and H, cresyl violet staining of the sections shown in A, B, C and E, F, G, respectively. A, strong labeling in the optic tract and in the diencephalic preoptic area is observed. E, strong labeling in some diencephalic areas, such as the posterior preglomerular complex, and in mesencephalic areas, such as the torus semicircularis. Scale bar represents 0.5 mm.

terior periventricular tuberculum, considered the diencephalic homolog in fish of the substantia nigra in tetrapod (Reiner and Northcutt, 1992; Kapsimali et al., 2001). In mammals, this latter nucleus presents the highest expression of DAT mRNA and a strong TH staining but is completely devoid of NET transcript as found in the medaka brain, using in situ hybridization. The fact that meNET transcripts were detected only by RT-PCR in the anterior brain further suggests that meNET mRNA expression is very weak in the anterior brain. Given the expression and pharmacological profile of meNET, there is little doubt that it transports NE in the noradrenergic synapses of the medaka brain. However, a role of this transporter in DA uptake is also probable in nuclei known to contain both DA-positive and NA-positive cell bodies, such as the area postrema and the interfascicular catecholaminergic neurons, as well as in all the anterior brain regions, which are innervated by noradrenergic fibers originating from the locus ceruleus. This possibility has already been proposed in the prefrontal cortex of the rat (Tanda et al., 1997; Yamamoto and Novotney, 1998). In addition, other high-capacity, low-affinity transport systems for monoamines exist in the fish brain. In particular, it has been shown that the paraventricular organ of the hypothalamus, the neurons of which are in contact with the cerebrospinal fluid, is able to concentrate very high levels of DA and other monoamines (Vigh and Vigh-Teichmann, 1998). Thus, the precise role of the several monoamine clearance systems in the fish brain needs to be more precisely investigated.

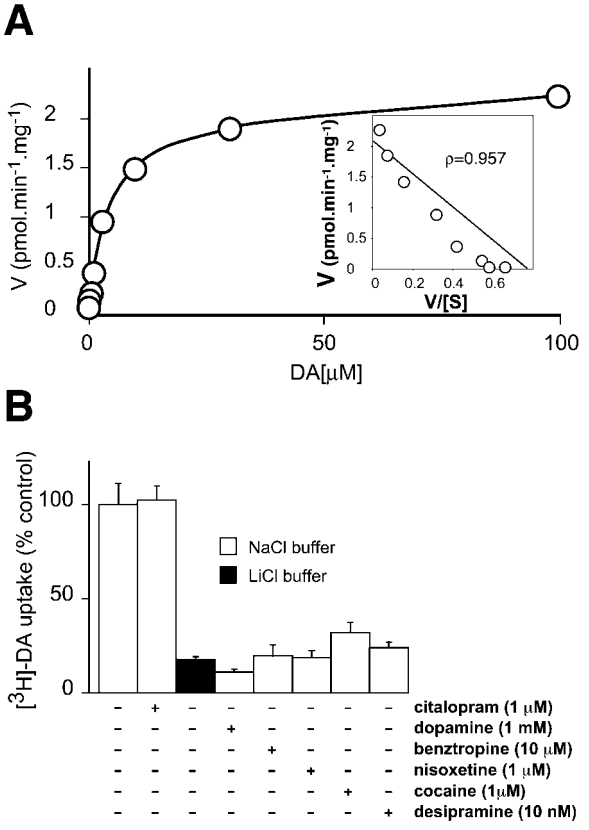

Fig. 6. Assessment of native [^{3}H]DA uptake from freshly prepared medaka brain synaptosomes. A, dopamine saturation curve and Eadie-Hofstee analysis. Mean value ± S.E.M. (n = 3) for K_M (nM) and V_{max} (pmol/min/mg protein) are 355 ± 80 and 2.1 ± 0.7, respectively. B, [^{3}H]DA uptake in the presence of various inhibitors and with substitution of NaCl by LiCl.

Is meNET the Unique Catecholamine Transporter in the Medaka Brain? The pharmacological characterization and anatomical data defined the medaka catecholamine transporter only as a bona fide NET. If the regulation of extracellular catecholamine levels in fish is comparable with that of mammals, a DAT should be present in the medaka brain. However, we were unable to provide any evidence of this.

First, neither cDNA cloning nor degenerate RT-PCR using low-stringency conditions, were able to reveal any additional catecholamine transporter besides meNET. This was also true for the anterior regions of the brain, which are devoid of meNET. It should be stressed that in both cases, we were able to identify GABA transporters (but not the serotonin transporter, in any experimental conditions), the primary sequences of which are more distant from NET than an expected DAT should be (see Fig. 2B). Although these experiments do not provide direct evidence for the absence of a DAT-like sequence in medaka brain, we can probably assume that, if present, it would be expressed at very low levels.

Second, we have shown that the pharmacological profile of [^{3}H]-DA uptake in synaptosomes prepared from medaka brain is very similar to what we observed with the cloned meNET transiently expressed in eukaryotic cells. Again, these findings suggest that, unless the hypothetical meDAT is pharmacologically close to meNET (mainly regarding the high affinity for tricyclic antidepressants), it is either absent or expressed at low levels.

Third, using [^{125}I]RTI-55 in autoradiographic experiments, we did not detect a DAT-like binding site (Fig. 5). A fair restriction to this conclusion would be to consider that such a DAT in medaka may either not bind [^{125}I]RTI-55 or display a high affinity for fluoxetine and desipramine, specific blockers of the mammalian SERT and NET. However, considering the remarkable conservation of the pharmacology of the amine transporters known to date in various species, this restriction may not apply. Thus, our results indicate that very few or no DAT-like binding sites are present in the medaka brain.

All these observations converge to the conclusion that if a meDAT exists, its expression level is lower than the detection abilities of these techniques. It may even suggest that meNET could be a unique catecholamine transporter in the medaka brain. Therefore, DAT will have emerged secondarily from this ancestor long after the divergence of teleost fish from the vertebrate phylum. Alternatively, some fish such as medaka may have lost the expression of a DAT, which exist in many other vertebrate groups. This hypothesis awaits isolation of catecholamine transporters from a large range of vertebrate species, but some evidence favors this contention. In the tree depicted in Fig. 2B, the phylogenetic distance is larger among DATs than in the other monoamine branches, indicating that DAT sequences diverge faster and supporting the fact that they are evolutionarily recent members of the monoamine transporter subfamily. This divergence correlates with significant changes in the transporter properties. DATs have gained more capacity while losing affinity for their specific substrate DA. Consistent with the late emergence of DAT is the fact that they have lost the sensitivity to tricyclic antidepressants exhibited by all the

known amine transporters, including those present in *C. elegans* and *D. melanogaster*.

In conclusion, whether DA neurons, which may be characterized as TH-positive, meNET-negative neurons, contain their proper transporter emerges as a major issue from our data. This is relevant not only to the phylogeny of this protein family, but also to the regulation of DA transmission in the mesencephalotelencephalic dopaminergic pathways in which DAT is concentrated in mammals.

The occurrence of fast and regulated uptake of DA in the nigrostriatal terminals of the limbic or striatal areas was certainly required by the massive increase in the number of dopaminergic cells that characterizes the mammalian nigrostriatal pathway compared with ray-finned fish or amphibians (Marín et al., 1998). In this case, the role of DA in several aspects of motivational processes or sensorimotor programming, but also the effect of addictive substances and many psychotropic drugs, will be rather specific for late-emerging vertebrates such as mammals. Elucidation of the evolutionary time when the duplication of the DAT/NET ancestor gene happened will be the next step in understanding the nature of the genetic mechanisms at the origin of this event, which has important physiological consequences.

Acknowledgments

We thank Dr. M. Hamon for providing a stimulating scientific environment and support, Dr. Bruno Gasnier for careful reading of the manuscript, and Dr. Catalina Betancur for her editorial work.

References

Amara SG and Kuhar MJ (1993) Neurotransmitter transporters: recent progress. *Annu Rev Neurosci* **16:**73–93.

Anken R and Bourrat F (1998) *Brain Atlas of the Medakafish* Oryzias latipes. INRA Editions, Paris, France.

Apparsundaram S, Galli A, DeFelice LJ, Harzell HC and Blakely RD (1998) Acute regulation of norepinephrine transport: I. Protein kinase C-linked muscarinic receptors influence transport capacity and transporter density in SK-N-SH cells. *J Pharmacol Exp Ther* **287:**733–743.

Apparsundaram S, Moore KR, Malone MD, Hartzell HC and Blakely RD (1997) Molecular cloning and characterization of an L-epinephrine transporter from sympathetic ganglia of the bullfrog *Rana catesbiana*. *J Neurosci* **17:**2691–2702.

Blakely RD, Berson HE, Fremeau RT, Caron MG, Peek MM, Prince HK and Bradley CC (1991) Cloning and expression of a functional serotonin transporter from rat brain. *Nature (Lond)* **364:**66–70.

Bockaert J and Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO (Eur Mol Biol Organ) J* **18:**1723–1729.

Bönisch H, Hammermann R and Brüss M (1998) Role of protein kinase C and second messengers in regulation of the norepinephrine transporter. *Adv Pharmacol* **42:** 183–186.

Borden LA, Smith KE, Hartig PR, Branchek TA and Weinshank RL (1992) Molecular heterogeneity of the gamma-aminobutyric acid (GABA) transport system. Cloning of two novel high affinity GABA transporters from rat brain. *J Biol Chem* **267:** 21098–21104.

Brüss M, Hammermann R, Brimijoin S and Bönisch H (1995) Antipeptide antibodies confirm the topology of the human norepinephrine transporter. *J Biol Chem* **270:**9197–9201.

Brüss M, Porzgen P, Bryan-Lluka LJ and Bönisch H (1997) The rat norepinephrine transporter: molecular cloning from PC12 cells and functional expression. *Mol Brain Res* **52:**257–262.

Buck KJ and Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* **91:**12584–12588.

Buck KJ and Amara SG (1995) Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. *Mol Pharmacol* **48:**1030–1037.

Burnham CE, Buerk B, Schmidt C and Bucuvalas JC (1996) A liver-specific isoform of the betaine/GABA transporter in the rat: cDNA sequence and organ distribution. *Biochim Biophys Acta* **1284:**4–8.

Chomczynski P and Sacchi N (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:**156–159.

Clark JA, Deutch AY, Gallipoli PZ and Amara SG (1992) Functional expression and CNS distribution of a beta-alanine-sensitive neuronal GABA transporter. *Neuron* **9:**337–348.

Comer AM, Qi J, L Christie D, Gibbons HM, Lipski J (1998) Noradrenaline transporter expression in the pons and medulla oblongata of the rat: localisation to noradrenergic and some C1 adrenergic neurones. *Mol Brain Res* **62:**65–76.

Corey JL, Quick MW, Davidson N, Lester HA and Guastella J (1994) A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc Natl Acad Sci USA* **91:**1188–1192.

Corio M, Peute J and Steinbusch HWM (1991) Distribution of serotonin and dopamine immunoreactivity in the brain of the teleost *Clarias gariepinus*. *J Chem Neuroanat* **4:**79–95.

Daniels GM and Amara SG (1999) Regulated trafficking of the human dopamine transporter: clathrin-mediated internalization and lysosomal degradation in response to phorbol esters. *J Biol Chem* **274:**35794–35801.

Demchyshyn LL, Pritsupa ZB, Sugamori KS, Barker EL and Blakely RD (1994) Cloning, expression and localisation of a chloride-facilitated, cocaine-sensitive serotonin transporter from *Drosophila megalonaster*. *Proc Natl Acad Sci USA* **91:**5158–5162.

Donovan DM, Vandenbergh DJ, Perry MP, Bird GS, Ingersoll R, Nanthakumar E and Uhl GR (1995) Human and mouse dopamine transporter genes: conservation of 5′-flanking sequence elements and gene structures. *Brain Res Mol Brain Res* **30:**327–335.

Ekström P, Honkanen T, Steinbusch HW (1990) Distribution of dopamine-immunoreactive neuronal perikarya and fibres in the brain of a teleost, *Gasterosteus aculeatus* L. comparison with tyrosine hydroxylase- and dopamine-beta-hydroxylase-immunoreactive neurons. *J Chem Neuroanat* **3:**233–260.

Ekström P, Reschke M, Steinbusch HW and van Veen T (1986) Distribution of noradrenaline in the brain of teleost *Gasterosteus aculeatus* L: an immunohistochemical analysis. *J Comp Neurol* **254:**297–313.

Eshleman AJ, Carmolli M, Cumbay M, Martens CR, Neve KA and Janowsky A (1999) Characteristics of drug interaction with recombinant biogenic amine transporters expressed in the same cell type. *J Pharmacol Exp Ther* **289:**877–885.

Freed C, Revay R, Vaughan RA, Kriek E, Grant S, Uhl GR and Kuhar MJ (1995) Dopamine transporter immunoreactivity in rat brain. *J Comp Neurol* **359:**340–349.

Fritz JD, Jahanthi LD, Thoreson MA and Blakely RD (1998) Cloning and chromosomal mapping of the murine norepinephrine transporter. *J Neurochem* **70:**2241–2251.

Giros B and Caron MG (1993) Molecular characterization of the dopamine transporter. *Trends Pharmacol Sci* **14:**43–49.

Giros B, El Mestikawy S, Bertrand L and Caron MG (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett* **295:**149–154.

Giros B, El Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T and Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. *Mol Pharmacol* **42:**383–390.

Giros B, Wang YM, Suter S, McLeskey SB, Pifl C and Caron MG (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J Biol Chem* **269:** 15985–15988.

Goridis C and Brunet JF (1999) Transcriptional control of neurotransmitter phenotype. *Curr Opin Neurobiol* **9:**47–53.

Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA and Kanner BI (1990) Cloning and expression of a rat brain GABA transporter. *Science (Wash DC)* **249:**1303–1306.

Guimbal C and Kilimann MW (1994) A creatine transporter cDNA from Torpedo illustrates structure/function relationships in the GABA/noradrenaline transporter family. *J Mol Biol* **241:**317–24.

Huff RA, Vaughan RA, Kuhar MJ and Uhl GR (1997) Phorbol esters increase dopamine transporter phosphorylation and decrease transport Vmax. *J Neurochem* **68:**225–232.

Ishikawa Y (2000) Medakafish as a model system for vertebrate developmental genetics. *Bioessays* **22:**487–495.

Javitch JA, D'Amato RJ, Strittmatter SM and Snyder SH (1985) Parkinsonism-inducing neurotoxin, *N*-methyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci USA* **82:**2173–2177.

Jayanthi LD, Apparsundaram S, Malone MD, Ward E, Miller DM, Eppler M and Blakely RD (1998) The *Caenorhabditis elegans* gene T23G5. 5 encodes an antidepressant- and cocaine-sensitive dopamine transporter. *Mol Pharmacol* **54:**601–609.

Joly JS, Bourrat F, Nguyen V and Chourrout D (1997) Ol-Prx 3, a member of an additional class of homeobox genes, is unimodally expressed in several domains of the developing and adult central nervous system of the medaka (*Oryzias latipes*). *Proc Natl Acad Sci USA* **94:**12987–12992.

Kapsimali M, Bourrat F and Vernier P (2001) Distribution of the orphan nuclear receptor Nurr1 in medaka (*Oryzias latipes*): cues to the definition of homologous cell groups in the vertebrate brain. *J Comp Neurol* **431:**276–292.

Kilty JE, Lorang D and Amara SG (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science (Wash DC)* **254:**578–579.

Kitayama S, Dohi T and Uhl GR (1994) Phorbol esters alter functions of the expressed dopamine transporter. *Eur J Pharmacol* **268:**115–119.

Kitayama S, Shimada S, Xu H, Markham L, Donovan DM and Uhl GR (1992) Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc Natl Acad Sci USA* **89:**7782–7785.

Lingen B, Brüss M and Bönisch H (1994) Cloning and expression of the bovine sodium- and chloride-dependent noradrenaline transporter. *FEBS Lett* **342:**235–238.

Ma PM (1997) Catecholaminergic systems in the zebrafish. III. Organization and projection pattern of medullary dopaminergic and noradrenergic neurons. *J Comp Neurol* **381:**411–427.

Marín O, Smeets WJAJ and González A (1998) Evolution of the basal ganglia in tetrapods: a new perspective based on recent studies in amphibians. *Trends Neurosci* **21:**487–494.

Meek J (1994) Catecholamines in the brains of Osteichthyes, in *Phylogeny and*

Development of Catecholamine Systems in the CNS of Vertebrates (Smeets W and Reiner A eds) pp 49–76, Cambridge University Press, London.
Melikian HE, Ramamoorthy S, Tate CG and Blakely RD (1996) Inability to *N*-glycosylate the human norepinephrine transporter reduces protein stability, surface trafficking, and transport activity but not ligand recognition. Mol Pharmacol **50:**266–276.
Nagatsu T and Stjarne L (1998) Catecholamine synthesis and release. Overview. *Adv Pharmacol* **42:**1–14.
Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ and Pickel VM (1996) The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J Neurosci* **16:**436–447.
Nishimura M, Sato K, Shimada S and Tohyama M (1999) Expression of norepinephrine and serotonin transporter mRNAs in the rat superior cervical ganglion. *Mol Brain Res* **67:**82–86.
Ordway GA, Stockmeier CA, Cason GW and Klimek V (1997) Pharmacology and distribution of norepinephrine transporters in the human locus coeruleus and raphe nuclei. *J Neurosci* **17:**1710–1719.
Pacholczyk T, Blakely RD and Amara SG (1990) Expression cloning of a antidepressant-sensitive human noradrenaline transporter. *Nature (Lond)* **350:**350–353.
Pifl C, Giros B and Caron MG (1996) The cloned target site of parkinsonism-inducing toxins and drugs of abuse. *Adv Neurol* **69:**235–238.
Pörzgen P, Park SK, Hirsh J, Sonders MS and Amara S (2001) The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Mol Pharmacol* **59:**83–95.
Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T, Chang AS, Ganapathy V and Blakely RD (1993) Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc Natl Acad Sci USA* **90:**2542–2546.
Reiner A, Medina L and Veenman CL (1998) Structural and functional evolution of the basal ganglia in vertebrates. *Brain Res Rev* **28:**235–285.
Reiner A and Northcutt RG (1992) An immunohistochemical study of the telencephalon of the senegal bichir (*Polypterus senegalus*). *J Comp Neurol* **319:**359–386.
Roberts BL, Meredith GE and Maslam S (1989) Immunocytochemical analysis of the dopamine system in the brain and spinal cord of the European eel, *Angilla angilla*. *Anat Embryol (Berl)* **180:**401–412.
Roubert C, Cox PJ, Brüss M, Hamon M, Bönisch M and Giros B (2001) Determination of residues in the norepinephrine transporter that are critical for tricyclic antidepressant affinity. *J Biol Chem* **276:**8254–8260.
Sas E, Maler L and Tinner B (1990) Catecholaminergic systems in the brain of gymnotiform teleost fish: an immunohistochemical study. *J Comp Neurol* **292:**127–162.
Shimada S, Kitayama S, Lin CL, Patel A, Nanthakumar E, Gregor P, Kuhar M and Uhl G (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science (Wash DC)* **254:**576–578.
Smeets W and Reiner A (1994) Catecholamines in the CNS of vertebrates: current concepts of evolution and functional significance, in *Phylogeny and Development of Catecholamine Systems in the CNS of Vertebrates*. (Smeets W and Reiner A eds) pp 463–481, Cambridge University Press, London.
Tanda G, Pontieri FE, Frau R and Di Chiara G (1997) Contribution of blockade of the noradrenaline carrier to the increase of extracellular dopamine in the rat prefrontal cortex by amphetamine and cocaine. *Eur J Neurosci* **9:**2077–2085.
Usdin TB, Mezey E, Chen C, Brownstein J and Hoffman BJ (1991) Cloning of a cocaine sensitive bovine dopamine transporter. *Proc Natl Acad Sci USA* **88:**11168–11171.
Valdenaire O and Vernier P (1997) G protein coupled receptors as modules of interacting proteins: a family meeting. *Prog Drug Res* **49:**173–218.
Vigh B and Vigh-Teichmann I (1998) Actual problems of the cerebrospinal fluid-contacting neurons. *Microsc Res Tech* **41:**57–83.
Yamamoto BK and Novotney S (1998) Regulation of extracellular dopamine by the norepinephrine transporter. *J Neurochem* **71:**274–280.
Ye W, Shimamura K, Rubenstein JL, Hynes MA and Rosenthal A (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93:**755–766.
Zhang L, Coffey LL and Reith EA (1997) Regulation of functional activity of the human dopamine transporter by protein kinase C. *Biochem Pharmacol* **53:**677–688.

Address correspondence to: Bruno Giros, INSERM U-513, Faculté de Médecine de Créteil 8, rue du Général Sarrail, F-94000 Créteil, France. E-mail: giros@im3.inserm.fr