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Ancestry of neuronal monoamine transporters in the Metazoa

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Summary

Selective Na^+-dependent re-uptake of biogenic monoamines at mammalian nerve synapses is accomplished by three types of solute-linked carrier family 6 (SLC6) membrane transporter with high affinity for serotonin (SERTs), dopamine (DATs) and norepinephrine (NETs). An additional SLC6 monoamine transporter (OAT), is responsible for the selective uptake of the phenolamines octopamine and tyramine by insect neurons. We have characterized a similar high-affinity phenoloamine transporter expressed in the CNS of the earthworm *Lumbricus terrestris*. Phylogenetic analysis of its protein sequence clusters it with both arthropod phenolamine and chordate catecholamine transporters. To clarify the relationships among metazoan monoamine transporters we identified representatives in the major branches of metazoan evolution by polymerase chain reaction (PCR)-amplifying conserved cDNA fragments from isolated nervous tissue and by analyzing available genomic data. Analysis of conserved motifs in the sequence data suggest that the presumed common ancestor of modern-day Bilateria expressed at least three functionally distinct monoamine transporters in its nervous system: a SERT currently found throughout bilaterian phyla, a DAT now restricted in distribution to protostome invertebrates and echinoderms and a third monoamine transporter (MAT), widely represented in contemporary Bilateria, that is selective for catecholamines and/or phenolamines. Chordate DATs, NETs, epinephrine transporters (ETs) and arthropod and annelid OATs all belong to the MAT clade. Contemporary invertebrate and chordate DATs belong to different SLC6 clades. Furthermore, the genes for dopamine and norepinephrine transporters of vertebrates are paralogous, apparently having arisen through duplication of an invertebrate MAT gene after the loss of an invertebrate-type DAT gene in a basal protochordate.

Supplementary material available online at
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Introduction

The molecular chemistry of the metazoan nervous system has a common evolutionary heritage. Serotonergic and dopaminergic neurons date back at least 600 million years to the stem metazoan, an ancient crawling worm proposed to be the common ancestor of the Cnidaria (radially symmetric organisms such as jellyfish, sea anemone and hydra) and the Bilateria (bilaterally symmetric animals that comprise most present-day animals) (Hay-Schmidt, 2000; Anctil et al., 2002; Bouchard et al., 2003; Raible and Arendt, 2004). In turn, all Bilateria are thought to share a common ancestor named Urbilateria (De Robertis and Sasai, 1996; Raible and Arendt, 2004). Modern bilaterian animals fall into either the Protostomia (major phyla Annelida, Mollusca, Arthropoda, Nematoda and Platyhelmintha) or the Deuterostomia (phyla Echinodermata and Chordata), based on molecular genetic and developmental characteristics (Phillipe et al., 2005). Analysis of genomic and transcriptome sequences from different bilaterian groups suggests that Urbilateria possessed a full complement of genes coding for neuronal proteins involved in the synthesis, reception and selective re-uptake of monoamine neurotransmitters such as serotonin and dopamine (DA). Two other monoaminergic pathways have a restricted distribution in the bilaterian nervous system. Neuronal pathways based on the phenolamines tyramine (TA) and octopamine (OA) are characteristic of protostomes (Pflüger and Stevenson, 2005; Evans and Maquiera, 2005), whereas those based on the catecholamines norepinephrine (NE) and epinephrine (E) are characteristic of deuterostomes (Vincent et al., 1998).

The neuronal expression of plasma membrane transporters involved in neurotransmitter re-uptake is a diagnostic component of monoaminergic systems in the Bilateria (Torres

et al., 2003). The fully sequenced genomes of animals representative of major bilaterian phyla, such as those of the flatworm, fruitfly, nematode and human, all contain serotonin transporters (SERTs) and dopamine transporters (DATs) (Pörzgen et al., 2001; Caveney and Donly, 2002). Other monoamine transporters expressed by monoaminergic neurons include an OA/TA transporter (OAT) associated with arthropod octopaminergic pathways (Malutan et al., 2002; Donly and Caveney, 2005) and the norepinephrine/epinephrine transporter (NET) associated with chordate adrenergic pathways (Apparsundaram et al., 1997; Roubert et al., 2001). These monoamine transporters are all metazoan members of the solute-linked carrier (SLC) family 6 [or alternatively sodium:neurotransmitter symporter (SNF) family] of Na^+-dependent nutrient transporters that are expressed in both prokaryotes (Androutsellis-Theotokis et al., 2003; Yamashita et al., 2005) and eukaryotes (Chen et al., 2004; Boudko et al., 2005b). Monoamine transporters are highly conserved and widely expressed in the bilaterian nervous system. Their protein structure and substrate kinetics may provide information on the origin and number of genes that encode SLC6 monoamine transporters in the Metazoa (Chen et al., 2004; Höglund et al., 2005). Do the genes encoding the transporter for each specific monoamine in the metazoan CNS derive from a common ancestor, and if not, are there differences in the gene complements present in the different bilaterian lineages? What was the ancestral monoamine transported by the different types of transporter? And what is the relationship between protostome OA and deuterostome NE transporters?

To address these questions, we have used both existing and new structural and functional data on SLC6 monoamine transporters representing many metazoan phyla. We have also cloned and characterized a phenolamine transporter from the CNS of the earthworm. The results suggest that OA and NE are ancient neurotransmitters in the protostome and deuterostome lineages, respectively, and that their transporters share a common origin.

Materials and methods

Reverse transcription-polymerase chain reaction (RT–PCR)

Template for amplification was obtained from each organism by dissecting the brain and/or nerve cord directly into RNAlater (Ambion, Austin, TX, USA). Total RNA was isolated using Trizol reagent (Invitrogen, Burlington, ON, Canada) and made up in DEPC-treated H_2O. Synthesis of cDNA was performed using 10 U Superscript II reverse transcriptase (Invitrogen) and 2.5 μg of RNA at 42°C for 50 min. PCR was then performed with 2 μl of cDNA as the template. Degenerate primers were designed from conserved amino acid sequences in known MATs (DAT0=5′-GCNGTNGAYYTNGCNAAYGTNTGG-3′ encoding AVDLANVW and DAT3=5′-GTNGCNGTDATC-CANACNACYTT-3′ encoding KVVWITAT) to amplify an internal segment of cDNA encoding from the first to the fifth transmembrane domain (TMD) of the protein. The PCR mix contained 0.2 mmol l^{-1} dNTPs, 2.5 mmol l^{-1} $MgCl_2$, 5 pmol μl^{-1} degenerate primers and 2.5 U Taq DNA Polymerase (Invitrogen). The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by one 5-min hold at 72°C.

Products from PCR were separated on agarose gels and bands estimated to be of the appropriate size were ligated into pGEM-T Easy (Promega, Madison, WI, USA) and transformed into XL1-blue cells (Stratagene, La Jolla, CA, USA). Plasmids were subjected to dideoxynucleotide chain-termination sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Kit and resolved with an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Cloning and expression of a Lumbricus MAT

Using the sequence of the internal DAT0/DAT3 fragment for *Lumbricus terrestris*, nested pairs of primers were synthesized for 5′ and 3′ rapid amplification of cDNA ends-PCR (RACE-PCR). RACE-PCR was performed using the FirstChoice RLM RACE kit (Ambion) as directed by the supplier. From the fully assembled sequence, primers were synthesized to amplify the complete open reading frame (ORF) (Lut8R: 5′-CGGCA-GCTCTCGAAGGATACCAT-3′ and Lut9F: 5′-GAAGCA-GAGACCGAGTGACGAGGA-3′). The resulting 2.4 kb product was amplified using EasyA polymerase (Stratagene) and cloned in pGEM-T Easy. Three independently amplified products were sequenced to avoid polymerase errors.

The cloned insert was transferred to the pIZ/V5-His vector for expression using the InsectSelect System (Invitrogen). Purified plasmid (6 μg) was used to transfect Sf9 cells in 60 mm culture plates using Cellfectin Reagent as instructed by the supplier (Invitrogen). Stably transformed lines derived from individual dishes were established and maintained in the presence of 100 μg ml^{-1} Zeocin.

Transport assays

Transport assays were performed in 12-well plates (Falcon; Becton-Dickinson, Franklin Lakes, NJ, USA) as previously described (Gallant et al., 2003). Sf9 cells stably expressing LutOAT were seeded at a density of 800 000 per well in 1 ml of SF-900 II SFM medium (Invitrogen) and used 24 h later. The wells were briefly rinsed and then incubated for an hour in a Na^+-containing saline (11.2 mmol l^{-1} $MgCl_2$, 11.2 mmol l^{-1} $MgSO_4$, 53.5 mmol l^{-1} NaCl, 7.3 mmol l^{-1} NaH_2PO_4, 55 mmol l^{-1} KCl and 76.8 mmol l^{-1} sucrose at pH 7). The cells were then exposed to 500 μl Na^+ saline containing either 29 nmol l^{-1} to 0.29 μmol l^{-1} ^{3}H-DA (specific activity 34.8 Ci mmol l^{-1}; 1 Ci=37 GBq), 18 nmol l^{-1} to 0.91 μmol l^{-1} ^{3}H-NE (specific activity 10.9 Ci mmol l^{-1}) (purchased from NEN Life Sciences Products, Inc., Boston, MA, USA), 20 nmol l^{-1} to 0.50 μmol l^{-1} ^{3}H-OA (specific activity 20 Ci mmol l^{-1}) or 10 nmol l^{-1} to 0.2 μmol l^{-1} ^{3}H-TA (specific activity 50 Ci mmol l^{-1}) (purchased from American Radiolabelled Chemicals, St Louis, MO, USA). Where required, unlabelled monoamine was added to the saline to give final concentrations over the range 10 nmol l^{-1} and 15.8 μmol l^{-1}. Uptake was stopped after 3 min by removing the radiolabelled solution and

washing the cells three times (2 ml/well/wash) with a Na^+-free saline. The plate was air dried and radiolabelled substrate accumulated by the cells extracted for 20 min with 500 μl 70% ethanol. A 400 μl aliquot was then removed from each well, added to Ready Safe scintillation fluid (Beckman Coulter, Fullerton, CA, USA) and the radioactivity counted on a scintillation analyzer (2900TR; Perkin-Elmer, Wellesley, MA, USA). All washes and incubations were done at 27–29°C. The radiolabelled monoamine solutions were kept on ice until immediately before use. Transport kinetics for each monoamine was determined by Eadie-Hofstee analysis of the uptake data, which provides an estimate of transporter affinity (K_m) for the radiolabelled substrate and the maximum velocity of its uptake ($\dot{V}_{max}$) (Jayanthi et al., 1998, Roubert et al., 2001). Na^+-dependent uptake of each radiolabelled monoamine was assessed on 3–5 plates of cells. The uptake data were corrected for Na^+-independent uptake by exposing the cells to an identical treatment except for the substitution of choline$^+$ for Na^+ in the saline. Sf9 cells also have an endogenous low-affinity Na^+-dependent uptake mechanism for amines that contributed less than 5% to the total Na^+-dependent uptake of OA in cells transformed with the high-affinity LutOAT cDNA. Its effect on the calculated K_m for OA was not significant over the range of OA concentrations used.

Bioinformatics

DNA sequences for the DAT0/DAT3 fragment of monoamine transporters from non-model organisms were obtained through cloning and sequencing as described above. These sequences, as well as the *Lumbricus* OAT RACE-PCR sequence, were assembled using SEQMAN II (DNASTAR, Madison, WI, USA). DNA sequences were also obtained for additional organisms by BLAST-searching public genome databases. Full lists of the deduced amino acid sequences obtained from all partial (supplementary material Table S1) and complete (supplementary material Table S2) transporter sequences are provided in the supporting information. We also used discontiguous Mega BLAST to search for exons representing fragments of monoamine transporter genes in metazoan whole genome shotgun (WGS) sequences deposited in the Trace Archive at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml).

The amino acid sequences obtained were aligned using the ClustalW facility in MEGA version 3.1 (Kumar et al., 2004). Phylogenetic trees were reconstructed using MEGA 3.1 by the distance neighbour-joining method (with complete deletion of gaps). The consensus trees resulting from bootstrapping the data (1000 replicates; seed 64884) are shown.

Results

We determined the distribution of monoamine transporter expression in groups of protostome Metazoa for which genome-level data are currently unavailable. Partial sequences were obtained from nervous tissue using RT-PCR to produce a 500–700 bp segment of monoamine transporter cDNA. The

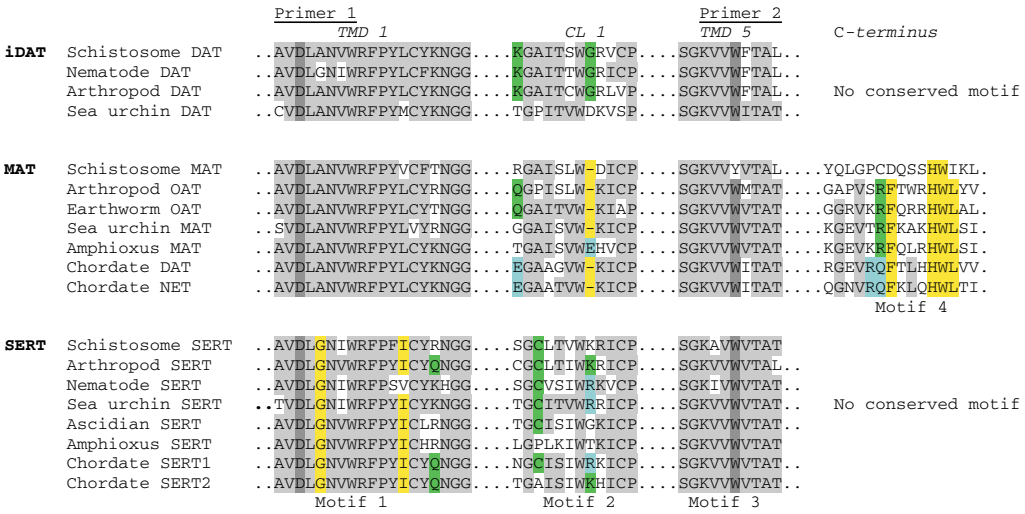

Fig. 1. Metazoan monoamine transporters belonging to the SLC6 family of membrane transporters have diagnostic polypeptide motifs. These conserved motifs helped identify three different monoamine transporter types, iDAT, a dopamine transporter restricted to invertebrates, and two widely distributed metazoan transporters, MAT, a catecholamine/phenolamine monoamine transporter, and SERT, a serotonin transporter. Motifs 1, 2 and 3 are present in all three transporter types, whereas motif 4 is a C-terminal sequence restricted to the MAT-type catecholamine/phenolamine transporter. Motif 1 is mainly in transmembrane domain 1 (TMD1), motif 2 in the cytoplasmic loop (CL1) between TMDs 2 and 3 and motif 3 is in transmembrane domain 5 (TMD5). Amino acid residues shaded in grey are consensus residues; the aspartate residue (D) in motif 1 and the tryptophan residue (W) (rarely tyrosine, Y) in motif 3 are not found in related nutrient amino acid transporters (NATs). The amino acids (or site of missing amino acid in MAT) shaded in yellow are largely restricted to one transporter type. Amino acids shaded in blue or green are restricted to subsets within a transporter type. Abbreviations for the other amino acids follow the single-letter code. The positions of the primer sites used in RT-PCR screening for partial transporter sequences are shown above the alignment.

primer pair used yielded a PCR product encoding the TMD1 to TMD5 region of the transporter protein (Fig. 1). The amino acid sequences encoded by these partial cDNAs and the corresponding regions of available full-length protostome and deuterostome monoamine transporter sequences were then aligned and shown as a phylogenetic tree. Fig. 2 shows that monoamine transporters are widely expressed in the metazoan CNS. Furthermore, these partial protein sequences suggest that there are four structural/functional types of monoamine transporter. The SERT and OAT clusters are clearly resolved, whereas the invertebrate DAT and chordate DAT (cDAT)/NET groups are less clearly resolved.

The relationship of these four clusters was then assessed. First, we aligned the full-length protein sequences derived from the limited number of SLC6-type monoamine transporter genes available online in complete or near-complete metazoan

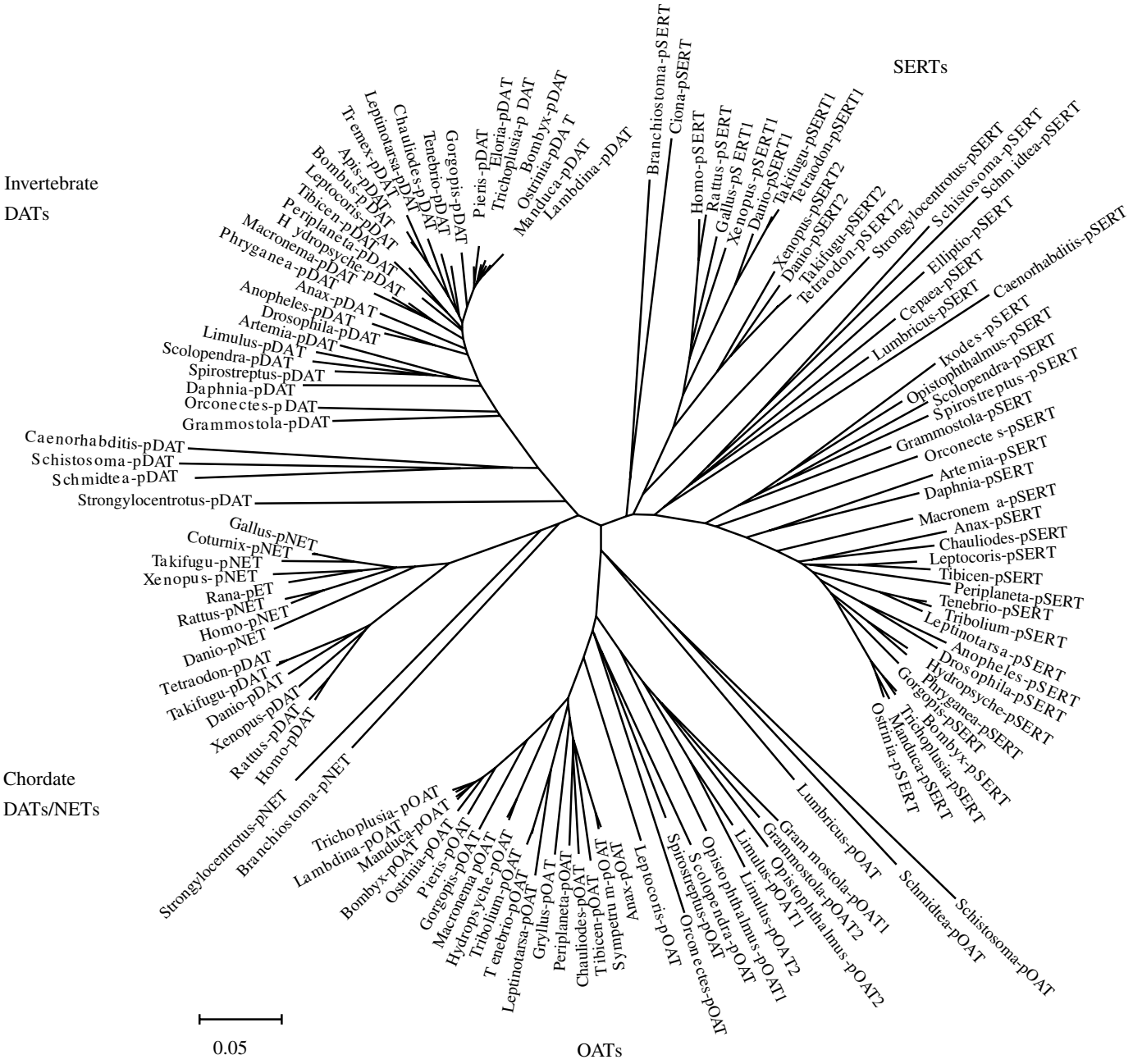

Fig. 2. Comparison of partial SLC6 monoamine transporter sequences in the Metazoa. The dendrogram combines our RT-PCR data with sequence data available online. The major resulting clusters are as labelled. Note that chordate DATs (cDATs) group more closely with vertebrate NETs than with invertebrate DATs (iDATs). The NCBI nucleotide and protein sequence accession numbers and other genome resource data used to assemble this figure are listed in supplementary material Table S1). Most of the invertebrate sequences are included in the NCBI PopSets #70797604, #113204555, #113204575 and #110734541. The scale bar shows evolutionary distance in number of substitutions per site.

genomes. To these sequences we added a new monoamine transporter encoded by a full-length cDNA cloned from the earthworm *Lumbricus terrestris* (Phylum Annelida) (Fig. 3). Second, we searched this set of full-length monoamine transporters for specific diagnostic amino acid residues and polypeptide motifs. SLC6 monoamine transporters have several diagnostic amino acid residues in their transmembrane and cytoplasmic domains (Nelson, 1998; Torres et al., 2003). As shown in Fig. 1, TMD1 contains a unique aspartate residue (D) in the conserved 'hallmark' consensus sequence $AVDL^{A}/_{G}N^{I}/_{V}WRFPY^{L}/_{I}CYxNGG$ (motif 1). This aspartate replaces a glycine residue (G) seen commonly in closely related SLC6 nutrient amino acid transporters (NATs) (Boudko et al., 2005a). In TMD5 a tryptophan residue (W) in the consensus sequence $SGK^{I}/_{V}VWxTA^{L}/_{T}$ (motif 3) normally replaces a tyrosine residue (Y) seen in NATs. These two highly conserved and unique motifs served as the sites (Fig. 1) for the PCR primers designed to amplify monoamine transporter fragments (see Materials and methods). The different types of monoamine transporters can be further distinguished by the presence of other amino acid residues (Fig. 1). Many serotonin transporters have an isoleucine (I) residue in motif 1 and a second cysteine (C) residue in motif 2 not seen in catecholamine/phenolamine transporters. Most SERTs also have a basic residue (K, R) in the first cytoplasmic loop between canonical tryptophan and proline residues in the sequence $W^{K}/_{R}X^{I}/_{V}CP$ (part of motif 2). A glycine residue (G in WGR) is seen in the corresponding position in protostome DATs. This amino acid residue is missing in invertebrate OA and chordate catecholamine transporters (amphioxus NET is an exception). Most notable in the full-length sequences, however, was a C-terminal HWL motif (FxxHWLxx) in chordate NETs, cDATs and invertebrate OATs (motif 4). Putative monoamine transporters in the flatworms *Schistosoma* and *Schmidtea* have variants of this motif. The four consensus motifs are shown in Fig. 1.

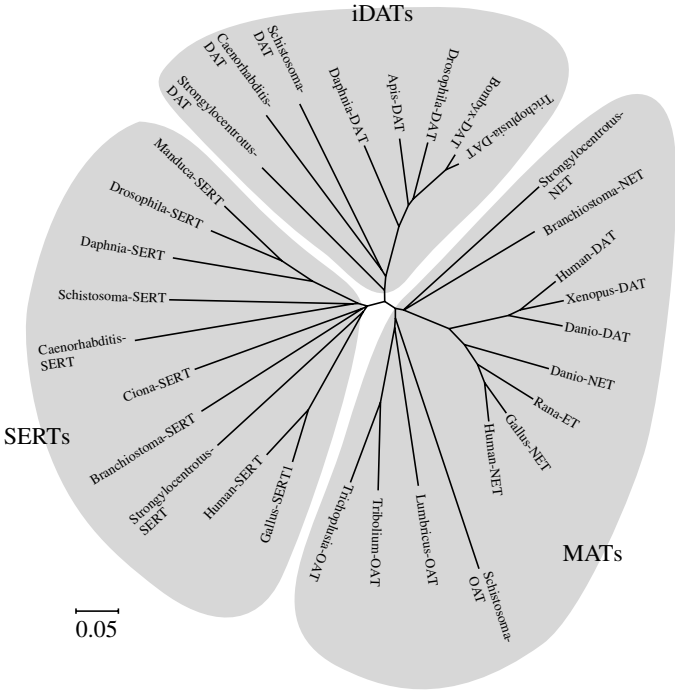

Fig. 3. Sequence comparisons of complete (or near-complete) metazoan monoamine transporter sequences obtained from BLAST searches online and a *Lumbricus terrestris* OAT sequence (NCBI accession #DQ813341) from this study. The dendrogram confirms our tentative groupings based on the presence or absence of certain diagnostic amino acid residues in the partial transporter sequences (Fig. 1). Note that protostome OATs and deuterostome DATs/NETs form a single cluster (MAT). A NET-like gene is apparently lacking from protostome genomes and an OAT-like gene from deuterostome genomes such as echinoderms and chordates. The NCBI nucleotide and protein accession numbers and other genome resources used are listed in supplementary material Table S2. The scale bar shows evolutionary distance in number of substitutions per site.

These peptide motifs suggest there are three clades of monoamine transporter belonging to the SLC6 transporter family. The first clade consists of SERTs. The second clade is a complex assemblage of monoamine transporters that, depending on organism and tissue context, may transport primarily catecholamines or phenolamines. We call this the MAT (monoamine transporter) clade. The protein structure of MATs includes the HWL motif. These two clades are represented throughout the bilaterian Metazoa. A third clade of DATs (iDAT) is apparently invertebrate-specific, being restricted to protostomes and invertebrate deuterostomes (i.e. non-chordates). iDAT is expressed in the nervous system of most protostome lineages (Fig. 2) and an iDAT variant is expressed in echinoderms. A phylogenetic reconstruction of the protein sequences constituting these three clades of monoamine transporter is shown in Fig. 3.

MAT clade

The MAT clade genes are present in major phyla at the apex of protostome and deuterostome evolution, namely the Arthropoda and Chordata. Neurally expressed MAT proteins fall into two structural and functional groups, invertebrate MATs (OATs) that appear to be primarily active in phenolamine uptake *in situ* and chordate MATs (NETs/DATs) primarily active in catecholamine uptake *in situ*. The first invertebrate MAT cloned was an octopamine transporter from the CNS of the moth *Trichoplusia ni* (Malutan et al., 2002). cDNAs coding for OAT have now been cloned or sequenced from several arthropods (supplementary material Table S1). Arthropod OATs have high affinity for TA, OA and DA. Chelicerate arthropods are unusual in expressing two OAT orthologues (Fig. 2). The schistosome genome contains a recognizable but as yet uncharacterized single-copy MAT gene that we provisionally name *Schistosoma* OAT (Fig. 3).

The CNS in the protostome and deuterostome lineages differs in having either octopaminergic or adrenergic neural pathways, respectively. Did the ancestral MAT have selective affinity for OA over NE? To investigate this possibility, we cloned and characterized a full-length MAT from the CNS of the earthworm *Lumbricus terrestris* (Phylum Annelida).

Annelids are 'lower' protostomes thought to possess a primitive anatomy and an ancestral gene inventory (Arendt et al., 2004). The annelid nervous system contains DA, OA and serotonin (Csoknya et al., 1996; Barna et al., 2001). Octopaminergic pathways (Csoknya et al., 1996, Barna et al., 2001) and dopaminergic pathways (Crisp et al., 2002) have been mapped in the annelid brain and stomatogastric ganglia. Octopaminergic signalling is associated with earthworm locomotion (Mizutani et al., 2002) and smooth muscle contractility in the foregut (Barna et al., 2001). Immunoreactivity for the NE biosynthetic enzyme DA β-hydroxylase is lacking in DA-containing neurons in the leech *Hirudo*, suggesting that NE-ergic pathways are absent from the annelid CNS (Crisp et al., 2002).

A full-length cDNA coding for the MAT transcript LutOAT (2019 bp, GenBank accession no. DQ813341) was cloned by RT-PCR using RNA extracted from the earthworm CNS (see Materials and methods). The sequence contained an ORF encoding a protein of 673 amino acids showing 55–57% identity with vertebrate MATs (cDAT and NET) and 73–77% identity with insect OATs. In phylogenetic analyses, this annelid transporter always clustered with OAT sequences, nearest the insect OATs using complete sequences (Fig. 3) and nearest the platyhelminth OATs using the internal DAT0/DAT3 PCR fragment (Fig. 2). The kinetic properties of the encoded transporter were determined using insect Sf9 cells after stable transformation with LutOAT cDNA and measuring dose-dependent uptake of either ^{3}H-labelled TA, OA, DA or NE (see Materials and methods). The earthworm MAT showed different affinities for the four phenylethylamine neurotransmitters tested (Fig. 4). The transporter has greatest affinity for TA (K_m^{TA}, 56 nmol l^{-1}), approximately 20-fold greater than for NE (K_m^{NE}, 1.1 μmol l^{-1}). A second phenolamine, OA, is also a high-affinity substrate (K_m^{OA}, 80 nmol l^{-1}). Dopamine, the other catecholamine tested, is also a moderately high-affinity substrate (K_m^{DA}, 282 nmol l^{-1}). Earthworm OAT displays an inverse relationship between substrate affinity and uptake capacity when expressed by Sf9 cells. Uptake of the high-affinity substrate TA saturates at a concentration almost two-orders lower than that for the low-affinity substrate NE. The kinetic data are summarized in Table 1. The high levels of OA and TA recorded in the earthworm CNS (Csoknya et al., 1996) support our contention that this transporter is associated with octopaminergic neurons where it primarily transports phenolamines. For these reasons we assigned this transporter the trivial name LutOAT. Earthworm OAT, like many other transporters in the MAT clade, has considerable affinity for DA. This is not unusual, in that fish NET (Roubert et al., 2001) and frog ET (Apparsundaram et al., 1997) have higher affinity for DA than their nominal substrates, and arthropod OAT (Gallant et al., 2003) and mammalian NET (Roubert et al., 2001) have approximately equal affinity for DA and their nominal substrates. Kinetic data from other cloned MATs imply that a high affinity for DA does not necessarily mean that a transporter's primary role *in situ* is in DA re-uptake. The apparent absence of an invertebrate-type DAT in the earthworm CNS does raise the possibility that LutOAT is also expressed by dopaminergic neurons where it might transport DA. To determine whether LutOAT is expressed preferentially by one or the other neuron type would require the identification of LutOAT RNA in OA- or DA-containing neurons that have been mapped in the earthworm brain (Barna et al., 2001).

Among basal deuterostomes, the genomes of the sea urchin *Strongylocentrotus* (Phylum Echinodermata) and the amphioxus *Branchiostoma* (Phylum Cephalochordata) contain

Table 1. *Properties of the earthworm monoamine transporter, LutOAT*

Monoamine transported	K_m (nmol l^{-1})	V_{max} (pmol 10^6 cells^{-1} min^{-1})
Tyramine	56±11	0.12±0.01
Octopamine	80±10	0.52±0.04
Dopamine	331±68	3.21±0.42
Norepinephrine	1108±190	8.25±1.85

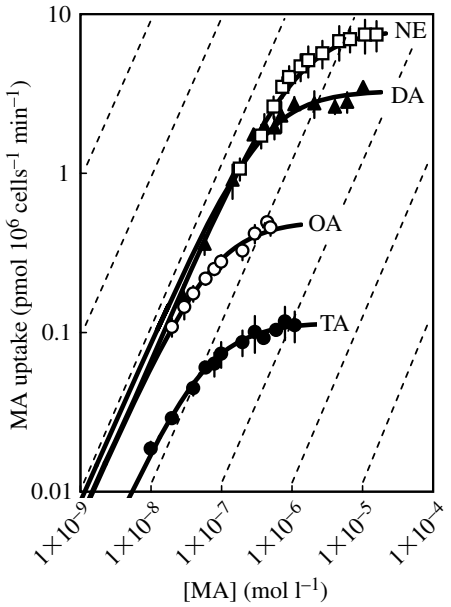

Fig. 4. Stable expression of *Lumbricus terrestris* OAT transporter RNA in transformed Sf9 insect cells. The relative affinity of LutOAT for neurotransmitter monoamines (MA) was determined by exposing transformed Sf9 cells to radiolabelled isotopes of the phenolamines tyramine (TA) and octopamine (OA), and the catecholamines dopamine (DA) and norepinephrine (NE) *in vitro*. Cells expressing LutOAT take up all four monoamines with high-affinity and in a Na^+-dependent manner. TA and OA uptake saturates at mid-nanomolar substrate concentrations, whereas DA and NE uptake saturates at low-micromolar concentrations. Uptake under non-saturating substrate concentrations is log-linear (as indicated by the slope of the broken lines). There is an inverse relationship between affinity and transport capacity. LutOAT has the highest affinity and the lowest transport capacity for TA; the opposite holds for NE. The estimated kinetic values are given in Table 1. Each point represents the mean ± s.d., *N*=3–5 independent trials.

single genes coding for recognizable NET-like MAT proteins (Fig. 3). Amphioxus is the nearest common invertebrate ancestor of the vertebrates (Shimeld and Holland, 2005). Motifs 2 and 4 of sea urchin NET and amphioxus NET (Fig. 1) suggest that these proteins derive from an ancestor in common with vertebrate NETs. The relative affinity of putative sea urchin NET and amphioxus NET for NE and DA is unknown, but both monoamines are pharmacologically active on echinoderm tissues (Shingyoji and Yamaguchi, 1995; Vanderlinden and Mallefet, 2004). Dopamine and OA, but not NE, are reportedly present in amphioxus tissues (Moret et al., 2004), implying that putative amphioxus NET is involved in the uptake of DA and/or OA rather than the uptake of NE. The genome of the ascidian *Ciona* (Dehal et al., 2002) appears to lack a MAT gene altogether, even though the ascidian larval brain has dopaminergic neurons and expresses the enzymes required for DA synthesis (Moret et al., 2005).

Analysis of the limited collection of lamprey (*Petromyzon,* Phylum Chordata Class Agnatha) WGS sequences in the NCBI Trace Archive suggests that the genome of this primitive vertebrate contains two MAT-type genes, unlike that of amphioxus, its nearest extant ancestor. In this way the lamprey genome conforms with those of higher vertebrates, which contain two MAT paralogues. Both vertebrate MAT genes encode catecholamine transporters. One codes for a NE transporter (e.g. mammalian NET) and the other a chordate-specific DA transporter (e.g. mammalian DAT). The latter has been extensively studied in mammals because of its link with cocaine addiction (Torres et al., 2003). Chordate DAT is present in all completely sequenced genomes of teleosts, birds and mammals (Fig. 3).

In general, metazoan MATs (as represented by earthworm OAT, insect OAT and mammalian NET) may be distinguished from iDAT and cDAT by their relatively high affinity for phenolamines in addition to catecholamines (Malutan et al., 2002; Gallant et al., 2003). Chordate NET, for instance, has a submicromolar affinity for both TA and OA, based on inhibition data (Pörzgen et al., 2001). *Drosophila* DAT and *Trichoplusia* DAT, by contrast, have lower affinity for TA and even less affinity for OA (Pörzgen et al., 2001; Gallant et al., 2003). Rat DAT and *C. elegans* DAT similarly have modest affinity for TA and little affinity for OA (Pörzgen et al., 2001).

It is important, however, to appreciate that sequence similarity between a functionally uncharacterized MAT and well-characterized MATs (Fig. 3) is no guarantee of its substrate preferences *in situ*. This is particularly true with respect to the MAT clade. Tentatively named *Branchiostoma* NET, *Strongylocentrotus* NET and *Lumbricus* OAT could *in situ* be involved in DA rather than NE or OA transport (although this is unlikely in the sea urchin, as its genome contains an iDAT gene, see below).

Invertebrate DA transporter clade

A second bilaterian SLC6-type gene encodes a DA transporter limited in distribution to protostome and echinoderm (sea urchin) genomes. This invertebrate-type DA transporter (iDAT) has some distinctive structural motifs (Fig. 1). Expression of iDAT in the invertebrate CNS is probably limited to small numbers of dopaminergic neurons, such as in the insect brain and ventral nerve cord (Pörzgen et al., 2001; Gallant et al., 2003). The iDAT protein has a selective affinity for DA (Jayanthi et al., 1998; Pörzgen et al., 2001; Gallant et al., 2003). Evidence of iDAT homologues can be found in many protostome genomes, such as those of the insects *Apis* and *Tribolium*, the flatworms *Schistosoma* and *Schmidtea* and the nematode *Caenorhabditis*. We isolated cDNAs for partial iDAT sequences from the CNS of many arthropod species representing the major lineages in the phylum, namely the Chelicerata, Myriapoda and Pancrustacea (Crustacea and Insecta). These iDAT genes appear to be direct descendants of an ancestral bilaterian DAT gene, although iDAT may not be expressed by all present-day protostome phyla. For example, dopaminergic neurons are present both in the annelid CNS (Barna et al., 2001) and mollusc CNS (Hiripi et al., 1998; Kiehn et al., 2001), yet iDAT was not detected in the earthworm CNS nor in the CNS of the molluscs examined. However, the NCBI Trace Archive for the sea hare *Aplysia californica* (Mollusca, Gastropoda) contains some WGS sequences suggestive of the presence of a catecholamine or phenolamine transporter-like gene. Among the deuterostome genomes examined, only *Strongylocentrotus* contains an invertebrate-type DAT homologue. Homologues of the iDAT gene are missing from the genomes of the lower chordates *Ciona* and *Branchiostoma* and from those of all higher chordates.

Serotonin transporter clade

The neurotransmitter serotonin was present in neurons in the CNS of all protostomes and deuterostomes examined (Hay-Schmidt, 2000). Serotonin transporters (SERTs), in contrast to invertebrate-type DA transporters, are near-ubiquitously expressed in Metazoa. SERT has the smallest and most conserved structure of the three known MATs in the SLC6 family. Representatives of all protostome phyla examined were found to express recognizable SERT homologues. This was most notable in species that lack genes encoding one or both of the other MATs. In the Mollusca, partial SERT cDNAs were obtained from the CNS of the snail *Cepaea nemoralis* and the clam *Elliptio dilatata* (Fig. 2). A small SERT fragment has also been cloned from *Aplysia* (NCBI accession #AAK94482). In the Annelida, we cloned a partial SERT cDNA from the earthworm CNS. The partial SERT sequences encoded by these molluscan and annelid cDNAs aligned with the corresponding region of full-length flatworm *Schistosoma* SERT (Fig. 1). The nematode *Caenorhabditis* expresses a SERT with structure least similar to those of the other protostome SERTs. The honeybee *Apis* genome (and probably those of other hymenoptera) is exceptional in that it lacks a SERT gene. Alignment of all available partial-SERT protein sequences obtained from genomic and RT-PCR sources supports a conventional protostome clade in metazoan phylogeny (Fig. 2).

All deuterostomes examined possess a SERT gene

homologue. The list includes the sea urchin *Strongylocentrotus*, the tunicate *Ciona*, the amphioxus *Branchiostoma* and many representative vertebrates (Phylum Chordata). The genomes of the lower chordates possess single-copy SERT genes, whereas analysis of higher chordate genomes suggests that two SERT genes existed in the stem ancestor of the subphylum Vertebrata. Two SERT paralogues (designated here as SERT1 and SERT2) are present in teleost and amphibian genomes (Wang et al., 2006). The *Petromyzon* (lamprey) WGS sequences in the NCBI Trace Archive indicate that this primitive agnathan also expresses two SERT genes. The *Gallus* (chicken) genome contains SERT1 and an additional SERT2-like sequence (XM_425275.1). Mammalian genomes, however, have single-copy SERT genes that are most similar to the lower vertebrate SERT1 (Fig. 2). The protein sequences of deuterostome SERTs align to form a distinct deuterostome clade, in which echinoderm SERT sits near the base in a position comparable to those occupied by the protein sequences of echinoderm DAT and echinoderm NET in the two other clades of deuterostome transporters (Fig. 3). The *Strongylocentrotus* genome is exceptional among the genomes of basal deuterostomes examined in that it contains genes representative of all three clades of SLC6 monoamine transporter.

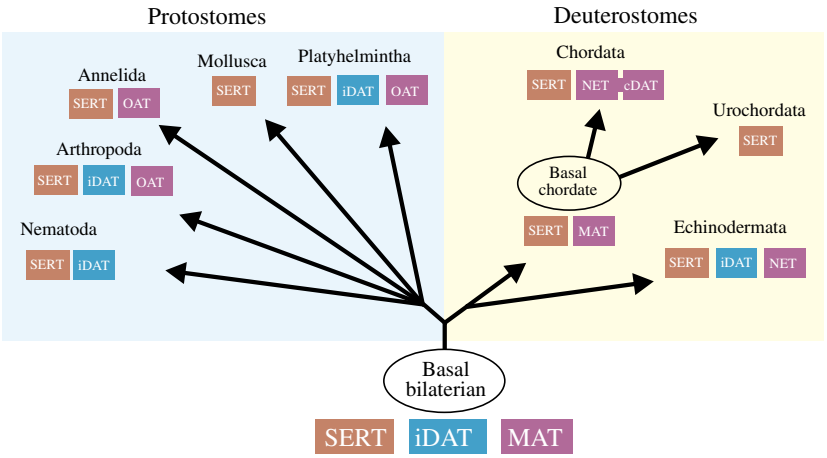

Fig. 5. Suggested origins of the three monoamine transporter types in the Bilateria. Homologues of the serotonin transporter gene, SERT, are expressed in all phyla examined to date. Although presumed to be ancestral, the invertebrate DAT gene (iDAT) may have been lost in some invertebrate phyla (Annelida, possibly Mollusca) and in the Chordata. The ancestral catecholamine/phenolamine transporter (MAT) persists today as invertebrate OAT and the chordate paralogues NET and cDAT. The chordate NET and cDAT genes appear to have arisen through duplication of a MAT gene following the loss of an invertebrate-type DAT gene in a basal protochordate. The loss, or absence of expression, of some monoamine transporter genes in different phyla (iDAT in Annelida, iDAT and MAT in Mollusca) or in sub-phyletic taxa (DAT in Urochordata, OAT in some Insecta, SERT in Hymenoptera) appears to be a commonly repeated theme in the Metazoa.

Discussion

The data presented here confirm that the metazoan nervous system expresses three distinct clades of Na^+-dependent monoamine transporters belonging to the SLC6 family of membrane co-transporters: (i) a pan-bilaterian clade of MATs (an assemblage of arthropod-type OAT and vertebrate-type NET and cDAT proteins), (ii) an invertebrate-specific DAT (iDAT) clade and (iii) a pan-bilaterian SERT clade. We presume that the hypothetical urbilaterian ancestor expressed genes representing all three clades of SLC6 monoamine transporter. The gene homologues of one or more of these ancestral transporters appear to have been subsequently lost in certain bilaterian lineages. Many protostome phyla, such as the Mollusca, Nematoda and Arthropoda, contain taxa that apparently lack genes encoding particular SLC6 monoamine transporters. For instance, genes encoding a MAT-type transporter are missing from the completely sequenced genomes of several protostome 'model systems', namely those of the fruitfly, mosquito, honeybee and (possibly all) nematodes. Nevertheless, these model invertebrates are at the centre of studies into the molecular genetic basis of animal behaviour. These animals all possess demonstrable octopaminergic neurons and retain OA-dependent behaviours (Alkema, 2005; Roeder, 2005). This does raise the issue, however, of how important OATs are to brain function in invertebrates. Presumably invertebrates lacking OAT possess alternative means to downregulate OA levels at octopaminergic synapses (Pörzgen et al., 2001).

Gene loss (in this instance loss of transporter genes) in the lineages leading to *Drosophila* and *Caenorhabditis* are well documented (Kortschak et al., 2003; Raible and Arendt, 2004). Gene loss has also occurred during the evolution of the chordates (Okamura et al., 2005). The urochordate *Ciona* lacks both an iDAT-type and MAT-type gene, and the cephalochordate *Branchiostoma* an iDAT-type gene. Despite the absence of these particular transporter protein sequences, an alignment of available full-length and partial transporter sequences provides a conventional view of the general relationships among metazoan phyla (Figs 2, 3).

The conservation of an HWL motif (and the schistosome HWI variant) at the MAT protein C-terminus in phyla expressing this protein (e.g. cDAT, chordate NET and protostome OAT) is particularly striking. The C-terminus of chordate NET and cDAT is proposed to interact with the PDZ-domains of cytoplasmic proteins involved in protein trafficking through the endoplasmic reticulum (ER) and in surface localization (Bjerggard et al., 2004). Other non-PDZ binding domains, such as the (R/H/Q)HW motif (overlapping the HWL above) seen in many metazoan MATs, could be equally important in the trafficking of MAT proteins (Bjerggard et al., 2004). The sea urchin and amphioxus genomes contain genes

encoding MAT-like proteins that have a structure more similar to vertebrate NET and cDAT than to protostome OAT. These MAT-type genes probably derive from a MAT-like ancestral urbilaterian MAT gene distinct from the ancestor of the iDAT lineage that lacks this C-terminal motif. The OAT gene, such as that seen in the earthworm, may represent an ancestral MAT gene shared by both chordate DAT and NET gene lineages.

Dopamine transporters expressed in the bilaterian CNS thus appear to have two origins (Fig. 5). The vertebrate cDAT gene appears to have arisen as a paralogue of the chordate NET gene, probably following the separation of the vertebrate lineage from the basal chordate groups, possibly through gene or genome duplication (see below). The fact that the *Drosophila* and human DAT genes are derived from differing lineages may be significant in assessing the correlative value of *Drosophila* studies to model human cocaine addiction (Pörzgen et al., 2001). The presence of both an iDAT gene and a MAT gene in the sea urchin genome suggests this duplication event occurred after the echinoderms branched off from the deuterostome line of evolution that leads to the chordates. In our opinion, the possibility that chordate NET derives from cDAT instead of the other way around is less likely. Both NE and E are thought to play a role in sea urchin embryogenesis (Anitole-Misleh and Brown, 2004) and NE has been shown to trigger brittlestar luminescence (Vanderlinden and Mallefet, 2004). Whether this catecholamine is a *bona fide* neurotransmitter in echinoderms is unresolved, however. Norepinephrine may be a very ancient neurotransmitter that existed in the urbilaterian CNS and possibly even in the nerve nets of pre-bilaterians such as ancestral cnidarians (Anctil et al., 2002). Similarly, OA is extensively employed as a neurotransmitter in the protostome Metazoa, as well as in the deuterostome branch at least as far as the Cephalochordata. The uptake data presented here suggest that the ancestor of earthworm OAT was 'pre-adapted' to serve as a transporter of either catecholamine or phenolamine neurotransmitters. The modest differences in protein structure of putative echinoderm NET and putative amphioxus NET do not allow us to predict with any confidence the nature of their neuronal transport substrate(s) *in situ*.

Given these reservations, the following scenario might explain the *de novo* evolutionary origins of the higher cDAT gene. The scenario is based on the premise that the cephalochordate ancestor of amphioxus (unlike that of the urochordate *Ciona*) is on the direct evolutionary line to modern-day vertebrates. The absence of an invertebrate-type DAT gene in the amphioxus genome then becomes evolutionarily significant, as it implies that the iDAT gene was lost in the protochordate ancestor of modern-day vertebrates, whereas the MAT homologue was retained. This protochordate ancestor might have been a largely sessile organism similar in form and/or behaviour to present-day urochordates and cephalochordates. Other neurally expressed genes, possibly not crucial to survival in this simplified lifestyle (or for reasons unknown), were also discarded by the first protochordates. Recent analysis of the *Ciona* genome has revealed that many genes encoding invertebrate-type voltage and ligand-gated ion channels/receptors have apparently been lost (Okamura et al., 2005), and the same may hold true for the amphioxus genome. During the subsequent evolution of higher chordates with more complex nervous systems, alternative genes coding for ion channels have been duplicated to replace some of the functions of those lost (Vincent et al., 1998).

The loss of an invertebrate-type DAT at dopaminergic synapses in the relatively simple brains of ancestral chordates, for instance, would have necessitated that passive diffusion or other non-transport-based mechanisms restore external DA to pre-activation levels (see Pörzgen et al., 2001). Genome duplication in the ancestor to the modern vertebrates, which is claimed to have allowed the vertebrate brain to evolve in size and functional complexity (Vincent et al., 1998), may have provided the opportunity for an ancestral deuterostome MAT-type gene to diverge into the paralogous NET and DAT genes expressed in the CNS of all modern vertebrates. Such a proposal is not novel, as a similar pattern is seen in the duplication of catecholamine receptor sequences in the vertebrates. Amphioxus expresses a single catecholamine receptor of the DA D1 β-adrenergic type (Vincent et al., 1998; Candiani et al., 2005) (NCBI accessions CAA06536 and AAQ91625). All higher vertebrates (lamprey, hagfish and beyond) express two or more catecholamine receptors of this type. Vincent et al. link the D1 β-receptor duplication to the origin of an adrenergic neural system in the vertebrate CNS, and propose that the adrenergic receptor evolved from a DA receptor (Vincent et al., 1998). However, studies on invertebrate OA-receptor and vertebrate-adrenoreceptor sequences suggest that these proteins share a common ancestry and that the adrenergic receptor evolved instead from an OA receptor (Evans and Maquiera, 2005; Roeder, 2005). For example, the human α-1 adrenoreceptor and β-adrenoreceptor are apparently vertebrate homologues of a pair of OA receptors found in arthropods and molluscs (Evans and Maquiera, 2005; Roeder, 2005; Pflüger and Stevenson, 2005). Both scenarios underscore the close evolutionary relatedness of the vertebrate noradrenergic and dopaminergic systems to the invertebrate octopaminergic system, in which both vertebrate monoamine receptor and transporter gene paralogues appear to have duplicated in tandem. A further neural component supports the idea of an urbilaterian (or even pre-bilaterian) origin of the phenolaminergic/catecholaminergic (MAT) pathway hinted at by the MAT and receptor sequences. Most metazoan genomes contain a gene encoding a monoamine β-hydroxylase needed to catalyze the synthesis of OA from TA (TβH) and/or NE from DA (DβH) (supplementary material Table S3). The phylogeny of this enzyme resembles that of MAT and of OA/NE receptors (data not shown). Furthermore, these β-hydroxylase protein sequences, as seen in the iDAT and SERT sequences figured above, question the validity of a close evolutionary relationship between the Nematoda and other invertebrate phyla, particularly the Arthropoda. Access to sequence data for proteins from unstudied lesser phyla may help resolve the peculiar ancestry of the Nematoda.

In short, our transporter sequence data support the argument that octopaminergic and adrenergic neurotransmitter pathways arose from a common ancestral pathway. The genes encoding the hallmark combination of transporter, receptor and β-hydroxylase proteins expressed by phenolaminergic and catecholaminergic neurons appear to have existed in the ancestor of all modern Bilateria, with the two pathways diverging along the protostome and deuterostome lineages. The full complement of ancestral SLC6 genes encoding all three monoamine transporter types appears to have existed in the basal urbilaterian stock prior to the 'explosive' (relatively simultaneous) appearance of the major metazoan phyla in the Cambrian period, approximately 530–570 million years ago. This could be the reason why the monoamine transporter sequences failed to endorse any particular scheme proposed to explain the inter-relationships among the protostome phyla. Recent proposals that the phyla Mollusca, Annelida and Platyhelmintha cluster in a lophotrochozoan clade of protostome animals and the phyla Arthropoda and Nematoda in an ecdysozoan clade of protostome animals (Aguinaldo et al., 1997; Adouette et al., 2000) are only partly supported by our analysis. Although similarity in the full and partial SERT sequences from flatworm, an annelid and two mollusc lophotrochozoans lend support to the concept of a lophotrochozoan clade (Fig. 2), comparison of the partial and full-length DAT and SERT sequences from the nematode *C. elegans* with those of several arthropods fails to suggest any affinity between these two so-called ecdysozoan phyla (Figs 2, 3). Indeed, the nematode DAT and SERT proteins appear not to be particularly closely related in structure to homologous sequences in other invertebrate phyla, in that they contain some unusual amino acid residues in the core motifs (shown in Fig. 1). The association of the Nematoda with the Arthropoda in an Ecdysozoa clade is currently disputed (Blair et al., 2002; Wolf et al., 2003; Philippe et al., 2005). Comparison of the arthropod SERT and OAT sequences, however, do endorse a recent model for the ancestry of the Arthropoda (Richter et al., 2002), in which a crustacean-insect lineage, the Pancrustacea, is proposed to be a sister group to a less well-defined lineage containing the myriapods and chelicerates, the Paradoxica (Nardi et al., 2003; Delsuc et al., 2003; Regier et al., 2005). In this model, the insects are in essence highly successful descendants of a basal terrestrial crustacean (Regier et al., 2005).

List of abbreviations

cDAT	chordate dopamine transporter
DA	dopamine
E	epinephrine
iDAT	invertebrate dopamine transporter
MAT	monoamine transporter
NE	norepinephrine
NET	norepinephrine transporter
OA	octopamine
OAT	octopamine transporter
SERT	serotonin transporter
SLC6	solute-linked carrier family 6
TA	tyramine

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Table S1. *Sequences used in the analysis of partial MATs shown in Fig. 2*

	Gene accession no. or location	Protein accession no.
Protostome DATs (*N*=33)		
Anax pDAT	new	new
Anopheles pDAT	XM_308462	XP_308462.2
Apis pDAT	XM_395746	XP_395746.1
Artemia pDAT	new	new
Bombus pDAT	new	new
Bombyx pDAT	DQ105979	AAZ17653
Caenorhabditis pDAT	NM_066642	NP-499043.1
Chauliodes pDAT	DQ097770	AAZ08601.1
Drosophila pDAT	AF439752	NP_523763.2
Eloria pDAT	DQ105980	AAZ17654.1
Gorgopis pDAT	DQ097771	AAZ08602.1
Grammostola pDAT	new	new
Hydropsyche pDAT	DQ097772	AAZ08603.1
Lambdina pDAT	new	new
Leptinotarsa pDAT	DQ097773	AAZ08604.1
Leptocoris pDAT	new	new
Limulus pDAT	new	new
Macronema pDAT	DQ097774	AAZ08605.1
Manduca pDAT	DQ097775	AAZ08606.1
Orconectes pDAT	DQ097776	AAZ08607.1
Ostrinia pDAT	DQ097777	AAZ08608.1
Periplaneta pDAT	new	new
Phryganea pDAT	new	new
Pieris pDAT	new	new
Schistosoma pDAT	*S. mansoni* Genome version 3 c20553, c20554, c20556	
Schmidtea pDAT	assembled from NCBI trace archive	
Scolopendra pDAT	new	new
Spirostreptus pDAT	new	new
Tenebrio pDAT	new	new
Tibicen pDAT	new	new
Tremex pDAT	new	new
Trichoplusia pDAT	AY154398	AAN52844.2
Deuterostome DATs (*N*=7)		
Danio pDAT	NM_131755	NP_571830
Homo pDAT	NM_001044	NP_001035
Medaka pDAT	–	Roubert et al., 2001
Rattus pDAT	NM_012694	NP_036826
Strongylocentrotus pDAT	XM_784053	XP_789146
Takifugu pDAT	*Fugu* Genome scaffold 616 (99611-102003)	
Tetraodon pDAT	*Tetraodon* chr 8 (4601781-4608646)	AF96720
Xenopus pDAT	*Xenopus* Genome v4.1 scaffold 46 (496255-507077)	
Deuterostome NETs (*N*=10)		
Branchiostoma pNET	assembled from NCBI trace archive	
Coturnix pNET	AF230787	AAF89166
Danio pNET	XM_689046	XP_694138
Gallus pNET	NM_204716	NP_990047
Homo pNET	NM_001043	NP_001034.1
Medaka pNET	–	Roubert et al., 2001
Rana pET	RCU72877	AAB67676
Rattus pNET	NM_031343	NP_112633
Strongylocentrotus pNET	XM_779389	XP_784482
	XM_784934	XP_790027
Takifugu pNET	*Fugu* Genome scaffold 29 (252280-262000)	
Xenopus pNET	*Xenopus* Genome v4.1 scaffold 458 (50984-103108)	

Protostome OATs (*N*=31)

Anax pOAT	new	new
Bombyx pOAT	DQ097788	AAZ08584.1
Chauliodes pOAT	DQ097789	AAZ08585.1
Gorgopis pOAT	DQ097790	AAZ08586.1
Grammostola pOAT1	new	new
Grammostola pOAT2	new	new
Gryllus pOAT	DQ097798	AAZ08594.1
Hydropsyche pOAT	DQ097791	AAZ08587.1
Lambdina pOAT	DQ097799	AAZ08595.1
Leptinotarsa pOAT	DQ097792	AAZ08588.1
Leptocoris pOAT	DQ097800	AAZ08596.1
Limulus pOAT1	new	new
Limulus pOAT2	new	new
Lumbricus pOAT	new	new
Macronema pOAT	DQ097793	AAZ08589.1
Manduca pOAT	DQ097794	AAZ08590.1
Opistophthalmus pOAT1	new	new
Opistophthalmus pOAT2	new	new
Orconectes pOAT	DQ097795	AAZ08591.1
Ostrinia pOAT	DQ097796	AAZ08592.1
Periplaneta pOAT	DQ097801	AAZ08597.1
Pieris pOAT	DQ097797	AAZ08593.1
Schistosoma pOAT	*S. mansoni* genome version 3 contigs 54424, 54428	
Schmidtea pOAT	assembled from NCBI trace archive	
Scolopendra pOAT	new	new
Spirostreptus pOAT	new	new
Sympetrum pOAT	new	new
Tenebrio pOAT	DQ097802	AAZ08598.1
Tibicen pOAT	DQ097803	AAZ08599.1
Tribolium pOAT	XM 970263	XP 975356.1
Trichoplusia pOAT	AF388173	AAL09578.1

Protostome SERTs (*N*=32)

Anax pSERT	new	new
Anopheles pSERT	XM_310113.2	XP_310113.2
Artemia pSERT	new	new
Bombyx pSERT	DQ097778	AAZ08609.1
Caenorhabditis pSERT	AF385631	AAK84832
Cepaea pSERT	new	new
Chauliodes pSERT	DQ097779	AAZ08610.1
Daphnia pSERT	assembled from NCBI trace archive	
Drosophila pSERT	NM_079122	NP_523846.2
Elliptio pSERT	new	new
Gorgopis pSERT	DQ097780	AAZ08611.1
Grammostola pSERT	new	new
Hydropsyche pSERT	DQ097781	AAZ08612.1
Ixodes pSERT	assembled from NCBI trace archive	
Leptinotarsa pSERT	DQ097782	AAZ08613.1
Leptocoris pSERT	new	new
Lumbricus pSERT	new	new
Macronema pSERT	DQ097783	AAZ08614.1
Manduca pSERT	AF384164	AAN59781.1
Opistophthalmus pSERT	new	new
Orconectes pSERT	DQ097785	AAZ08616.1
Ostrinia pSERT	DQ097786	AAZ08617.1
Periplaneta pSERT	new	new
Phryganea pSERT	new	new
Schistosoma pSERT	DQ220811	ABA60792.1
Schmidtea pSERT	assembled from NCBI trace archive	

Scolopendra pSERT	new	new
Spirostreptus pSERT	new	new
Tenebrio pSERT	new	new
Tibicen pSERT	new	new
Tribolium pSERT	XM 963624	XP 968717.1
Trichoplusia pSERT	DQ097787	AAZ08618.1
Deuterostome SERTs (*N*=15)		
Branchiostoma pSERT	assembled from NCBI trace archive	
Ciona pSERT	Ensembl v3	ENSCINT00000015924
Danio pSERT1	XM_692577	XP_697669
Danio pSERT2	XM_679581	XP_684673
Gallus pSERT1	AY573844	AAS79016.1
Homo pSERT	NM-001045	P31645
Strongylocentrotus pSERT	XM_785856	XP_790949
	AF230787	XP_789146.1
Takifugu pSERT1	*Takifugu* Genome v3.0 scaffold 742 (74766-77904)	
Takifugu pSERT2	*Takifugu* Genome v3.0 scaffold 2 (675469-677555)	
Tetraodon pSERT1	chromosome Un_random (15087618-15091676)	
Tetraodon pSERT2	chromosome 12 (6411316-6412715)	
Xenopus pSERT1	*Xenopus* Genome v4.1 scaffold 1378 (934-14438)	
Xenopus pSERT2	*Xenopus* Genome v4.1 scaffold 17 (1054495-1078484)	

The new data have been submitted to the NCBI in PopSet format.

Bullfrog (*Xenopus tropicalis*) MAT sequences were identified in genome scaffolds (version 4.1) at the DOE Joint Genome Institute Genome Project website (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Tiger Pufferfish (*Takifugu rubripes*) MAT sequences were identified in genome scaffolds produced by the Institute of Molecular Biology Fugu Genome Project website (http://www.fugu-sg.org). Green Pufferfish (*Tetraodon nigroviridis*) MAT sequences were identified in genome scaffolds produced by Genoscope, the French National Sequencing Center, at http://www.genoscope.cns.fr/externe/tetranew. *Schistosoma* MAT sequences were constructed from contigs in the *Schistosoma mansoni* genome assembly (version 3) at the Wellcome Trust Sanger Institute website (www.sanger.ac.uk/Projects/S_mansoni).

Table S2. *Sequences used in the analysis of full-length MATs shown in Fig. 3*

	Gene accession no. or location	Protein accession no.
iDATs		
Apis DAT	XM_395746	XP_395746.1
Bombyx DAT	DQ105979	AAZ17653
Caenorhabditis DAT	NM_066642	NP-499043.1
Daphnia DAT	assembled from the NCBI trace archive	
Drosophila DAT	AF439752	NP_523763.2
Schistosoma DAT	*S. mansoni* genome version 3 c20553, c20554, c20556	
Schmidtea DAT	assembled from NCBI trace archive	
Trichoplusia DAT	AY154398	AAN52844.2
MATs		
Branchiostoma NET	assembled from NCBI trace archive	
Danio DAT	NM_131755	NP_571830
Danio NET	XM_689046	XP_694138
Gallus NET	NM_204716	NP_990047
Homo DAT	NM_001044	NP_001035
Homo NET	NM_001043	NP_001034.1
Lumbricus OAT	DQ813341	
Rana ET	RCU72877	AAB67676
Strongylocentrotus NET	XM_779389, XM_784934	XP_784482, XP_790027
Tribolium pOAT	XM_970263	XP_975356.1
Trichoplusia pOAT	AF388173	AAL09578.1
Xenopus DAT	*Xenopus* Genome scaffold 46 (496003-518802)	
SERTs		
Branchiostoma SERT	assembled from NCBI trace archive	
Caenorhabditis SERT AF385631	AAK84832	
Ciona SERT	Ensembl v3	ENSCINT00000015924
Daphnia SERT	assembled from NCBI trace archive	
Drosophila SERT	NM_079122	NP_523846.2
Gallus SERT1	AY573844	AAS79016.1
Homo SERT	NM_001045	P31645
Manduca SERT	AF384164	AAN59781.1
Schistosoma SERT	DQ159205	ABA39884
Strongylocentrotus SERT	XM_785856	XP_790949
Tribolium SERT	XM_963624	XP_968717.1

*Unless indicated below, these sequences were obtained from the NCBI GenBank database.

Amphioxus (*Branchistoma*) sequences were identified in genome scaffolds at the DOE Joint Genome Institute Amphioxus Genome Project website (http://shake.jgi-psf.org/Brafl1/Brafl1.home.html). Bullfrog (*Xenopus tropicalis*) DAT sequences were identified in genome scaffolds (version 4.1) at the DOE Joint Genome Institute *Xenopus* Genome Project website (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). *Schistosoma* MAT sequences were constructed from contigs in the *Schistosoma mansoni* genome assembly (version 3) at the Wellcome Trust Sanger Institute website (www.sanger.ac.uk/Projects/S_mansoni).

Table S3. *Sequences used in the analysis of partial- and complete β-hydroxylase protein sequences*

	Gene accession # or location	Protein accession # (Protein ID)
Protostome TβHs		
Schistosoma TβH	contig_0024863 (8358-537)	
	contig_0024864 (3767-1862)	
Caenorhabditis TβH		
C. elegans	NM_078161.1	181639
C. briggsae	CAAC01000032.1	CBG07714
C. remanei	AAGD01001630.1 (1479-399)	
	AAGD01001629.1 (7355-6670)	
Drosophila TβH	NM_176711	NP_788884.1
Anopheles TβH	AAB01008795	EAA03588.2
Aedes aegyptae TβH	AAGE 02000000 (53965-52190 & 35976-35659)	
Apis TβH	AADG05007658 (48577-46732)	
Tribolium TβH	AAJJ01002068 (10642-8352)	
Deuterostome DβHs and predicted TβHs		
Strongylocentrotus TβH	XM_788265.1	XP_793358
	XM_786108	XP_791201.1
Ciona TβH		
C. intestinalis	AABS01000424.1 (28961-27338)	
C. savignii	AACT01065467.1 (2235-6145)	
Rattus DβH	NM_013158.1	(Locus) NP_037290
Danio DβH	XM_694081.1	(Locus) XP_699173
Homo DβH	NM_000787.2	(Locus) NP_000778
Mus DβH	NM_138942.2	(Locus) NP_620392
Gallus NβH	XM_415429	XP_415429.1
Tetraodon DβH	CAAE01014752	CAG04874.1
Xenopus DβH	*Xenopus* Genome scaffold_953 (155802-139426)	
Takifugu DβH	*Fugu* Genome contig CAAB01004949 (6155-13757)	

Bullfrog (*Xenopus tropicalis*) MAT sequences were identified in genome scaffolds (version 4.1) at the DOE Joint Genome Institute Genome Project website (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Pufferfish (*Takifugu rubripes*) MAT sequences were identified in genome scaffolds (version 4.0) at the Institute of Molecular Biology Fugu Genome Project website (http://www.fugu-sg.org). Flour beetle (*Tribolium castaneum*) MAT sequences were constructed from genome scaffolds at the Tribolium Genome Database (http://www.bioinformatics.ksu.edu/BeetleBase). Schistosome MAT sequences were constructed from contigs in the *Schistosoma mansoni* genome assembly (version 3) at the Wellcome Trust Sanger Institute website (www.sanger.ac.uk/Projects/S_mansoni).