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Abstract

The Caenorhabditis elegans genome encodes nine homologues of mammalian glycoprotein-associated amino acid transporters. Two of these C. elegans proteins (AAT-1 and AAT-3) have been shown to function as catalytic subunits (light chains) of heteromeric amino acid transporters. These proteins need to associate with a glycoprotein heavy chain subunit (ATG-2) to reach the cell surface in a manner similar to that of their mammalian homologues. AAT-1 and AAT-3 contain a cysteine residue in the second putative extracellular loop through which a disulfide bridge can form with a heavy chain. In contrast, six C. elegans members of this family (AAT-4 to AAT-9) lack such a cysteine residue. We show here that one of these transporter proteins, AAT-9, reaches the cell surface in Xenopus oocytes without an exogenous heavy chain and that it functions as an exchanger of aromatic amino acids. Two-electrode voltage clamp experiments demonstrate that AAT-9 displays a substrate-activated conductance. Immunofluorescence shows that it is expressed close to the pharyngeal bulbs within C. elegans neurons. The selective expression of an aat-9 promoter-green fluorescent protein construct in several neurons of this region and in wall muscle cells around the mouth supports and extends these localization data. Taken together, the results show that AAT-9 is expressed in excitable cells of the nematode head and pharynx in which it may provide a pathway for aromatic amino acid transport.
Aromatic amino acid transporter AAT-9 of Caenorhabditis elegans localizes to neurons and muscle cells

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SUMMARY

The *C. elegans* genome encodes nine homologues of mammalian glycoprotein-associated amino acid transporters. Two of these *C. elegans* proteins (AAT-1 and -3) have been shown to function as catalytic subunits (light chains) of heteromeric amino acid transporters. These proteins need to associate with a glycoprotein heavy chain subunit (ATG-2) to reach the cell surface in a similar manner to their mammalian homologues. AAT-1 and 3 contain a cysteine residue in the second putative extracellular loop through which a disulfide bridge can form with a heavy chain. In contrast six *C. elegans* members of this family (AAT-4 to -9) lack such a cysteine residue. We show here that one of these transporter proteins, AAT-9, reaches the cell surface in *Xenopus* oocytes without an exogenous heavy chain and that it functions as an exchanger of aromatic amino acids. Two-electrode voltage clamp experiments demonstrate that AAT-9 displays a substrate-activated conductance. Immunofluorescence shows that it is expressed close to the pharyngeal bulbs within *C. elegans* neurons. The selective expression of an *aat-9* promoter-GFP construct in several neurons of this region and in wall muscle cells around the mouth supports and extends these localization data. Taken together, the results show that AAT-9 is expressed in excitable cells of the nematode head and pharynx in which it may provide a pathway for aromatic amino acid transport.
INTRODUCTION

The genome of Caenorhabditis elegans contains more than 750 genes encoding proteins thought to be involved in transmembrane transport processes. This represents ~4% of the total estimated number of genes. Most of these proteins are functionally not characterized and their roles are predicted based on phylogenetic inference (1). Among these putative transporter genes, nine encode homologues of heteromeric amino acid transporter catalytic subunits. Mammalian heteromeric amino acid transporters (HAT) are composed of two subunits. Besides a catalytic subunit (the light chain), they contain a type II glycoprotein subunit (the heavy chain). The light chain must associate with the heavy chain for functional expression at the cell surface (2-7). This association is stabilized by a disulphide bridge formed between two highly conserved cysteine residues (7). Only three out of the nine C. elegans catalytic subunit homologues display this conserved cysteine. In a previous study (8), we characterized two of these three catalytic subunits, AAT-1 and AAT-3. These functionally interact with ATG-2, a C. elegans glycoprotein subunit homologue. Since the remaining six light chain homologues, AAT-4 to 9, do not have the conserved cysteine, we hypothesize that these may not need interaction with a heavy chain for functional surface localization. In addition, these six light chain homologues are phylogenetically more distant than AAT-1 to 3 from the mammalian catalytic subunits. Figure 1. illustrates the phylogenetic relationship between members of the AAT protein family in C. elegans and selected human homologues.
We have now observed that AAT-9 (Wormbase gene no. Y53H1C.1) expressed alone in Xenopus oocyte localizes to the cell surface. We have therefore investigated its putative transport function in this expression system. Our results show that it transports aromatic amino acid substrates most likely by obligatory exchange and displays a substrate activated anion conductance. Localization experiments in C. elegans showed that it is expressed in some neurons and in muscular cells.

**EXPERIMENTAL PROCEDURES**

**cDNA cloning and cRNA synthesis**

Extraction of RNA form mixed stage C.elegans, cDNA synthesis, cloning into Xenopus oocytes cRNA expressing vector and *in vitro* cRNA synthesis were all performed as described previously (8). The full length cDNA for aat-9 (Wormbase gene no. Y53H1C.1) was amplified by RT-PCR using following primers: aat-9 fwd: 5’-GAT CGG CGC GCC ATG TCA TCA ATA GAA GAT CTC-3’; aat-9 reverse: 5’-CTT GCG GCC GCT TAC AGA CGA GTC ATT GGC-3’, designed according to the predicted initiation and termination sequence of the aat-9 gene. Primers contained restriction sites to facilitate cloning into the cRNA expression vector pSD5easy (9).

**Functional experiments in Xenopus laevis oocytes**
Uptakes: oocyte preparation and cRNA injection were carried out according to the previously described procedures (8,10). Briefly, injected oocytes were incubated for 72 h in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES pH 7.4 adjusted with KOH) supplemented with tetracycline (50 mg/l). Then, seven oocytes per condition were washed 6 times either in +Na\(^+\) (100 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES pH 7.4 adjusted with KOH) or -Na\(^+\) (100 mM CholineCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES pH 7.4 adjusted with KOH) buffer and pre-incubated for 2 min at 25°C. Subsequently, oocytes were transferred to +Na\(^+\) or -Na\(^+\) buffer containing substrate at the indicated concentration, with corresponding radiolabeled tracer, for the given time (Hartmann Analytic, Braunschweig, Germany). Further oocyte treatment, washing and individual counting of radioactivity incorporated has been described elsewhere (8). The mean uptake rate of endogenous oocyte transporters (H\(_2\)O-injected control oocytes) was subtracted (with the exception of results presented in Fig. 4A) from each uptake value obtained from oocytes expressing AAT-9.

Concentration dependence: uptake experiments to determine concentration-dependence were undertaken at five different concentrations of L-Phe. Values obtained in two different experiments were normalized to the mean uptake rate at 3 mM L-Phe. The Michaelis-Menten equation was fit to the experimental data to obtain an estimate of the apparent substrate affinity using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California.
**Efflux:** for efflux experiments, oocytes were injected with 5 nmol of L-Phe dissolved in water, containing 1 nCi of L-[³H]Phe as tracer. Injected oocytes were transferred to +Na⁺ solution that was supplemented with 1 mM L-Phe, as indicated. Five µl aliquots of the incubation solution were taken at 0, 1, 3, 10, 30 and 60 min and counted by liquid scintillation. The percentage of effluxed L-Phe was calculated as described earlier, subtracting the efflux measured in oocytes not expressing the transporter (8).

**Transstimulation:** oocytes expressing AAT-9 were injected with increasing amounts of cold L-Phe (in 50 nl of H²O ) and incubated 10 min in ND96 buffer at 16ºC. Oocytes were then washed six times with ND96 and the uptake (1 min) of 1 mM L-Phe was measured as described above. The values obtained in three different experiments were normalized to the uptake measured in the absence of transstimulation (H₂O-injected oocytes) and pooled.

**Electrophysiology:** oocytes expressing the AAT-9 transporter were placed in a small plexiglas superfusion chamber (0.2 ml volume) and continuously superfused (5 ml/min) with +Na⁺, -Na⁺ (see above), or -Cl⁻ buffer (equimolar replacement of Cl⁻ by morpholinoethane sulfonic acid (MES)). All experiments were performed at 20-22 ºC. The substrate application did not exceed 20 s to avoid possible loading of the cell. The long-term stability of the preparations was monitored and each new test solution was applied only when the holding current had returned to the previous control value. Oocytes were voltage-clamped using a custom-built two-electrode voltage clamp. Cells were normally clamped at a holding potential of -50 mV (11). I-V curves were obtained using a stair case
protocol in which the holding potential was stepped from -120 mV to + 40 mV in 20 mV steps, each of 100 ms duration. Data were normalized to a values obtained at -100 mV in the +Na+ buffer.

**Immunocytochemistry on oocyte cryosections**

Polyclonal rabbit antibody was raised against the synthetic peptide (NH$_2$-SSIEDLPTVGDVAATRHQC-COOH) corresponding to the NH$_2$- terminus of AAT-9. Peptide preparation, rabbit immunization and antibody purification were made as described (12). Oocyte treatment, freezing, cutting and immunostaining were carried out as detailed elsewhere (8,13,14). An antibody dilution of 1:30 was used. Sections were viewed using an epifluorescence microscope (Nikon ECLIPSE TE300/200 Inverted microscope).

**Plasmid construction and generation of transgenic C. elegans**

The *aat-9::gfp* transcriptional reporter gene was produced by PCR amplification of a 2.67kb genomic fragment containing 2.4 kb of 5' promoter sequence and 270 bp of the AAT-9 open reading frame using following primers: AAT-9 ffd: 5'-TTG CAT GCA GAA TCC GA C GCC TTC TTG G-3'; AAT-9 rev: 5'-AAG TCG ACC TCG AAT CGA CGT GCC TAG C-3'. Conditions for the amplification were: 98°C for 10 sec followed by 68 °C for 10 min, 30 cycles. The PCR product was cloned into Sphl and Sall cut pPD95.69 in frame with the green fluorescence protein (GFP) coding DNA (kindly provided by A. Fire). Transgenic animals were generated by injecting the DNA at the concentration 100 ng/µl into both arms of
the syncytial gonad as described (15). The plasmid pUNC119 (20 ng/µl) was used as a transformation marker (16).

**Immunostaining of C. elegans**

Standard methods were used for laboratory cultivation of the N2 variety of *C. elegans* Bristol strain (17). Animals from one plate were washed three times with 1 ml 1 X PBS buffer. To break the cuticle and permeabilize the animals, 500 µl of freshly prepared paraformaldehyde solution (2% paraformaldehyde, 12 mM NaOH, 1 mM HCl in 1 X PBS) and 500 µl of 2X RWB buffer (160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine-HCl, 30 mM PIPES ph7.4, 50 % metOH) were added and worms were frozen and thawed three times in liquid nitrogen and incubated for 30 min at 4ºC. Animals were subsequently washed three times with TTB buffer (100 mM TRIS pH 7.5, 0.1% Triton X 100, 1 mM EDTA). To permeabilize the cells, worms were then incubated in TTB buffer containing 0.5 % Triton X 100 and 2 % β-mercapto ethanol for at least 6 hours at 37ºC. Worms were washed twice in 4 X borate buffer (80 mM H₃BO₃, 40 mM NaOH) and incubated with shaking for 15 min at room temperature in 4 X borate buffer containing 10 µl 1 M DTT. Then, animals were washed twice with 4 X borate buffer and incubated with shaking for 15 min at room temperature in 4 X borate buffer containing 10 µl of H₂O₂. After washing two times with 4 X borate buffer, worms were incubated with shaking for 15 min at room temperature in ABA solution (1 X PBS with 2 % BSA). Overnight incubation in 200 µl of ABA containing antibody against AAT-9 (1:50 dilution) was performed at +4ºC. Next
day, worms were washed three times in PBST (1X PBS with 0.05 % Triton X 100), incubated for two hours at room temperature in 200 µl ABA with 2 µl of secondary anti-rabbit antibody (donkey anti-rabbit, Alexa) and again washed three times in PBST. In order to stain nuclei, 1:1000 DAPI was added in the last washing step. Worms were mounted in Moviol.

**Microscopy of C. elegans**

Fluorescent images were recorded with a Leica DMRA wide-field fluorescent microscope equipped with coded CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software package (Improvision). Localization of the AAT-9 fusion protein was analyzed with a Leica TCS4 confocal microscope.

**RESULTS**

**AAT-9 transports amino acids when expressed alone in Xenopus oocytes**

Unlike mammalian light chain subunits of HATs and *C. elegans* AAT-1 and 3 that must associate with an exogenous heavy chain subunit to reach the membrane surface, AAT-9 localizes to the plasma membrane of *Xenopus laevis* oocytes when expressed alone. This is shown by immunofluorescence on cryosections of oocytes expressing AAT-9 (Fig. 2). This finding was corroborated by the results of functional uptake experiments (see below). These facts do however not exclude the possibility that AAT-9 interacts with an endogenous oocyte protein to reach the oocyte surface.
Neuronal and muscle cell localization of AAT-9 in *C. elegans*

The localization of AAT-9 *in vivo* was investigated introducing a construct containing a GFP gene under the control of the *aat-9* promoter region by injecting it into the syncytial gonad of *C. elegans*. The transgenic progeny containing this extrachromosomal *aat-9 :: gfp* fusion construct showed a strong specific signal in the neuronal body, the position of which (lateral to the anterior pharyngeal bulb) and morphology (Fig. 3B, arrow), resembles that of the specifically stained neuron detected by immunofluorescence (see below). In addition, a prominent signal was present in a symmetrical pair of neurons also located in the vicinity of the anterior pharyngeal bulb. With processes running towards the tip of the animals extreme anterior (Fig. 3C, arrow), this pair of neurons displays a typical feature of chemosensory neurons exposed to the environment through specialized structures in the cuticle. A less prominent signal was observed in several additional neuronal bodies localized between the two pharyngeal bulbs. In addition, a signal was visible in the body wall muscles around the worm mouth (Fig. 3B and C, arrowhead). The signal detected in the posterior region (Fig. 3B, asterisk) was considered autofluorescence (as visible in other animals). Immunostaining of AAT-9 in whole wild type worms (Fig. 3D) showed a weak, but reproducible signal at least in one anterior neuronal body (arrow) and its process. A strong non-homogenous, presumably nonspecific staining (Fig. 3D asterisk) was also observed in the head of the worm (as with other antibodies). The extended localization observed with the transcriptional construct suggests that
AAT-9 is expressed in several additional neurons and muscle cells, possibly at a lower level than in the neuron labeled by immunofluorescence.

**AAT-9 exchanges aromatic amino acids and L-dopa**

Uptake of the amino acid L-Phe was observed in *Xenopus laevis* oocytes expressing only the AAT-9 transporter (Fig. 4A). Co-expression of *C. elegans* heavy chain homologues (ATG-1 or ATG-2) further increased this uptake by up to 85%. Substrate selectivity of AAT-9 was investigated using various labeled amino acids. To exclude the effect of endogenous amino acid transporters from *Xenopus* oocytes and to analyze only the transport induced by AAT-9, the mean values measured for H$_2$O injected oocytes from the same donor frog were subtracted. Figure 4B shows the uptake rate of different amino acids relative to that of L-Phe. (The mean L-Phe uptake rate was ~ 200 pmol/ hour per oocyte). L-Phe was found to be the preferred amino acid substrate of AAT-9. This protein also transported other aromatic amino acids, namely L-Trp, L-Tyr and L-dopa (a non-proteogenic neurotransmitter precursor) (Fig 4B,C). In contrast to L-dopa, L-dopamine was not a substrate for AAT-9. The uptake of L-Val, L-Met, L-Leu and L-His was not significantly above background and other proteogenic amino acids did not appear to be transported. The uptake of L-Phe in the absence of sodium (Fig. 4D) was reduced by 50 % compared to that observed in the presence of 100 mM NaCl. Interestingly, as detailed later, a reduction of the substrate induced conductance was also observed in the absence of sodium (Fig. 7B).
The time-course of L-Phe uptake at a concentration of 1 mM is shown in Fig. 5A. These data indicated that 1 min was an appropriate incubation time (linear phase) for measuring concentration-dependence. The uptake rate of L-Phe was determined at five different concentrations, background values were subtracted, and the data were normalized to those obtained for 3 mM L-Phe. Since saturation could not be reached, only an approximate apparent $K_m$ of $\geq 0.7$ mM could be derived from fitting of the Michaelis Menten function (Fig. 5B).

**Efflux of L-Phe via AAT-9 is an obligatory exchange**

Figure 6A shows the results of efflux experiments on oocytes expressing AAT-9. Oocytes were injected with 5 nmole L-Phe with tracer (this results in an intracellular concentration of injected L-Phe of $\sim 12.5$ mM, assuming a distribution volume of $\sim 400$ nl). The amount of effluxed substrate was measured at different time points in the presence (continuous line) or absence (dotted line) of 1 mM L-Phe in the extracellular buffer. In the absence of extracellular substrate, the amount of effluxed L-Phe was negligible. In contrast, a rapid efflux of a substantial amount of substrate was observed in the presence of extracellular L-Phe.

The question whether substrate influx could take place by facilitated diffusion (without exchange) was addressed using transstimulation, as described by Meier *et al* (18) and shown in Fig. 6B. The substantial increase in L-Phe uptake with increasing intracellular L-Phe suggests that inward facilitated diffusion is not as efficient as exchange. However, we can not exclude that facilitated diffusion
could take place in the absence of intracellular ligand (empty carrier switching from inward facing binding site configuration to outward facing configuration in the absence of ligand) since oocytes, as every cell, always contain endogenous amino acids.

**Substrate activated ion conductance of AAT-9 transporter uncoupled from its transport function**

Application of a near-saturating concentration of L-Phe to oocytes that expressed the AAT-9 protein and voltage clamped to $V = -50 \text{ mV}$, resulted in the activation of an inward current ($I_{\text{L-Phe}}$) that was typically 10-fold larger than that observed in non-injected oocytes from the same donor frog (Fig. 7 A). To determine the ionic components of this substrate-dependent current, we performed ion-substitution experiments over a range of membrane potentials ($-120 \text{ mV} < V < 40 \text{ mV}$) (Fig. 7 B). Recordings obtained in the absence of L-Phe were subtracted and the currents for the same oocyte were normalized to $I_{\text{L-Phe}}$ in the standard external bath solution at $V = -100 \text{ mV}$ (see Experimental procedures). First, substitution of external $\text{Na}^+$ ions with an equimolar concentration of choline resulted in approximately 50 % reduction in the current at all potentials, without a significant shift in the reversal potential. This indicated that although external $\text{Na}^+$ may contribute to $I_{\text{L-Phe}}$, the conductance pathway was not exclusively selective for this ion. Second, substitution of 94% of external $\text{Cl}^-$ with MES, resulted in a clear depolarising shift in the reversal potential from $-36 \text{ mV}$ to $+20 \text{ mV}$, as would be expected if the pathway was selective for $\text{Cl}^-$ ions and followed a Nernstian
relationship for the reversal potential. Moreover, we did not observe a correlation between membrane potential and L-Phe flux by measuring L-Phe uptake on voltage clamped oocytes (data not shown). This suggested that $I_{\text{L-Phe}}$ was thermodynamically uncoupled from the substrate flux. Similar findings with respect to the Cl$^-$ component of $I_{\text{L-Phe}}$ have been reported for the ASCT-1 amino acid exchanger (19) and glutamate transporters (20,21).

**DISCUSSION**

We have characterized a novel *C. elegans* amino acid transporter, AAT-9 that is a homologue of mammalian heretomeric amino acid transporter light chain subunits. AAT-9 exhibits 28-33% identity with several vertebrate homologues (8). Unlike the *C. elegans* proteins AAT-1 and -3 and all mammalian and non-mammalian members of the HAT family characterized so far, AAT-9 can reach the *Xenopus* oocyte cell membrane and perform transport without interacting with an exogenous heavy chain. The possibility that the AAT-9 transporter interacts with unknown endogenous *Xenopus laevis* protein(s) that might mediate its functional surface localization is not excluded. The AAT-9 transporter belongs to the subgroup of the AAT family that does not have a conserved cysteine in the second putative extracellular loop. In mammalian homologues, this cysteine forms a disulphide bridge with the heavy chain and stabilizes the subunit interaction. However, while AAT-9 does not exhibit this prominent feature of HATs, our results show a further increase in substrate uptake (70-85%, Fig. 4A).
by oocytes co-expressing ATG-1 or ATG-2 heavy chains with the AAT-9 light chain. This increase might be due to a non-covalent interaction between heavy and light chain that stabilizes AAT-9 in the membrane. The nature of the interaction of AAT-9 with *C. elegans* heavy chains was not investigated in the present study.

AAT-9 expressed in *Xenopus* oocytes specifically transports the aromatic amino acids, L-Phe, L-Trp and L-Tyr as well as L-dopa. Therefore, in terms of substrate selectivity, AAT-9 resembles the mammalian transporter TAT1 that is expressed mostly in kidney, skeletal muscle, placenta and heart (in humans) and in the small intestine (in rats) (22,23). Sequence similarities between AAT-9 and this transporter are, however, negligible. The previously characterized *C. elegans* transporters AAT-1/ATG-2 and AAT-3/ATG-2 transport aromatic neutral amino acids as well, but not exclusively as AAT-9. Transport of these substrates from the inside to the outside (efflux) is a common feature of AAT-9 and rat TAT1. But, in contrast to TAT1, the efflux of substrate mediated by AAT-9 is detectable only in the presence of extracellular substrate, indicating that efflux via AAT-9 corresponds to an obligatory exchange. This property of AAT-9 is common with AAT-1/ATG-2 and AAT-3/ATG-2 that function as nearly obligatory exchangers. Its approximate apparent affinity of ≥ 0.7 mM for its preferred substrate L-Phe is similar to that determined for human TAT1 (Km ~0.45 mM for L-Trp), and probably higher than that of rat TAT1 (Km ~5 mM for L-Trp,).

Using two independent approaches to identify the *in vivo* localization of AAT-9, immunofluorescence and expression of a transcriptional GFP fusion construct,
we detected a signal in several neurons positioned between the two pharyngeal bulbs and in body wall muscles of the mouth region. The neuronal localization of AAT-9 together with its selectivity for the transport of aromatic amino acids suggests a possible function in providing the cells with precursors of transmitters such as serotonin and/or dopamine. The fact that AAT-9 is localized in possible chemosensory neurons positioned in the vicinity of the anterior pharyngeal bulb suggests a potential role in sensing food or food breakdown products. The role of the AAT-9 amino acid transporter in muscle cells is not yet understood. It is, however noteworthy that neurons and muscle cells are electrically active cells that conduct action potentials. In this context, AAT-9 acting as a substrate-activated ion channel might play a role in stabilizing the membrane potential, as has been suggested for the anion conductance of excitatory amino acid transporters (EAAT) (24).

No obvious phenotype was revealed either in our attempt to knock down AAT-9 by feeding wild type worms with bacteria containing an AAT-9 RNA interference (RNAi) construct (data not shown), or in a large genome wide screening by Kamath et al. (25). This is not a definitive finding since it has been recognized that gene silencing by RNAi feeding is mostly not successful in the case of genes expressed in neurons (Alex Hajnal, personal communication) and some subtle phenotypes might not have been detected. Creating a real knock out for aat-9 and establishing particular physiological assays would be challenging, but necessary to determine more precisely the physiological role of AAT-9 in C.elegans.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.**
Phylogenetic relationship of the AAT-9 transporter and other *C. elegans* and human amino acid transporters. The phylogenetic tree is based on amino acid sequence alignment and bootstrap analysis using Clustal W and viewed using TREE software. The tree includes the predicted amino acid sequences of 9 *C.elegans* and 7 human transporters. The *C. elegans* proteins are highlighted by ellipses and AAT-9 is shaded.

**Figure 2.**
*C. elegans* AAT-9 transporter expressed alone in *Xenopus* oocytes is localized at the cell membrane. Immunofluorescence with anti-AAT-9 antibody on cryosections of *Xenopus* oocytes injected with AAT-9 cRNA (left panel) or H₂O (control, right panel) shows AAT-9 at the cell surface.

**Figure 3**
AAT-9 expression in the head region of wild type C. elegans. A. Nomarski image of the animal shown in the two next panels. B and C. The GFP fluorescence signal (green) is observed in neuronal bodies (arrows) and in body wall muscles (arrowheads) of aat-9 :: gfp transgenic animals. The upper arrow in panel B indicates a neuron possibly corresponding to the one labeled by immunofluorescence (below, panel D). The signal in the posterior region (panel B, asterisks) is considered as autofluorescence. In a different focal plane of the same worm (panel C), a specific GFP signal is seen in a symmetrical pair of presumably sensory neurons (upper arrow) localized ventral to the pharynx. D. Immunofluorescence with AAT-9 antibody (red) shows a signal at the level of a neuronal body (arrow). Nuclei are stained with DAPI (blue). The punctuated signal in the anterior head region (asterisk) is considered non specific. Scale bars: 50 µm

**Figure 4**

Uptake of aromatic amino acids by AAT-9. A. Expression of AAT-9 induces the uptake of L-Phe (1 mM) also in the absence of exogenous heavy chain. Means (+/- SEM) of 12-15 oocytes from two experiments are shown. The difference between control values (H₂O injected oocytes) and test values was evaluated using one way ANOVA followed by Bonferroni’s multiple comparison test (** P ≤ 0.01, *** P ≤ 0.001). L-Phe uptake rate was further stimulated in the presence of either heavy chain ATG-1 or -2 (by 70 - 85%). B and C. The uptake of L-amino acids (50 µM, panel B), L-dopa and L-dopamine (100 µM, panel C) was
measured for 6 min in oocytes expressing AAT-9 in the presence of Na\(^+\) (100 mM). Absolute values were normalized to the uptake rate of L-Phe. Means of values normalized to the L-Phe uptake rate obtained from 12-14 oocytes pooled from two experiments are shown. D. The uptake of 50 µM L-Phe in the presence and absence of Na\(^+\) was measured for 6 min. Means of normalized data (n=27) from 4 experiments are shown.

**Figure 5**

Time course and concentration-dependence of L-Phe uptake in the presence of sodium by oocytes expressing AAT-9 transporter. A. The uptake of 1 mM L-Phe was measured after 1, 3, 6 and 12 min incubation at 25 °C. The results from one of three replicate experiments are shown. A one minute incubation period was chosen for the concentration dependence experiments (B). L-Phe uptake by oocytes expressing AAT-9 was measured at 5 different concentrations and results from two experiments were pooled after normalizing them in each experiment to the value obtained for 3 mM L-Phe. The Michaelis Menten function was fitted to the data using a non-linear regression routine (Prism 3.0). The Km is ≥ 0.7 mM. Error bars represent SEM of values obtained using 14 oocytes.

**Figure 6**

Stimulation of efflux and transstimulation of uptake of L-Phe. A. Oocytes were injected with 5 nmole L-Phe containing tracer amounts of L-[\(^3\)H]Phe and efflux was measured in the presence or absence of 1 mM unlabeled L-Phe in the buffer
Background efflux from oocytes not expressing the transporter (water injected oocytes) was subtracted. Data are means (+/- SEM) of 10 oocytes from two independent experiments. B. Oocytes were injected with the indicated amounts of L-Phe, incubated for 10 min at 16ºC in ND96 and the uptake of L-Phe (1 mM) measured for one min. Data from three independent experiments were pooled after normalizing them to the uptake rate in the absence of transtimulation. Bars represent the mean of 21 oocytes and error bars the SEM.

**Figure 7**

Substrate induced conductance. A. Challenging an oocyte expressing AAT-9 with a near saturating concentration of L-Phe (2 mM) induced inward current when the voltage was clamped at –50 mV (upper trace). A representative trace from a non-injected oocyte from the same donor frog under the same conditions showed a substantially smaller induced current.

B. Current-voltage (I-V) curves showing $I_{L-Phe}$ determined from the difference between the holding current in the presence and absence of of 2 mM L-Phe using a voltage staircase protocol (See Materials and Methods). Three superfusion conditions are shown: normal bath solution (filled squares); Na⁺-free (filled triangles) and low Cl⁻ (100 mM MES, 7 mM Cl⁻, open triangles). Data were pooled from 9 oocytes from 3 donor frogs and are shown as mean ±SEM. For each oocytes, currents were normalized to $I_{L-Phe}$ in normal bath solution at –100 mV.
FOOTNOTE

1The abbreviations used are: HAT, heteromeric amino acid transporters; AAT-1, AAT-3, *C. elegans* amino acid transporter catalytic subunit; ATG-1, ATG-2, *C. elegans* amino acid transporter glycoprotein subunit

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