A Screen for Neurotransmitter Transporters Expressed in the Visual System of *Drosophila melanogaster* Identifies Three Novel Genes

Rafael Romero-Calderón,[1] Ratula M. Shome,[2] Anne F. Simon,[2] Richard W. Daniels,[3] Aaron DiAntonio,[3] David E. Krantz[2]

[1] Interdepartmental Ph.D. Program in Neuroscience, University of California, Los Angeles, California

[2] Department of Psychiatry and Biobehavioral Sciences, The David Geffen School of Medicine at UCLA, Gonda (Goldschmied) Center for Genetic and Neuroscience Research, Los Angeles, California 90095-1761

[3] Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 12 June 2006; revised 15 September 2006; accepted 16 September 2006

ABSTRACT: **The fly eye provides an attractive substrate for genetic studies, and critical transport activities for synaptic transmission and pigment biogenesis in the insect visual system remain unknown. We therefore screened for transporters in *Drosophila melanogaster* that are down-regulated by genetically ablating the eye. Using a large panel of transporter specific probes on Northern blots, we identified three transcripts that are down-regulated in flies lacking eye tissue. Two of these, *CG13794* and *CG13795*, are part of a previously unknown subfamily of putative solute carriers within the neurotransmitter transporter family. The third, CG4476, is a member of a related subfamily that includes characterized nutrient transporters expressed in the insect gut. Using imprecise excision of a nearby transposable P element, we have generated a series of deletions in the *CG4476* gene. In fast phototaxis assays, *CG4476* mutants show a decreased behavioral response to light, and the most severe mutant behaves as if it were blind. These data suggest an unforeseen role for the "nutrient amino acid transporter" subfamily in the nervous system, and suggest new models to study transport function using the fly eye.**
Develop Neurobiol 67: 550–569, 2007

Keywords: **SLC6; amino acid permeases; Northern blot; fast phototaxis; CG4476**

INTRODUCTION

Plasma membrane neurotransmitter transporters terminate synaptic transmission following the regulated release of neurotransmitter (Masson et al., 1999; Amara and Fontana, 2002; Torres et al., 2003; Blakely et al., 2005). Mutations that reduce transport function increase the synaptic concentration of transmitter and can significantly influence behavior (Gainetdinov et al., 2002; Hahn and Blakely, 2002), and, in some cases, reduced function may also promote excitotoxicity (Amara and Fontana, 2002; Kanai and Hediger, 2003).

Correspondence to: D.E. Krantz (dkrantz@ucla.edu).
Contract grant sponsor: National Institute of Health; contract grant numbers: R21 DK60857 (DEK), NS051453 (AD), T32 GM08151 (RWD).
Contract grant sponsor: EJLB (DEK).
Contract grant sponsor: Edward Mallinckrodt, Jr. Foundation (DEK).
Contract grant sponsor: Shirley & Stefan Hatos Neuroscience Research Foundation.
Contract grant sponsor: American Psychological Association (R.R-C.).

Published online 9 February 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/dneu.20342

Most drugs used to treat depression and anxiety, as well as psychostimulants such as cocaine and methylphenidate target neurotransporters (Blakely and Bauman, 2000; Rothman and Baumann, 2003), and additional drugs that target transporters may be useful for neurological diseases (Schousboe et al., 2004). Despite their scientific and clinical importance, the regulation of most neurotransmitter transporters remains poorly understood (Torres et al., 2003). Model genetic organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* may be useful to study the genetics of transporter function and to identify novel pathways involved in their regulation (Nass and Blakely, 2003; Wu and Gu, 2003; Nass et al., 2005; Chang et al., 2006). However, the study of neurotransmitter transporters in model invertebrates has been relatively limited compared with studies in mammals.

Most currently characterized neurotransmitter transporters belong to one of two structurally distinct solute-linked carrier (SLC) families: SLC1 and SLC6 (Hediger et al., 2004). Both families drive transport by using electrochemical gradients across the plasma membrane that include sodium, and in some cases chloride, potassium, or proton exchange (Masson et al., 1999; Chen et al., 2004). Characterized SLC6 members include the serotonin (SERT), dopamine (DAT), and GABA transporters (GAT) (Chen et al., 2004). SLC1 is composed of the excitatory amino acid transporters (EAATs), which are responsible for glutamate uptake (Kanai and Hediger, 2003). Additional amino acids known to function as neurotransmitters include glycine (Zafra et al., 1997) and possibly proline (Takemoto and Semba, 2006). In mammals, sodium-dependent transporters for both of these amino acid substrates have been identified as members of the SLC6 family, and are structurally similar to SERT, DAT, and GAT (Fremeau et al., 1992; Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992).

Multiple additional SLC families contain the remaining amino acid transporters. The members of these families are structurally and bioenergetically distinct from SLC6 transporters, and in some cases function as obligatory amino acid exchangers (Castagna et al., 1997). In addition, recent studies have identified novel invertebrate members of SLC6 that form distinct subfamilies (Boudko et al., 2005), and shown that some SLC6 members previously classified as orphans function as broad-substrate amino acid transporters (Broer, 2006). Although some are expressed in the brain, the functional role in the nervous system for most amino acid permeases and the more recently described members of SLC6 (Boudko et al., 2005; Broer, 2006) remain poorly defined. Indeed, most have been characterized primarily as nutrient transporters in the gut, kidney, and other non-neuronal tissues (Verrey et al., 2004; Boudko et al., 2005; Broer, 2006).

The anatomy of the *Drosophila* eye and optic ganglia is well characterized (Campos-Ortega, 1980; Meinertzhagen, 2000; Hsiung and Moses, 2002) and vision is not required for either viability or fertility. These and other advantages have made the *Drosophila* visual system a favored model to study a number of neuronal processes (Clandinin and Zipursky, 2002; Marsh and Thompson, 2004; Nagaraj and Banerjee, 2004; Pearson and Doe, 2004; Wernet and Desplan, 2004; Chen and Mardon, 2005; Schweisguth, 2005). We are interested in similarly using the *Drosophila* visual system to study the function and regulation of neurotransmitter transporters. Although genetic methods are available to restrict the expression of ubiquitous genes to the eye, transporters that are endogenously expressed in visual pathways may provide particularly useful genetic reagents to study transporter function and regulation. Furthermore, the molecular identities of several predicted transport activities thought to be essential for visual function remain unknown (Melzig et al., 1998; Borycz et al., 2002). We have therefore performed a screen to identify transporter-like genes that are either expressed in the eye or in downstream pathways that may be regulated by visual function.

Using Northern blots to differentially screen mRNA from wild type and eyeless flies, we identified three putative transporters whose expression is down-regulated by genetically ablating the eye. Two of these, *CG13794* and *CG13795*, correspond to a previously unknown SLC6 subfamily. The third, *CG4476*, belongs to the "nutrient amino acid transporter" (NAT) subfamily of SLC6 that have been previously defined based on their expression and function in the gut (Boudko et al., 2005). We have generated a series of deletions in *CG4476* and found that the flies are visually impaired. Our data suggest an unforeseen role for the NAT family of transporters in the nervous system, and suggest potential models for the study of neurotransmitter transporters using the fly eye.

METHODS

Phylogenetic Analysis

All sequences of the transporter gene candidates were obtained in the public database at www.flybase.net. Sequence alignments were performed using Clustal X, version 1.83 with a gap opening penalty of 10.00, a gap extension penalty of 0.20, a delay of divergent sequences of 30%, and the IUB DNA weight matrix. Phylogenetic relationships were constructed by the neighbor joining (NJ) method and

bootstrapped with 2000 iterations and a random number generator seed of 111. Unrooted phylograms were drawn using TreeView 1.6.6. Internal references included *dSERT*, *dDAT*, *blot* and *dGAT* (*CG1732*) for Figure 1(A), and *slif*, *mnd*, *JhI-21* and the *dEAATs* for Figure 1(B).

Northern Blots

For mRNA extraction, population cages containing Oregon-R and GMR-*hid Drosophila* stocks were cultured as previously described (Sisson, 2000) at room temperature (23°C) with a relative humidity of 20–40% in a 12 h dark/light cycle. Adult male and female flies were harvested by freezing over dry ice and stored at −80°C until use. Heads, bodies (thorax and abdomen), and appendages were mechanically separated by vigorous shaking of the frozen adult fly stocks over dry ice. Purification of heads and bodies was achieved by sequential passage through 0.71 mm (No. 25) wire mesh to retain the bodies and 0.38 mm (No. 40) wire mesh to retain the heads. Poly-A mRNA was isolated sepa-

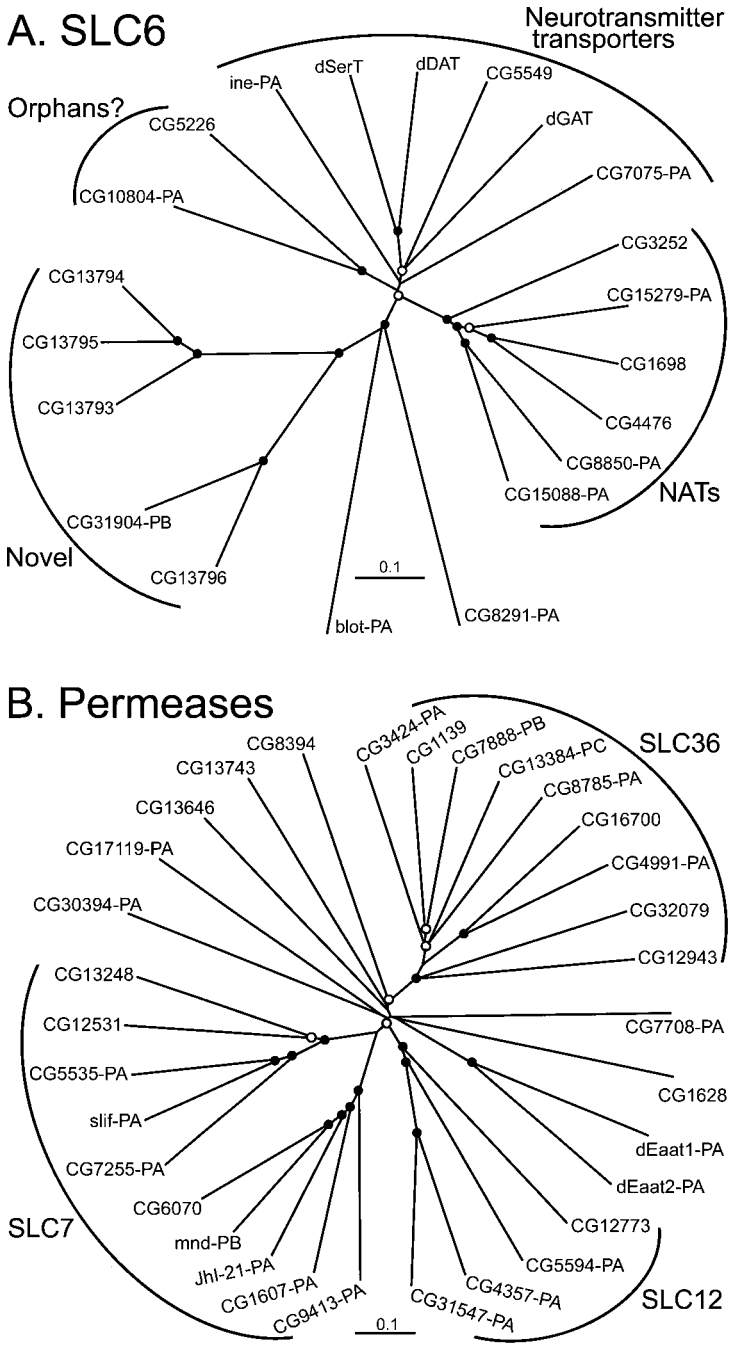

Figure 1

rately from heads and bodies from both wild type and GMR-*hid* flies using the MicroPoly(A) Pure kit (Ambion).

For Northern blotting, 2.5 μg/lane of Poly-A mRNA was electrophoresed through agarose containing formaldehyde, and transferred to Hybond XL filters (Amersham-Pharmacia). To generate probes, we amplified a $\sim$500 bp exonic region representing each transporter candidate using wild type (Oregon-R) genomic DNA as template. PCR reactions were carried out in a PTC-200 Peltier thermal cycler (MJ Research) using 20 ng genomic DNA, 2.5 U of Taq polymerase (NEB), 0.1 μM of each primer and 0.2 mM dNTP mixture with an annealing temperature of 55°C, and an extension time of 30 s for 35 cycles. Probes were synthesized from gel-purified PCR products using random priming (Rediprime II, Amersham-Pharmacia) and α-^{32}P-dCTP, separated from free nucleotides by filtration (Qiagen Nucleotide Removal Kit) and hybridized to blots in Rapid-hyb Buffer (Amersham-Pharmacia) for at least 12 h. After one 10 min wash in 2× SSC, 0.1% SDS, and two 10 min washes in 1× SSC, 0.1% SDS, blots were exposed on Kodak Biomax XAR film for 0.5–3 h at −80°C, using a Fisher biotech L-plus screen for signal amplification. All membranes were stored at 4°C for 8 half lives (4 months), then reprobed with an *actin-5C* control probe generated from the oligonucleotides (5′–3′) AAGTTGCTGCTCTGGTTGTCG and AGGGCATAACCCTCGTAGATG as a control for loading. The Northern blots for the control genes *chaoptin* and *histidine decarboxylase* and for the three gene candidates preferentially expressed in the eye were done in triplicate. An additional nine gene candidates were randomly chosen to replicate, and in all cases showed comparable results.

The exposures were digitized as 300 dpi grayscale images using an Epson Perfection 2450 Photo scanner, and the adjusted for brightness and contrast using Adobe Photoshop 6.0. Quantitation of band intensity was measured using Image J (freeware, http://rsb.info.nih.gov/ij/) and the data processed using Microsoft Excel. Graphs were obtained with Sigma Plot 8.0.

Reverse Transcriptase Polymerase Chain Reactions

For reverse transcriptase polymerase chain reactions (RT-PCR), poly-A mRNA was isolated as described above for Northern blotting, and single stranded cDNA was synthesized from 1 μg of mRNA template using Transcriptor reverse transcriptase (Roche), 1 mM dNTP mix (NEB), and 0.625 μM poly-dT primer incubated at 55°C for 1 h. The transcriptase was heat inactivated for 5 min at 85°C, and the cDNA was used directly in PCR reactions without further purification. PCR reactions were prepared and carried out as described above, using 1 μL of cDNA product. $\sim$500 base pair diagnostic fragments of *CG13794* and *CG13795* were selectively amplified using oligonucleotide pairs (5′–3′) GGGCTATACTAACGTCAC/AAAGTGTGGTCTGTGAAA and CGGTAATATTGTGGGTAG/ATGGAGCAGCTGGTGAGA, respectively.

The full-length products for *CG4476*, *CG13794*, and *CG13795* were obtained using oligonucleotide pairs (5′–3′) GAAACTGGAGACCAGCAG/GTCATCCTGGTGTACAGC, ACGCTCGCGAAATGTTTG/AGCACACTAATATTCCTA, and CGGTAATATTGTGGGTAG/CTCTTCGAGGTTACTGAG, respectively. The PCR reactions were prepared as described above, using 1 μL of cDNA product and the cycling parameters were modified for an extension time of 2 min.

Gene expression patterns were analyzed using a *Drosophila* Rapid-Scan gene expression panel (OriGene Technologies). Duplicate panels were prepared as described by the manufacturer using oligonucleotide pair (5′–3′) GAAACGGATTCCCTGGAG/GATGAAAGCCAATCCAGG for *CG4476* and the oligonucleotide pairs listed above for *CG13794* and *CG13795*.

Figure 1 *Drosophila* neurotransmitter transporter and amino acid permease candidates cluster into distinct subfamilies. (A) Unrooted phylogram of 17 neurotransmitter transporter-like candidates found in the *Drosophila* genome. The known *Drosophila serotonin transporter* (*dSERT*), *Drosophila dopamine transporter* (*dDAT*), *bloated tubules* (*blot*), and *inebriated* (*ine*) are used as internal references. Three distinct subfamilies of transporters are identified, which include the neurotransmitter transporters, the insect specific nutrient amino acid transporters (NATs), the orphan-class amino acid transporters and a novel subfamily composed of five members probably formed by gene duplication (Fig. 5). Two genes, including *blot*, appear to be distinct from the other members. (B) Unrooted phylogram of 27 different amino acid permease-like candidates, using the *Drosophila* excitatory amino acid transporters (*dEAAT* 1 and 2), *mini discs* (*mnd*), *slimfast* (*slif*), and *juvenile hormone inducing gene 21* (*JhI-21*) as references. The electroneutral cation-Cl^- cotransporter family (SLC12), the H^+-coupled amino acid transporter family (SLC36), and the cationic amino acid permease family (SLC7) are readily identifiable. As in mammals, SLC7 forms two distinct clusters. Seven other genes with putative transporter function appear to be distinct from the other members. Where more than one polypeptide sequence was available in the public database for a given gene, the actual peptide used in the phylogenetic analysis was denoted with the suffix -P. Bootstrap values are denoted on the nodes as filled circles for $\geq$0.90 and open circles for 0.70–0.90 (see Materials and Methods section for details). Bars represent the average substitutions per site along the branches.

Expression in S2 Cells

The Drosophila serotonin transporter (*dSERT*; *CG4545*) and *CG4476* were subcloned using PCR into the pMT/V5-His A vector (Invitrogen) vector using EST clones RE10485 (accession no. AY071023) and RE33779 (accession no. BT003509), respectively. A *Drosophila* transcription initiation sequence (CAAC) (Cavener, 1987) and an HA tag (5′-ATGTACCCCTACGACGTCCCCGACTACGCC-3′) were added to the 5′ primers. Plasmids were purified using the NucleoBond Plasmid Mini Kit (Clontech) as per manufacturer's instructions, and all constructs sequenced at the UCLA sequencing facility.

Drosophila S2 cells were cultured at room temperature (23°C) in 10 cm cell culture plates using Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 I.U./mL penicillin/streptomycin (Cellgro). Stably transfected S2 cell lines were prepared by cotransfecting pMT constructs containing the gene of interest and pcHYGRO vector (Invitrogen), using FuGENE 6 transfection reagent (Roche) as per the manufacturer's instructions. Following an initial 2- to 3-day incubation in standard S2 cell media, colonies were selected in media containing 400 μg/mL hygromycin for 1–3 weeks. Up to five colonies with >80% positively stained cells were used for subsequent experiments, and stored in liquid nitrogen or maintained in hygromycin containing media.

Immunofluorescent Labeling

Drosophila S2 cells ($\sim 2 \times 10^5$ cells) were plated over poly-L-lysine coated coverslips in 24-well plates and induced using 0.7 m*M* $CuSO_4$ for 24–48 h. The cells were fixed in 4% PFA, 0.1 *M* Na_2HPO_4, pH 7.4 (fix) for 20 min, washed 3× in PBS, blocked in PBS, 0.2% TritonX-100, 5% FBS (block) and incubated in 1:400 mouse α-HA tag 1° antibody for 2 h and in 1:500 α-mouse-FITC conjugated 2° antibody for 45 min, at room temperature. The cover slips were then washed and mounted using ProLong Antifade (Molecular Probes).

The stained *Drosophila* S2 cells were imaged using a Zeiss Axioskop 2 fluorescent upright microscope, the pictures obtained with an AxioCam HRc digital camera, digitized with the AxioVision 4.0 picture analysis software, and processed with Adobe Photoshop 6.0. The S2 cell staining procedure was done a minimum of five independent times in duplicate, and the pictures shown here are representative of at least 100 different S2 cells visualized using a Plan-Neofluar 100×/1.30 oil pol, ∞/0.17 objective.

Electroretinograms

Electroretinograms (ERGs) were preformed as described previously (DiAntonio and Schwarz, 1994). Flies were briefly anesthetized on ice and then mounted in modeling clay so that one eye was exposed to the light. ERGs were recorded using a sharp glass electrode with access resistance of ~40 Mohms when filled with 3 *M* KCl placed in the eye and a tungsten reference electrode placed in the back of the head. Dark-adapted flies were exposed to multiple pulses of white light and a representative trace was chosen for presentation. We did not observe a loss or rundown of on- or off-transients after exposure to rapid light pulses.

P-Element Induced Imprecise Excisions

A survey of the public databases revealed a previously generated line in which a P element XP had been inserted into the 5′ UTR of *CG4476* (http://flymap.lab.nig.ac.jp/getdb.html). Line 19350 was obtained from the Bloomington *Drosophila* Stock Center and we confirmed that the P element was located 774 bp upstream of the predicted initiating methionine of *CG4476* (data not shown). The P element was excised using standard genetic methods (Greenspan, 1997). Briefly, the parent line was mated to a standard source of transposase (Δ2–3) and the F2 progeny screened for a loss of the mini-*white* gene indicating loss of all or part of the P element. F2 flies were mated individually to the balancer line *yw; + ;TM2/TM6B* and then to siblings to generate homozygous lines. All homozygous *CG4476* mutant lines were viable. To characterize the excisions in each line, we used the primer pair (5′–3′) CTACACAAAGCTGACAGG/TTGGCAGAGACGTTTGGT, which encode sequences 433 bp upstream (f1) and 2707 bp downstream (r2) of the parent P insertion site, respectively. Additional amplicons from each line generated with the primer pair (5′–3′) CTACACAAAGCTGACAGG/GATCACACCGAAACAGAC were subjected to automated DNA sequence analysis to confirm the extent of the deletions. In addition, we identified five lines in which the P element had been excised precisely resulting in a wild type DNA sequence. These precise excision lines were used as controls and include pe15, pe25, pe41, pe51, and pe52.

Positive (Fast) Phototaxis Assay

The *CG4476* P-element excised flies were prepared for positive phototaxis assays as previously described (Connolly and Tully, 1998) with minor modifications. Briefly, 3- to 7-day-old flies were gently sedated over ice and placed in fresh cornmeal–molasses–agar food vials at a density of 70–120 flies/vial. The flies recovered overnight at room temperature (23°C) before passing them to new cornmeal–molasses–agar food vials, and allowing them to acclimatize to the testing room (25°C, 50–70% relative humidity) for 2 h.

Positive (fast) phototaxis was assessed in the countercurrent apparatus designed by Benzer (Benzer, 1967), as described by Connolly and Tully (Connolly and Tully, 1998). Briefly, when testing locomotion toward the light, the flies were sharply tapped to the bottom of the first tube, and the apparatus was laid horizontally with the distal tubes 5 cm away from a 15 W fluorescent light. The flies were given 15 s to reach the distal tube, and this procedure was repeated five times per trial. Thus, the flies can choose to go toward the light a maximum of five times. The flies were collected, anesthetized under CO_2, and manually counted. To test reactivity in the dark, identical conditions were used but without

a light source. Manipulation of the apparatus occurred under a photo-safe dim red light (Kodak, wavelength >650 nm), which flies do not detect (Connolly and Tully, 1998). Experiments were performed three independent times in triplicate. Since the five precise excisions behaved similarly, their performances were pooled. Since males and females behaved similarly in the counter-current apparatus (data not shown), data representing each gender also was pooled. Statistical analyses were performed and graphs prepared using the Prism 4.0 statistical package.

RESULTS

To define neurotransmitter transporters and amino acid permeases that might be involved in visual function, we first surveyed the *Drosophila* genome for orthologs of known neurotransmitter transporters, amino acid permeases and additional, structurally similar genes. Our *in silico* search was performed using data from both the Berkley *Drosophila* Genome Project (www.fruitfly.org) and the *Drosophila* Genomes Annotation and Sequences Database (www.flybase.net). The results were cross-referenced to the Gene Ontology Consortium (www.geneontology.org) to help identify the putative functions of the gene candidates (Consortium, 2001). Based on their overall homology to previously characterized transporters, at least 49 genes in *Drosophila* are likely to express neurotransmitter and/or amino acid transporters/permeases. Of these, 11 have been characterized. These include transporters for which the substrates have been identified such as *dDAT* (Porzgen et al., 2001), *dSERT* (Corey et al., 1994; Demchyshyn et al., 1994), *dGAT* (Neckameyer and Cooper, 1998), the *dEAATs* 1 and 2 (Seal et al., 1998; Besson et al., 1999), the proton-dependent permease *pathetic* (Goberdhan et al., 2005), and the cation transporter *slimfast* (*slif*) (Colombani et al., 2003). Others have been defined genetically, but their substrates remain unclear. These include *inebriated* (*ine*) (Huang and Stern, 2002), *bloated tubules* (*blot*) (Johnson et al., 1999), *mini discs* (*mnd*) (Martin et al., 2000), and *Juvenile hormone inducing gene 21* (*JhI-21*) (Dubrovsky et al., 2002). Figure 1 displays the unrooted phylograms depicting these and all of the other putative neurotransmitter transporters (A) and putative amino acid permeases (B) that appear in the *Drosophila* genome.

Putative neurotransmitter transporter-like genes (the SLC6 family) in *Drosophila* cluster into several subfamilies [Fig. 1(A)]. The largest includes the *bona fide* neurotransmitter transporters *dSERT*, *dDAT*, and *dGAT* (*CG1732*). In addition, although *CG5549* has not been characterized in *Drosophila* or any other insect species, it is highly similar to mammalian glycine transporters [data not shown, see also (Boudko et al., 2005)]. This subfamily also includes the gene *ine*, widely believed to be a transporter, although the substrate remains undefined (Huang and Stern, 2002), and *CG7075*, which may be expressed in *Drosophila* germ cells (Bazinet, 2000). Related genes include *CG5226* and *CG10804*, both of which are similar to a subfamily of mammalian SLC6 transporters previously classified as orphans (Masson et al., 1999), but recently shown to function as amino acid transporters (Broer, 2006).

A second subfamily of SLC6 in *Drosophila* consists of those identified in other insect species as comprising the amino acid "nutrient amino acid transporter" group, or "NATs" thought to function primarily in the insect gut (Boudko et al., 2005). These include putative transporters contained in the fly genome that are similar to two index members first identified in *Manduca sexta*: the cation–anion-activated amino acid transporter/channel (CAATCH1) (Feldman et al., 2000) and the K^+-coupled amino acid transporter (KAAT1) (Castagna et al., 1998). This subfamily also contains the, mosquito, *Aedes aegypti* amino acid transporter 1 (aeAAT1), which primarily transports phenylalanine, and the *Aedes gambiae* amino acid transporters 6 and 8 (agAAT6 and 8), which transport tryptophan and tyrosine/phenylalanine, respectively (Boudko et al., 2005).

Our phylogenetic analysis of the *Drosophila* genome identifies a third SLC6 subfamily. This group is composed entirely of novel transporters showing up to 70% identity to each other, and includes *CG13793*, *4*, *5*, *6*, and *CG31904*. Strikingly, all five cluster together on the second chromosome. No function has yet been ascribed to any of these genes, and an extensive search of the public databases indicates that similar to the NATs, this novel subfamily is quite likely to be confined to insect genomes (data not shown). Interestingly, we find that the expression of two genes in this subfamily is enriched in the visual system (see below).

We constructed a separate phylogram for genes most likely to represent *Drosophila* putative amino acid permeases, that is, transporters that recognize amino acids and are not members of SLC6 [Fig. 1(B)]. Amino acid permeases in other animals belong to several distinct families (Hediger et al., 2004) and the phylogeny of the corresponding *Drosophila* families is similarly complex. Subfamilies previously characterized in vertebrates include the proton-coupled amino acid permeases (SLC36, PATs) and the cationic amino acid permeases (SLC7), both of which are found in *Drosophila* [Fig. 1(B)]. Similar to vertebrates, the

SLC7 subfamily in *Drosophila* appears to be composed of two distinct clusters, which may represent distinct cationic amino acid transporter (CAT) and the glycoprotein-associated/heterodimeric amino acid transporter (gpaAT/HAT) subfamilies (Verrey et al., 2004). In mammals, members of the gpaAT/HAT subfamily of SLC7 couple with a glycoprotein subunit (SLC3) to mediate the uptake of neutral and cationic amino acids (Verrey et al., 2004).

Mammals express five *EAATs* that make up the SLC1 family (Kanai and Hediger, 2003), whereas *Drosophila* contain only two (*dEAAT1* and *2*) (Seal et al., 1998; Besson et al., 1999). Interestingly, unlike mammalian EAATs, dEAAT2 shows a higher apparent affinity for aspartate than glutamate, and also transports taurine (Besson et al., 2000, 2005). In mammals, members of SLC38, systems N and A, are thought to mediate glutamine efflux from glia and neuronal glutamine uptake, respectively (Chaudhry et al., 2002). The *Drosophila* gene candidates *CG13646*, *CG13743*, and *CG30394* appear similar to the system A/N amino acid transporters, but do not show a statistically significant relationship on our phylogram.

CG7708, a member of the Na^+ glucose transporter family (SLC5) and *CG1628*, a member of the mitochondrial carrier family (SLC25) are structurally similar to amino acid permeases and were therefore included on the phylogram and in our screening efforts (see below). We note that the SLC5 family is known to transport substrates other than sugars (Wright et al., 2004) and SLC25 carriers are responsible of shuttling many kinds of solutes into the mitochondria, including amino acids (Palmieri, 2004). Finally, we included the electroneutral cation-Cl^- cotransporters (SLC12) in our analysis (Hebert et al., 2004). Although previously characterized members of this family transport small ions rather than amino acids, they are structurally similar to the SLC7 family and it is conceivable that the two orphan members in mammals could transport larger substrates.

To identify neurotransmitter transporters potentially regulated by the visual system, we used differential mRNA expression to determine which of the *Drosophila* genes we categorize above might be down-regulated in flies lacking eyes. We chose to use Northern blots rather than other methods for several reasons. First, neither microarrays nor RT-PCR would allow us to visualize alternative splice variants that might be differentially expressed. Second, since we were specifically interested in screening a limited number of genes, we reasoned that the benefits of internally validating the specificity of the bands based on size outweighed the potential benefits of a microarray. *In situ* hybridization represents a robust method for visualizing expression in *Drosophila* embryos; however, relative to embryonic tissues, *in situ* hybridization using adult fly heads is less efficient, more variable and highly probe-dependent, making screening multiple genes cumbersome.

To generate eyeless flies, we used a previously constructed transgene that includes the proapoptotic gene *head involution defective* (*hid*) (Grether et al., 1995) driven by the glass multimer reporter (GMR) (Hay et al., 1994). GMR drives expression primarily if not exclusively in eye tissue (Hay et al., 1994), and because of the induction of apoptosis by *hid* in the developing eye, GMR-*hid* flies lack photoreceptor cells, cone cells, basal cells, and pigments cells (Grether et al., 1995). Importantly, anterograde trophic interactions between the retina and the target area, the optic lobes, are ablated in eyeless mutants (Fischbach and Technau, 1984), broadening the use of our screen to identify genes that are both directly and indirectly involved in visual function.

We reasoned that comparing mRNA levels between eyeless GMR-*hid* flies and wild type control flies (Oregon-R) would identify genes that are preferentially expressed in either the eye itself or in the optic lobes, which receive anterograde trophic interactions from the retina. To test the feasibility of this screening method, we used probes representing (1) the eye-specific cell adhesion molecule chaoptin (*chp*) (Van Vactor et al., 1988) and (2) the *histidine decarboxylase* (*hdc*) gene (Burg et al., 1993). Hdc is the sole enzyme responsible for biosynthesis of histamine, the major neurotransmitter in arthropod photoreceptor cells, and is expressed in all histaminergic cells, including the several thousand photoreceptor cells in each *Drosophila* eye (Burg et al., 1993; Monastirioti, 1999). In comparison, the fly head contains only 20–24 other *hdc* expressing histaminergic neurons (Monastirioti, 1999; Hamasaka and Nassel, 2006). Thus, ablation of the eye should markedly decrease the expression of *hdc* in the head. Specific bands representing both *chp* and *hdc* are evident in wild-type heads at the size consistent with previous reports (Van Vactor et al., 1988; Burg et al., 1993) [Fig. 2(A)]. As predicted, the expression of mRNA representing both *chp* and *hdc* are significantly reduced in heads derived from flies lacking eyes. As a control for nonspecific changes in expression, we reprobed blots for the ubiquitously expressed gene *actin-5C* (Fyrberg et al., 1983). In contrast to *chp* and *hdc*, we observe robust expression of *actin-5C* in both GMR-*hid* and wild type heads. Quantification of the expression levels of both *chp* and *hdc* using *actin-5C* expression to normalize these values show that both *chp* and *hdc* are decreased 15–25 fold in eyeless heads

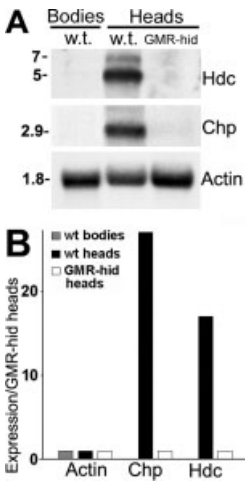

Figure 2 Northern blots efficiently detect differences in transcript expression levels between GMR-*hid* (eyeless) flies and Oregon R (wild type) flies. (A) Both chaoptin (*chp*), an eye-specific cell adhesion molecule, and histidine decarboxylase (*hdc*), highly expressed in photoreceptor cells, appear exclusively in the wild type heads lane, while *actin-5C*, which is ubiquitously expressed, appears in all three tissue samples. One of three representative blots is shown using mRNA from wild type and eyeless (GMR-*hid*) heads and wild type bodies. Molecular weight markers (kb) are shown at the left side of the blot. (B) Quantitation of panel A for expression of *chp*, *hdc*, and *actin-5C* normalized to GMR-*hid* (eyeless) heads. *Chp* and *hdc* are expressed ×26 and ×18 fold over GMR-*hid* head levels respectively.

relative to wild type heads vs. [Fig. 2(B)]. These data indicated that a similar strategy could be used to detect transporters preferentially expressed in the visual system.

We constructed probes representing each member of the SLC6 family shown in Figure 1(A). We then used Northern blots to determine the relative level of

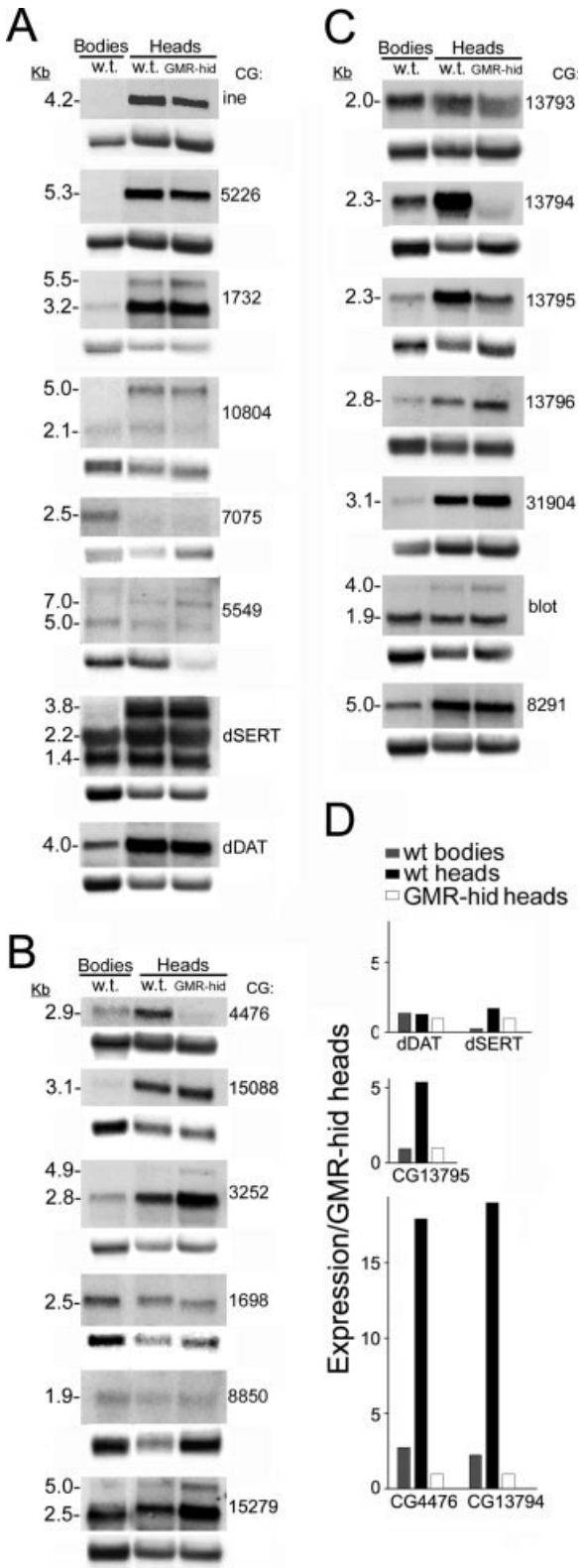

Figure 3 Expression patterns of the neurotransmitter transporter family (SLC6) transcripts in *Drosophila melanogaster* (see Figure 1A). The expression patterns of (A) the neurotransmitter transporter subfamily members, (B) the 'insect specific' nutrient amino acid transporter subfamily members, and (C) the novel subfamily shown in Figure 1A plus the two outliers are shown. The expression patterns of *ine* and *blot* are also shown here. Note that three transcripts (*CG4476*, *CG13794*, *CG13795*) show enrichment in wild type heads vs. GMR-*hid* (eyeless) heads. See Table 1 for expression pattern summary. The actin loading controls are included under each corresponding blot. (D) *CG4476*, *CG13794*, and *CG13795* are expressed ×16, ×23, and ×5 above GMR-*hid* head levels, respectively. For comparison, *dSERT* and *dDAT* show little enrichment in wild type heads. All values for expression in heads and bodies are ratios normalized to the expression of the corresponding gene in GMR-*hid* heads (open bars), which were arbitrarily assigned a value of 1. For each gene, quantitation of one out of three representative blots is shown.

Table 1 Summary of Expression Patterns of Transporter Gene Candidate Transcripts as Determined from the Northern Blots in Figures 3(A–C) and 4(A–C)

	Expression Patterns			
	Ubiquitous	Bodies	Heads	Visual
Neurotransmitter transporters	14	1	11	3
Amino acid permeases	10	6	15	0

The neurotransmitter transporters and amino acid permeases analyzed by Northern blots in Figures 3 and 4, respectively, show expression patterns that were classified as (1) "Ubiquitous", if visible bands of the same size were detected in both heads and bodies of wild type flies; (2) "Bodies", or (3) "Heads" if the transcripts were primarily expressed in mRNA from bodies or heads, respectively, and (4) "Visual", if expression in wild type heads was >5-fold greater than in GMR-*hid* (eyeless) heads. A total of 49 genes were probed, of which 9 show ≥2 transcripts (see Table 2).

expression in the heads of wild type and eyeless (GMR-*hid*) flies [Figs. 3(A–C)]. In addition, we probed Northern blots using mRNA isolated from bodies of wild type flies. The head contains a relatively large number of neuronal cells, whereas the body contains a proportionally larger number of gut-derived cells. Therefore, comparison of expression in heads vs. bodies gives, as a first approximation, an estimate of the relative expression of each transporter in the nervous system vs. other tissues that express amino acid transporters such as the gut. We detect four major expression patterns as summarized in Table 1. These include (1) ubiquitous expression in both heads and bodies, (2) enrichment in body, (3) enrichment in the head, and (4) a substantial decrease in eyeless vs. wild type heads (listed as "visual" in Table 1). To preclude the selection of false positives in the last class based on experimental variability, we chose to focus on genes that were reproducibly enriched ≥5-fold in wild type heads vs. eyeless heads. We find that *CG4476* [Fig. 3(B)], *CG13794*, and *CG13795* [Fig. 3(C)] are ≥5-fold decreased in eyeless heads (three independent blots from three independent mRNA preparations), suggesting that these novel transporters are either directly expressed in eye tissue or in downstream pathways that require visual input. Quantitation of representative blots indicates that *CG13795* was decreased 5- to 6-fold in eyeless flies, whereas *CG4476* and *CG13794* were decreased ∼20-fold [Fig. 3(D)]. For comparison, we also show quantitation of representative blots for *dSERT* and *dDAT*.

We similarly probed for the expression of the amino acid permeases shown in Figure 1(B) including the putative H^+-coupled amino acid [SLC36, Fig. 4(A)], cationic amino acid [SLC7, Fig. 4(B)], electroneutral cation-Cl^- (SLC12), and the remaining seven unclustered amino acid permeases [Fig. 4(C)]. We omitted the previously characterized *EAATs* (Seal et al., 1998; Besson et al., 1999), *mnd* and *JhI-21* (Dubrovsky et al., 2002). As for transporters in the SLC6 family, we detect several patterns of expression (Table 1); however, no additional candidates were found to be substantially decreased (≥5-fold) in eyeless vs. wild type heads.

The three genes identified in our screen that show decreased expression in eyeless flies have not been characterized previously in either *Drosophila* or any other species. One, *CG4476* maps to the left arm of chromosome 3 [Fig. 5(A), bottom], and is a member of the insect nutrient amino acid transporter family, which are thought to function primarily in the insect gut (Castagna et al., 1998; Feldman et al., 2000; Boudko et al., 2005). The two others, *CG13794* and *CG13795*, map to the left arm of chromosome 2 [Fig. 5(A), top]. As shown in Figure 1(A), *CG13794* and *CG13795* cluster together with a novel subfamily of transporters that includes three other genes, *CG13793*, *CG13796*, and *CG31904*. In contrast to *CG13794* and *CG13795*, the other three genes in this subfamily of SLC6 are expressed either ubiquitously (*CG13793*), or primarily in heads [*CG31904* and *CG13796*, Figure 3(C) and Table 2]. All five of these structurally similar genes are located in tandem within a ∼25 Kb region of chromosome 2.

Homologous genes are thought to be formed by gene duplication (Lewin, 1994), and most gene duplicates appear to degrade into pseudogenes (Rodin et al., 2005). Therefore, to confirm that *CG13794* and *CG13795* are expressed, we performed reverse transcriptase PCR (RT-PCR) using gene-specific primers for each candidate. Amplification of specific products when using both gene specific primers [Fig. 5(B), +] but not when interchanged [Fig. 5(B), −] confirm our results using Northern blots and indicate that transcripts from both *CG13794* and *CG13795* are expressed. Additional RT-PCR reactions using gene-specific primers directed to the 5′ and 3′ untranslated regions of *CG4476*, *CG13794*, and *CG13795* results in a single product for each gene [Fig. 5(C)], consistent with our observation of a single band for each using Northern blots, and suggesting that each gene expresses a single mRNA splice variant.

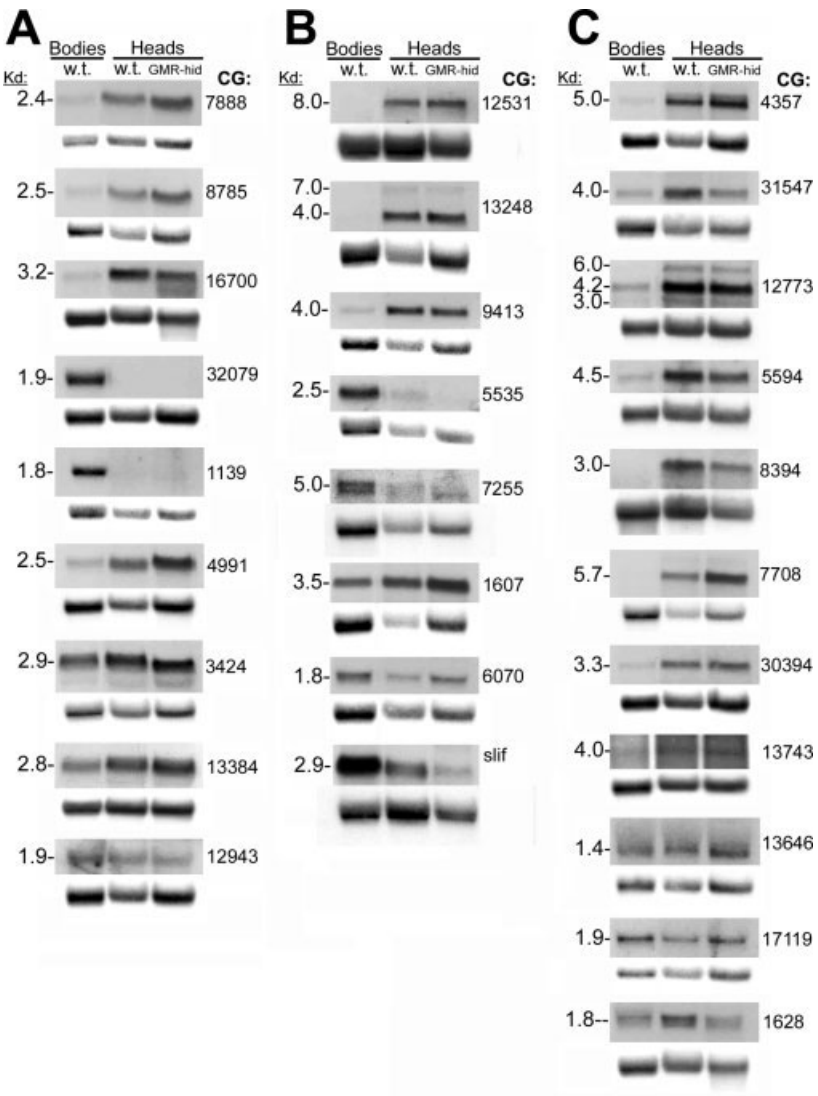

Figure 4 Expression patterns of the amino acid permease-like transcripts in *Drosophila melanogaster*. The expression patterns of the H^+-coupled amino acid permease family (SLC36; A), cationic amino acid permease family (SLC7; B), and the Electroneutral cation-Cl^- cotransporter family (SLC12) plus remaining unclustered members (C) are shown. The *actin* loading controls are included under each corresponding blot. Although several distinct expression patterns are evident (Table 1), none of these genes show enriched expression in wild type heads vs. GMR-*hid* (eyeless) heads.

To determine the temporal pattern of gene expression for *CG13794*, *CG13795*, and *CG4476*, we used a *Drosophila* Rapid-Scan gene expression panel [Fig. 5(D)]. This panel contains normalized amounts of cDNA, thus allowing the semi-quantitative analysis of gene expression throughout development. *CG13795* is expressed during most stages, including the embryo, the 3rd instar larval stage, and in the adult whereas *CG13794* is expressed almost exclusively in the adult. Similar to the results of Northern blots, *CG13794* and *CG13795* appear to be enriched in the heads vs. bodies. Importantly, the gene expression patterns of *CG13794* and *CG13795* are distinct, further indicating that they are independent, functional genes.

Using the Rapid-Scan panel, we find that *CG4476* shows abundant expression in the 3rd instar through the adult stage including the adult head and body. These results are consistent with our screen using Northern blots, although the Rapid-Scan panel shows a higher level of expression in the adult body relative to the data shown in Figure 3. In contrast to our screen using Northern blots, which included tissue from genetically modified (eyeless) flies, the Rapid-Scan panel uses only wild type heads. Thus, the Rapid-Scan panel

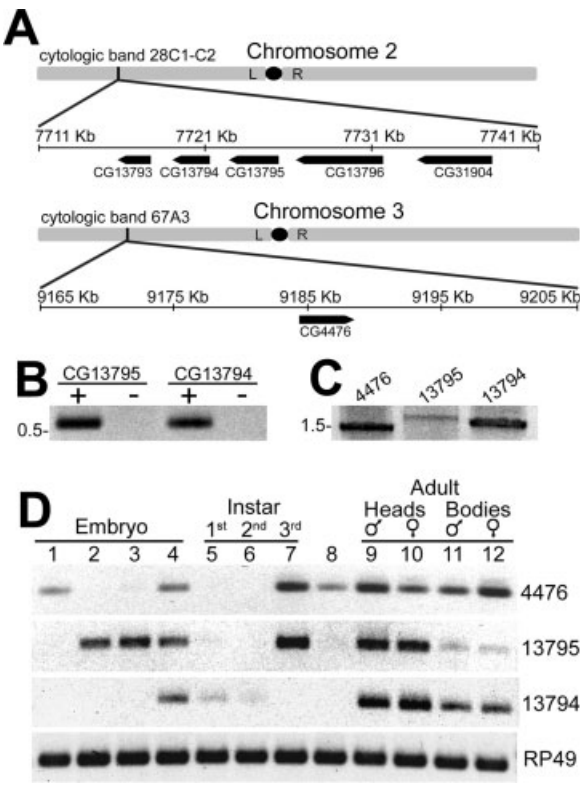

Figure 5 Expression of *CG4476*, *CG13794*, and *CG13795*. The coding sequence for genes *CG13794* and *CG13795* share 70% identity and are adjacent to each other on the left arm of chromosome 2 near three other similar gene candidates (A, top). *CG4476* is located on the left arm of chromosome 3 (A, bottom). (B) RT-PCR was performed using poly-A tailed mRNA extracted from Oregon-R (wild type) flies. Forward and reverse specific oligonucleotides for *CG13794* and *CG13795* amplify a product of expected size when added together (+) but not when interchanged (−), suggesting that both genes are expressed. (C) Additional PCR reactions using oligonucleotides at the ends of the predicted genes show a single band for *CG4476*, *CG13794*, and *CG13795* suggesting a single mRNA variant for each gene. (D) A *Drosophila* Rapid-Scan gene expression panel (OriGene Technologies) was used to semiquantitatively determine the developmental pattern of expression for *CG4476*, *CG13794*, and *CG13795*. *RP49*, representing a ubiquitous ribosomal protein, was used as a loading control. Lane numbering: 1–4→ embryos aged for (1) 0–4, (2) 4–8, (3) 8–12, and (4) 12–24 h; 5–7→ larval stages as indicated; 8→ pupae; 9–12→ adult. One of two similar experiments is shown. We show the reversed (negative) image of a transilluminated 1% agarose gel stained with ethidium bromide.

cannot be used to differentiate between genes that are enriched in the eye vs. head as opposed to those that are more generally enriched in the head vs. the body.

In contrast to *CG13794* and *CG13795*, *CG4476* belongs to previously characterized family, the insect NATs [Fig. 1(A)]. The established functions for other members of this family (Castagna et al., 1998; Feldman et al., 2000; Boudko et al., 2005) will facilitate the study of *CG4476*, and its use as a genetic model to study transporter function and regulation. We therefore chose here to extend our analysis of the CG4476 gene product.

To aid our analysis of the CG4476 protein, an HA epitope tag was inserted at the amino terminus just distal to the initiating methionine (see Methods section). Western blots of S2 cells overexpressing the HA-tagged CG4476 and probed with an antibody to HA show a band migrating at ~62 kD, similar to the predicted size of 69 kD for the 629 amino acid CG4476 protein [Fig. 6(A)]. To determine the subcellular localization of CG4476, we stained *Drosophila* S2 cells expressing HA-tagged CG4476 in parallel with cells expressing HA-tagged vesicular monoamine transporter (dVMAT) and dSERT. dVMAT shows a punctate staining pattern consistent with its previously described localization to endosomes in S2 cells [Fig. 6(B)] (Greer et al., 2005). In contrast, dSERT shows a ring-like staining pattern consistent with its localization to the plasma membrane [Fig. 6(C)]. CG4476 displays a staining pattern similar if not identical to dSERT [Fig. 6(D)]. These data suggest that CG4476 protein is likely to localize to the plasma membrane.

A search of the public databases revealed a P element in the 5′ UTR of gene *CG4476* (also see Methods section). This parent P line was viable and fertile, and presented no gross morphological eye defects. Preliminary tests also determined that the parent P line presented no discernible phototactic impairments, and behaved similarly to Canton-S wild type flies (data not shown). To help determine the function of *CG4476*, we generated a series of deletions in the *CG4476* gene using imprecise excision (Daniels et al., 1985; Ryder and Russell, 2003) of a P{XP}element within the predicted 5′ UTR of gene CG4476 (Thibault et al., 2004) [Fig. 7(A)]. PCR was performed on genomic DNA from individual lines [Fig. 7(B)] using oligonucleotides representing sequences flanking the original P{XP} insert [f1 and r2 in Fig. 7(A)]. Three mutants with increasingly large deletions were identified [Figs. 7(A,B), Δ0.8, Δ1.2, and Δ1.6]. DNA sequence analysis showed deletions of 831, 1206, and 1631 bp for each of these mutants, respectively. The mutant lines were fertile and viable, and presented no evident morphological eye defects or obvious behavioral deficits.

Prediction of the secondary structure for CG4476 using the online program SOSUI (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosui_submit.html) (Hirokawa et al., 1998) suggests that CG4476 forms 12 predicted transmembrane domains (TMDs) and a

Table 2 Expression Patterns of the Transporter Gene Candidate Transcripts

Gene Identification (CG Number)	Family (subfamily)	Expression Pattern	BDGP Embryonic *In Situ*
7708	SLC5	H	Ventral nerve cord, sensory system head
1732 (GAT)	SLC6 (neurotransmitter transporter)	U^{lb}, H^{ub}	CNS
4545 (SERT)	SLC6 (neurotransmitter transporter)	$U^{lb, mb}$, H^{ub}	CNS, neurons
5549	SLC6 (neurotransmitter transporter)	U^{lb}, H^{ub}	–
7075	SLC6 (neurotransmitter transporter)	B	–
8380 (DAT)	SLC6 (neurotransmitter transporter)	H	CNS, neurons
15444 (ine)	SLC6 (neurotransmitter transporter)	H	CNS, glia, hind/midgut, malpighian tubules
1698	SLC6 (insect a.a. transporter)	U	Stomatogastric NS
3252	SLC6 (insect a.a. transporter)	U^{lb}, H^{ub}	Hindgut, malpighian tubules
4476	SLC6 (insect a.a. transporter)	E	Stomatogastric NS, endocrine system
8850	SLC6 (insect a.a. transporter)	U	Malpighian tubules
15088	SLC6 (insect a.a. transporter)	H	–
15279	SLC6 (insect a.a. transporter)	$U^{lb, ub}$	–
13793	SLC6 (novel)	U	–
13794	SLC6 (novel)	E	–
13795	SLC6 (novel)	E	–
13796	SLC6 (novel)	U	–
31904	SLC6 (novel)	H	
5226	SLC6 (orphan)	H	Brain, lateral cord glia and neurons
10804	SLC6 (orphan)	U^{lb}, H^{ub}	–
3897 (blot)	SLC6 (outlier)	$U^{lb, ub}$	–
8291	SLC6 (outlier)	H	–
5535	SLC7 (cationinc a.a. transporter)	B	–
7255	SLC7 (cationinc a.a. transporter)	B	–
11128 (slif)	SLC7 (cationinc a.a. transporter)	B	–
12531	SLC7 (cationinc a.a. transporter)	H	–
13248	SLC7 (cationinc a.a. transporter)	$H^{lb, ub}$	CNS, neurons, glia
1607	SLC7 (heterodimeric a.a. transporter)	U	–
6070	SLC7 (heterodimeric a.a. transporter)	B	CNS, endoderm
9413	SLC7 (heterodimeric a.a. transporter)	H	–
17119	SLC7 (heterodimeric a.a. transporter)?	U	–
4357	SLC12	H	–
5594	SLC12	U	Lateral cord glia, midgut
12773	SLC12	U^{lb}, $H^{mb, ub}$	–
31547	SLC12	H	Midgut
1628	SLC25	U	–
8394	SLC32	H	–
1139	SLC36	B	–
3424	SLC36	U	Brain, muscle, endocrine system, trachea
4991	SLC36	H	–
7888	SLC36	H	–
8785	SLC36	H	Midgut
12943	SLC36	U	–
13384	SLC36	U	–
16700	SLC36	H	–
32079	SLC36	B	–
13646	SLC38	U	–
13743	SLC38	U	–
30394	SLC38	H	Salivary glands

The 49 different transporter gene candidates that were screened are listed here; 21 belong to the "neurotransmitter transporter" family (SLC6) shown in Figure 1(A), and 28 belong to the amino acid permease families (SLC7, 32, 36, and 38) or related families (SLC5, 12 and 25) as shown in Figure 1(B). The major function of the SLC families shown here include SLC5, Na^+ glucose cotransport; SLC7, cationic amino acid permeases, which includes the "cationic transporter subfamily", and the catalytic or light chain of the "heterodimeric transporter subfamily", which form functional dimers with the heavy chains of the SLC3 family; SLC12, electroneutral cation-Cl^- cotransport; SLC25, mitochondrial carrier; SLC36, H^+-coupled amino acid permease; SLC32, vesicular inhibitory amino acid transport (VGAT/VIAAT); SLC38, system A/N neutral amino acid transport. The listed expression patterns are based on data shown in Figures 3 and 4; and include "U", ubiquitous expression; "B", enriched in bodies; "H", enriched in heads; and "E", enriched in eyes/visual system. For genes expressing multiple transcripts, the relevant bands are indicated in superscript as: "lb", lower band; "mb", middle band; "ub", upper band. The column labeled embryonic *in situ* summarizes currently available data from the Berkeley *Drosophila* Genome Project (BDGP) at http://www.fruitfly.org/cgi-bin/ex/insitu.pl.

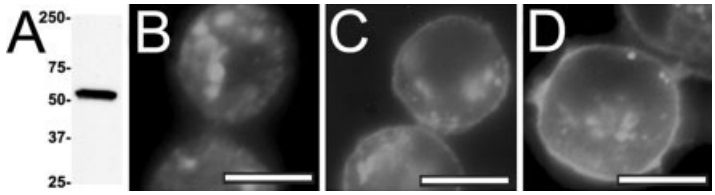

Figure 6 CG4476 localizes to the plasma membrane in *Drosophila* S2 cells. (A) A Western blot of *Drosophila* S2 cells stably transfected with an HA-tagged version of CG4476 shows a single band migrating at ~62 kD relative to the indicated markers. S2 cells transfected with HA-tagged versions of dVMAT (B), dSERT (C), and CG4476 (D) were immunofluorescently labeled using a primary antibody to the HA-tag. Labeling for dVMAT (A) shows a punctate, intracellular pattern of expression. In contrast, dSERT (C) and CG4476 (D) show an additional, prominent ring-like pattern consistent with localization to the plasma membrane. Scale bars: 10 μm.

large second extracellular loop, similar to all other members within the SLC6 family of neurotransmitter transporters (Masson et al., 1999), including a recently crystallized prokaryotic transporter (Yamashita et al., 2005). The TMDs of SLC6 family members are highly conserved relative to other domains suggesting little tolerance for mutation of these regions. Mapping the deletion mutants onto this predicted structure suggests that the 0.8 kb deletion removes only 19 amino acids of the extreme N-terminus but does not disrupt any of the TMDs. In contrast, the Δ1.2 mutation deletes 97 amino acids that encompass the first TMD and a portion of the second predicted TMD. The most severe Δ1.6 allele deletes 173 amino acids, which includes all of the first three TMDs and a portion of the second extracellular loop [Fig. 7(C)].

Since our original screen was designed to detect transporters that were associated with the visual system, we tested the effects of mutating *CG4476* on visual function. We first performed electroretinogram (ERG) recordings, a standard electrophysiological measure of photoreceptor function in *Drosophila* (Hengstenberg and Gotz, 1967; Hotta and Benzer, 1969). The traces representing depolarization of the photoreceptors [Fig. 8(A), white arrow] were unchanged by mutation of *CG4476* and ERGs of the Δ1.6 allele were essentially identical to that obtained from precise excision lines [Fig. 8(A)]. The on- and off-transient currents that occur before and after the larger sustained depolarization are thought to reflect synaptic transmission between photoreceptors and the first downstream neuron (Hotta and Benzer, 1969). The on- and off-transients of *CG4476* mutants [Fig. 8(A), black arrows] were indistinguishable from those of the precise excision controls, suggesting that neurotransmitter release from the photoreceptors and neurotransmission at the first synapse in the visual system is essentially intact.

We next tested the behavioral response to visual stimuli of the *CG4476* mutants. Flies are highly sensitive to visual stimuli (Connolly and Tully, 1998), and have a natural tendency to move toward a light source when mechanically stimulated or otherwise disturbed (Markow and Merriam, 1977). The response of adult flies to a light stimulus can be easily quantitated using a well-described fast phototaxis assay (Benzer, 1967) (see Methods section). This assay employs a countercurrent apparatus to measure the number of times that a fly will chose to move toward light following a brief mechanical stimulus. We tested the three deletion mutants (Δ0.8, Δ1.2, and Δ1.6) as well as five precise excision control lines. The results using the five precise excisions were similar (data not shown), and therefore the data were pooled.

The precise excision control lines displayed a robust attraction to light similar to wild type flies [Fig. 8(B), dotted line] and consistently move toward the light an average of four times in the countercurrent apparatus. Similarly, the relatively modest deletion of 19 amino acids in the Δ0.8 allele does not cause an appreciable in difference from the behavior of controls and these flies also choose to move toward the light four times [Fig. 8(B), light gray line]. In contrast, the behavioral patterns of both the Δ1.2 and Δ1.6 alleles are significantly different from the controls, and the Δ0.8 allele (2-way ANOVA followed by a Bonferroni's post-hoc test comparing each genotype, $p < 0.001$) with the largest deletion (Δ1.6) choosing to move toward light on average 0–1 times [Fig. 8(B), black line] and Δ1.2 moving toward light an average of 2–3 [Fig. 8(B), dark gray line]. These data strongly suggest that mutations of *CG4476* that affect the conserved TMDs cause defects in visual behavior.

The fast phototaxis assay that we used, and most other visual assays, require (1) an additional stimulus to initiate the phototactic behavior, in this case sharply tapping the apparatus and (2) the ability to locomote toward the light stimulus (Benzer, 1967; Connolly and Tully, 1998). Therefore, to rule out the possibility that the phenotype of Δ1.2 and Δ1.6 was primarily the result

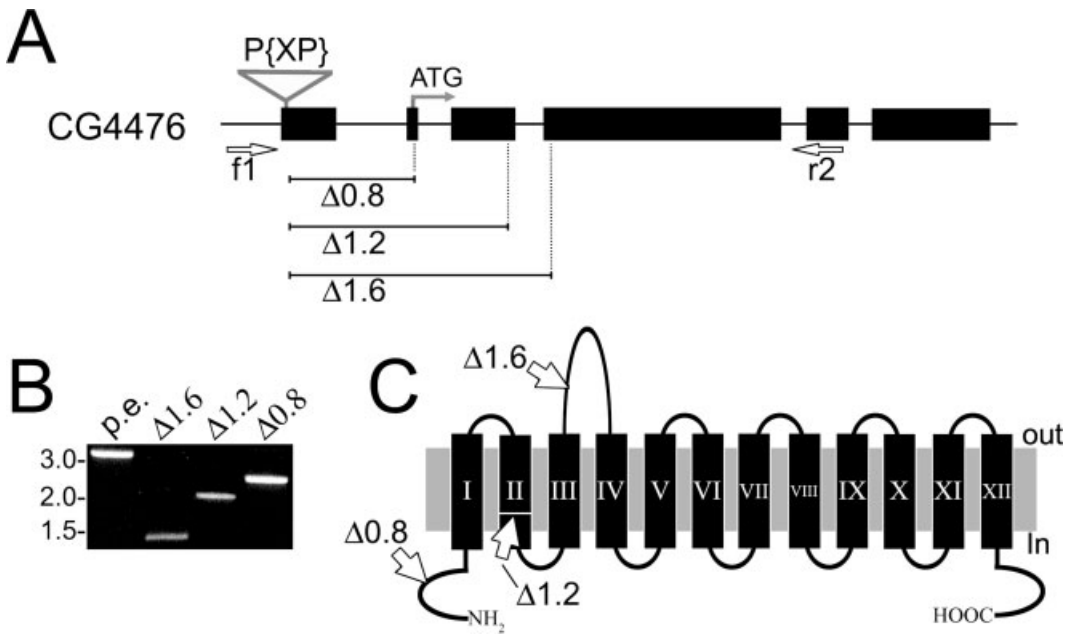

Figure 7 P-element mediated imprecise excision generates three different deletions in the 5′ region of *CG4476*. (A) A P-element (P{XP}) in the 5′ UTR of gene *CG4476* was imprecisely excised to generate three deletion alleles. Black boxes represent the six exons in the *CG4476* gene. Open arrows (f1 and r2) represent the oligonucleotides used to screen for deletions. (B) Using f1 and r2 as primers, PCR across the indicated deletions generates products that are 1.6, 1.2, and 0.8 kb smaller than those obtained using DNA from precise excision (p.e.) lines. Numbers at the left of the panel indicate kb markers. (C) The cartoon represents the membrane topology of *CG4476* as predicted by SOSUI. Black bars represent the 12 predicted transmembrane domains. Both termini are assumed to be localized intracellularly. Open arrows indicate the 3′ ends of the deletions relative to the predicted primary structure.

of either a defect in their response to a mechanical stimulus or an inability to move, we performed the identical assay in the dark [Figs. 8(C–F), black lines]. All the mutant and control lines display locomotor reactivity, with 58–82% moving out of the initial tube in response to the mechanical stimulus depending on the genotype. These data support the idea that locomotor impairments alone do not account for the defective response to light of the Δ1.2 or Δ1.6 alleles We note, however, that the Δ1.6 allele is less active than either of the other alleles or the precise excision controls (2-way ANOVA, followed by Bonferroni's post-hoc test comparing the distribution of all four genotypes, $p < 0.001$).

To better assess the visual defect of the mutants, we compared the data obtained in the dark vs. those obtained using the standard light stimulus. We find that the Δ1.6 allele shows statistically identical behaviors in the light and the dark [Fig. 8(C)]. When compared with previously described visual mutants, these results suggest that the Δ1.6 flies are functionally blind in this assay (Benzer, 1967). In contrast, the Δ1.2 flies show statistically different responses in light vs. dark conditions [Fig. 8(D)], indicating that although they are visually impaired compared with controls (see above), they are not blind. Similarly, the Δ0.8 allele [Fig. 8(E)] and the controls [Fig. 8(F)] show very different locomotor responses in the presence vs. the absence of the light stimulus. Together, these results show that the severe disruption of the *CG4476* coding sequence in Δ1.6 causes a behavioral deficit equivalent to blindness in the fast phototaxis assay, despite preserved function of the photoreceptor cells and the first downstream synapse.

DISCUSSION

To identify novel transporters that may be expressed in the fly visual system, we have examined the expression of 49 transporter genes predicted from the sequence of the *Drosophila* genome. We used Northern blots to compare transporter expression between heads derived from wild type flies and flies in which the eyes were genetically ablated. We have identified three transcripts that are down-regulated by ablation of the eye: *CG4476*, *CG13794*, and *CG13795*. Muta-

tions in *CG4476* show impairment in fast phototaxis indicating that our screening strategy was successful in identifying transporters involved in vision. However, since the electroretinograms of the mutants are not impaired, we speculate that *CG4476* mutants may be visually impaired because of defects in the central processing of visual information, similar to the clinical phenomenon of cortical blindness (Stoerig and Cowey, 1997). *CG4476* as well as the other candidates will provide useful genetic models to study transporter function using the large number of previously identified mutants and assays developed to study vision in *Drosophila*.

Our data also provide general information on the expression of most if not all of the neurotransmitter transporter-like genes in the fly genome (Table 2). Previously characterized transporters in *Drosophila* include *dSERT*, *dDAT*, *dGAT*, *ine, blot* from the SLC6 family of transporters and *slif*, *mnd*, *JhI-21*, *pathetic*, and two *dEAATs* from amino acid permease families. The function of a relatively small number of other candidates, such as *CG5549* and *CG8394*, can be inferred from their clear similarity to previously characterized transporters. *CG5549* is similar to mammalian glycine transporters while *CG8394* is likely to be the *Drosophila* ortholog of VGAT/VIAAT (Gasnier, 2004). However, limited information is available for other related transporters in the fly.

We find that 9 of the 49 transporter genes we probed show mRNA splice variants (Table 2). Variants in coding or regulatory regions of the mammalian glycine transporter (Hanley et al., 2000), EAATs (Reye et al., 2002; Rauen et al., 2004; Berger et al., 2005), NET (Kitayama and Dohi, 2003); and the insect proline transporter (Sandhu et al., 2002) have been described previously and may regulate both activity and expression. Similarly, we observe three splice variants in *dSERT*, only one of which is preferentially expressed in heads. Pan-neuronal expression of dSERT

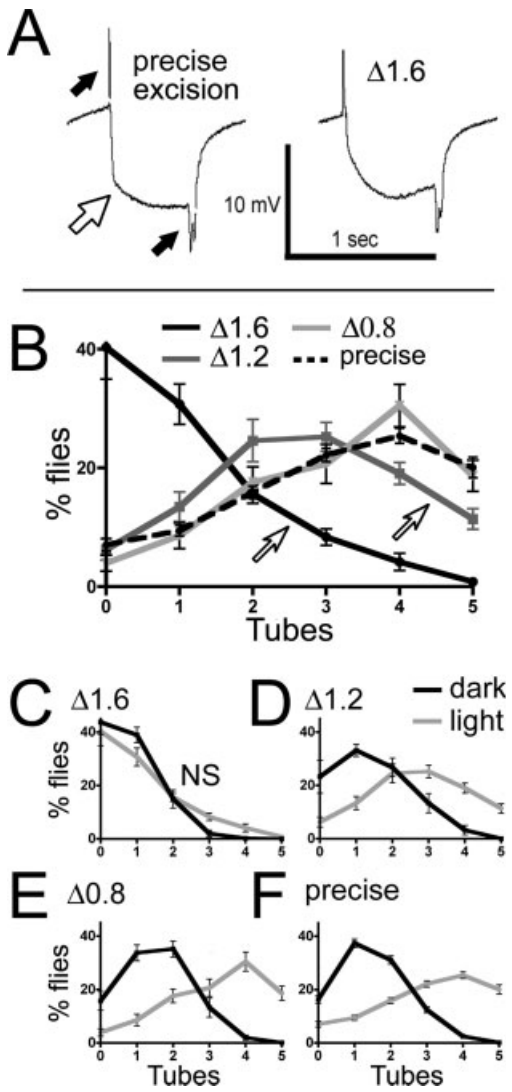

Figure 8 *CG4476* mutants show normal ERGs but decreased phototaxis. (A) Synaptic transmission between the photoreceptors and laminar cells is intact in *CG4476* mutant flies. ERGs recorded from the *CG4476* Δ1.6 mutant and precise excision control flies exhibit normal retinal depolarization and on-/off-transients when exposed to a brief pulse of light. The white arrow indicates the photoreceptor depolarization response to a pulse of light and the black arrows show the on-/off-transients. (B) Response of *CG4476* deletion mutants and precise excision controls to light in a fast phototaxis assay. The Δ1.6 and Δ1.2 mutants, but not the Δ0.8 flies show defective phototactic behavior, when compared with precise excision controls. Each point represents the average percentage (±SEM) of nine trials of ~50 flies showing the indicated behavior. Three independent trials were done in triplicate for Δ1.6, Δ1.2, and Δ0.8. For the control plot, three independent trials done in triplicate for five different precise excisions were pooled. The pattern of choices displayed by both Δ1.6, Δ1.2 (arrows) are statistically different from Δ0.8 and the precise excision controls ($p < 0.001$, 2-way ANOVA followed by Bonferroni's post-hoc test comparing the distribution shown by each genotype). In addition, Δ1.6 and Δ1.2 differ from each other ($p < 0.001$). Panels (C)–(F): a comparison of locomotor reactivity with light vs. without a light stimulus (i.e. behavior in the dark). Fast phototaxis experiments were performed using a standard light stimulus (gray line) and in the dark (black line). (C) Δ1.6 is not responsive to light and 2-way ANOVA reveals no statistical difference (NS) between the distribution of choices taken with and without the light stimulus. In contrast Δ1.2 (D), Δ0.8 (E) and the precise excision controls (F) each show some response to light (2-way ANOVA with Bonferroni's post-hoc test, $p < 0.001$) despite the decrease in phototaxis for Δ1.2.

in *Drosophila* shows a surprisingly restricted uptake of 5HT into aminergic cells (Park et al., 2006). It is possible that alternative splicing could contribute to this apparent cell-specific regulation of activity.

Of the 60 transcripts that we observe (49 genes, with 11 additional splice variants), 26 are primarily expressed in heads (Tables 1 and 2). Since most cells in the head are associated with the CNS, some of these transporters are likely to function in neurons and/or glia. Conversely, transporter transcripts expressed primarily in the body are more likely to be expressed in the gut or other tissue confined to the body, such as the gonads. Although additional expression data will of course be needed to validate these possibilities, embryonic *in situ* hybridizations performed by BDGP and covering a portion of the genes we have examined are essentially consistent with our data (Table 2).

Many members of the neurotransmitter and amino acid permease families act primarily in the gut and kidney for the uptake of nutrient amino acids (Verrey et al., 2004; Boudko et al., 2005; Broer, 2006). In contrast, some such as systems A and N are required in the nervous system for the uptake and recycling of amino acids (Chaudhry et al., 2002). The relative specificity of particular transporters also varies: some mediate the broad-spectrum transport of multiple amino acids. In SLC6, these include the B^0 subclass (the former 'orphan' transporters), and KAAT1 or CAATCH1 of the insect NAT subfamily (Castagna et al., 1998; Feldman et al., 2000; Broer, 2006), to which *CG4476* belongs. Others mediate the relatively more specific uptake of molecules previously characterized as neurotransmitters, which include both amino acids and monoamines. These transporters/permeases include the EAATs (SLC1), DAT, SERT, GAT, and the glycine transporter. The potential roles in the nervous system for many other transporters in the SLC6 and amino acid permease families remain unknown.

We describe here a new subfamily of SLC6 including two genes that appear to be enriched in the visual system, *CG13794* and *CG13795*. Both *CG13794* and *5 cluster with three* related genes on the second chromosome (*CG13793*, *CG13796*, and *CG31904*), suggesting that all may be derived from a common precursor. Similar to the recently described NATs (Boudko et al., 2005), the subfamily of SLC6 containing *CG13794* and *CG13795* appears to be restricted to insect genomes, and we speculate that CG13794 and CG13795 may be relevant to two distinct transport processes in the insect visual system which remain poorly understood. First, they may be involved in signaling at the synapse between photoreceptors and neurons in the first (lamina) and second (medulla) optic ganglia. Histamine is the major neurotransmitter in insects and all arthropod photoreceptors, and also an important neuromodulator in mammals (Schwartz et al., 1991; Brown et al., 2001). For both insects and mammals, it is possible that histamine may be metabolized prior to reuptake (Huszti, 1990; Barnes and Hough, 2002; Borycz et al., 2002; Richardt et al., 2003; True et al., 2005). However, a transporter for neither histamine nor a histamine metabolite has been identified in any species, and it remains unclear how histamine is recycled at the synapse.

A second potential role for CG13794 and CG13795 is the transport of eye pigments. Despite the intense study of eye pigmentation in *Drosophila*, the mechanism by which precursors and metabolites are transported into photoreceptor and pigment cells is not yet clear (Ewart and Howells, 1998). In *Drosophila*, the two pigments, ommochromes and pteridines, are synthesized from tryptophan and guanine, respectively (Yamamoto et al., 1976). Although the gene product of *white*, a member of ABC cassette class has been previously described as a tryptophan transporter, *white*, as well as its partners *brown* and *scarlet* are likely to localize to the membrane of pigment granules rather than the cell surface (Mackenzie et al., 2000). Thus, the plasma membrane transporters that serve to transport pigment precursors such as tryptophan into photoreceptors and pigment cells remains unknown. Since *CG13794* and *CG13795* are highly enriched in eye tissue, we speculate that one or both may be involved in either histamine homeostasis, or the uptake of pigment precursors. However, further localization and *in vitro* transport studies will be needed to test these hypotheses.

The WRFPY/F motif is diagnostic of the SLC6 family, and is evident in prokaryote SLC6 transporters (Androutsellis-Theotokis et al., 2003; Beuming et al., 2006), suggesting a conserved role in transport function. Interestingly, the subfamily in SLC6 containing CG13794, 5, and 6 lacks this canonical motif in the predicted first TMD. In addition, two other SLC6 members, blot and CG8291, lack the full canonical motif. Although the significance of this motif is still unclear, the crystal structure of a similar amino acid transporter in prokaryotes suggests that the first transmembrane domain containing this motif may mediate both substrate recognition and Na^+ binding (Yamashita et al., 2005). It is possible that transporters lacking the conserved motif in the first TMD recognize substrates that are very different from the amines or amino acids that are transported by other members of SLC6. Alternatively, these putative transporters might not mediate transport at all and function rather as receptors, similar to SGLT3/SLC5A4 of the sugar transporter family (Diez-Sampedro et al., 2003).

The sequence of NAT subfamily members, including CG4476, contain the conserved motifs of other SLC6 subfamilies, and the function of several other members of the NAT subfamily have been previously characterized (Castagna et al., 1998; Feldman et al., 2000; Boudko et al., 2005). The *Manduca* transporters KAAT1 and CAATCH1 recognize a broad spectrum of amino acids including leucine, phenylalanine, proline, and threonine. Interestingly, these transporters also function as substrate gated channels, and large Na^+ and K^+ based currents have been recorded in oocytes (Quick and Stevens, 2001). Similarly, it is possible that uncoupled ion currents could be important for the function of CG4476. Indeed, genes once considered true transporters have now been shown to be exclusively substrate gated ion channels (Diez-Sampedro et al., 2003), and a number of neurotransmitter transporters, including SERT, support both transport function and uncoupled ion currents that may be physiologically important (Petersen and DeFelice, 1999; Chaudhry et al., 2001; Bergles et al., 2002). In addition, a recent report on the *Drosophila* amino acid permease pathetic (CG3424) indicates relatively low transport of substrate, suggesting that the signaling properties of the transporter may in fact play a more prominent role than its activity as a transporter (Goberdhan et al., 2005).

In contrast to KAAT1 and CAATCH1, other members of the NAT subfamily characterized in *A. aegypti* and *A. gambiae* appear to recognize a more circumscribed set of substrates. aeAAT1 transports phenylalanine, whereas agNAT6 and 8 transport phenylalanine/L-DOPA/tyrosine and tryptophan, respectively (Boudko et al., 2005). The localization of CG4476 to the cell surface of S2 cells suggests that it mediates the uptake of substrate across the plasma membrane, and its apparent similarity to agNAT6 and 8 suggests that it may transport a derivative of phenylalanine or tryptophan. However, in contrast to the previously described roles for CAATCH1, KAAT1, aeAAT1, agNAT6 and 8 in the gut, we show that CG4476 functions in the nervous system. Our data thus suggest a previously unsuspected role for the NAT subfamily in neurons and/or glia. It is possible that an amino acid such as tryptophan or phenylalanine might serve as a *bona fide* transmitter in insects. Alternatively, it is possible that CG4476 could function to increase uptake of a specific precursor amino acid into a subset of neurons with a particularly high demand. Interestingly, melatonin is synthesized from tryptophan, and transport activities for this evolutionarily conserved neurohormone have not been identified.

The future localization of CG4476 *in vivo* and further behavioral tests will help elucidate both its potential substrates and the cellular pathways involved in the CG4476 mutant phenotype described here. For now, our data suggest a role for CG4476 in the modulation of visual information in the optic ganglia rather than in the eye itself. Previous screens for visual phenotypes have similarly identified mutations that do not affect retinal function, both in *Drosophila melanogaster* (Markow and Merriam, 1977) and in the zebrafish *Danio rerio* (Neuhauss et al., 1999). In humans, the well-known phenomenon of cortical blindness is caused by an inability of the cerebral cortex to process visual information, despite the perception of light by the eye (Stoerig and Cowey, 1997). The robust genetics of the fly eye are quite likely to facilitate our ability to determine how mutation of *CG4476* causes a similar phenomenon in the fly. Moreover, all three candidates will serve as useful models for the study of neurotransmitter and amino acid transporters and their regulation.

NOTE ADDED IN PROOF

A complementary analysis of SLC6 expression in Drosophila was recently published. See Thimgan MS, Berg JS, Stuart AE. 2006. Comparative sequence analysis and tissue localization of members of the SLC6 family of transporters in adult Drosophila melanogaster. J Exp Biol 209:3383–404.

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