

Drosophila Glial Glutamate Transporter *Eaat1* Is Regulated by Fringe-Mediated Notch Signaling and Is Essential for Larval Locomotion

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In the mammalian CNS, glial cells expressing excitatory amino acid transporters (EAATs) tightly regulate extracellular glutamate levels to control neurotransmission and protect neurons from excitotoxic damage. Dysregulated EAAT expression is associated with several CNS pathologies in humans, yet mechanisms of EAAT regulation and the importance of glutamate transport for CNS development and function *in vivo* remain incompletely understood. *Drosophila* is an advanced genetic model with only a single high-affinity glutamate transporter termed *Eaat1*. We found that *Eaat1* expression in CNS glia is regulated by the glycosyltransferase Fringe, which promotes neuron-to-glia signaling through the Delta-Notch ligand–receptor pair during embryogenesis. We made *Eaat1* loss-of-function mutations and found that homozygous larvae could not perform the rhythmic peristaltic contractions required for crawling. We found no evidence for excitotoxic cell death or overt defects in the development of neurons and glia, and the crawling defect could be induced by postembryonic inactivation of *Eaat1*. *Eaat1* fully rescued locomotor activity when expressed in only a limited subpopulation of glial cells situated near potential glutamatergic synapses within the CNS neuropil. *Eaat1* mutants had deficits in the frequency, amplitude, and kinetics of synaptic currents in motor neurons whose rhythmic patterns of activity may be regulated by glutamatergic neurotransmission among premotor interneurons; similar results were seen with pharmacological manipulations of glutamate transport. Our findings indicate that *Eaat1* expression is promoted by Fringe-mediated neuron–glial communication during development and suggest that *Eaat1* plays an essential role in regulating CNS neural circuits that control locomotion in *Drosophila*.

Introduction

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. Extracellular glutamate levels are tightly regulated for precise control of neurotransmission at glutamatergic synapses, and to prevent neuronal cell death from excitotoxicity (Danbolt, 2001). Certain astrocyte populations take up and recycle extracellular glutamate by expressing high-affinity, sodium-dependent excitatory amino acid transporters (EAATs) known as GLAST (alternate name EAAT1) and GLT-1 (alternate name EAAT2), the primary transporters for glutamate in the mamma-

lian CNS (Matthias et al., 2003). GLAST mutations are found in patients with type 6 episodic ataxia (EA6), a rare form of the disease that variably involves seizures, migraine, cerebellar atrophy, and hemiplegia (Jen et al., 2005; de Vries et al., 2009). Furthermore, expression of EAATs is dysregulated in amyotrophic lateral sclerosis, stroke, epilepsy, schizophrenia, and Alzheimer's and Huntington's diseases, among others (Beart and O'Shea, 2007). However, the mechanisms of EAAT regulation and the consequences of aberrant glutamate transport for CNS function and pathology remain to be fully understood.

Drosophila provides an advanced genetic model system to study EAAT regulation and function *in vivo* because the differentiation of glial cell subtypes can be studied in an intact nervous system. Moreover, the importance of glutamate transport for CNS development and function can be assessed *in vivo* with complementary behavioral studies and electrophysiology. In *Drosophila*, many functional, morphological, and molecular features of glial cells are conserved with mammals (Freeman and Doherty, 2006; Murai and Van Meyel, 2007), including the selective expression of the *Eaat1* gene in a subpopulation of glial cells (Soustelle et al., 2002; Freeman et al., 2003). *Drosophila* *Eaat1* has 41 and 35% amino acid identity, respectively, to human EAAT1 and EAAT2 (Besson et al., 1999). *Eaat1* is the only high-affinity

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glutamate transporter in flies (Besson et al., 2000), and so studies of *Eaat1* mutations are unlikely to be complicated by redundancy with the related protein *Eaat2*, which is a selective high-affinity transporter for taurine and aspartate (Besson et al., 2000, 2005). Previous research in adult flies has shown that reduction of *Eaat1* in glia using RNA interference (RNAi) increases sensitivity to oxidative stress, and results in fewer dopaminergic neurons, degeneration of the brain neuropil, and decreased life span (Rival et al., 2004). However, our understanding of the importance of this glutamate transporter for CNS development and function remains incomplete since RNAi approaches to study *Eaat1* function did not reveal phenotypes at embryonic and larval stages (Rival et al., 2004, 2006).

Here, we show that *Eaat1* expression in embryogenesis is regulated by the glycosyltransferase Fringe (Fng), which we have shown previously to promote neuron-to-glia signaling through the Delta–Notch ligand–receptor pair (Thomas and van Meyel, 2007). We generated *Eaat1* loss-of-function mutations and found that mutant larvae have severe defects of locomotion. Our electrophysiological and genetic approaches provide evidence that *Eaat1* acts in a limited subpopulation of CNS glial cells to influence glutamatergic neurotransmission controlling the rhythmic patterning of motor neuron activity. Thus, we have identified cellular and molecular interactions during development that affect the emergence of a functionally distinct glial subtype capable of influencing glutamatergic neurotransmission in the CNS, and discovered an essential role for the *Eaat1* glial glutamate transporter in locomotor behavior.

Materials and Methods

Fly stocks and genetics. Fly stocks were obtained from the Bloomington Stock Center and the Vienna *Drosophila* RNAi Center (VDRC), or alternative published sources as noted: *P{EPgy2}Eaat1^{EY20741}*; *Df(2L)30A-C* (here denoted *Eaat1^{DF}*); *UAS-mCD8::RFP*; *UAS-Eaat1*; *UAS-Pros RNAi* (VDRC ID 101477); *Eaat1-Gal4* (Rival et al., 2004); *htl-Gal4* (Shishido et al., 1997); *Repo-Gal4* (Sepp and Auld, 2003); *tub-Gal80^{ts}* (McGuire et al., 2003); *UAS-N^{lCD}* (Go et al., 1998); *UAS-Hairless* (Maier et al., 1999); *UAS-Tom* (Lai et al., 2000); *UAS-Eaat1::GFP* (Rival et al., 2004); *UAS-mCD8::GFP* (Lee and Luo, 1999); *UAS-nuclearGFP* (van Meyel et al., 2003); *Su(H)-lacZ* (Griffiths et al., 2007); and *fng¹³* and *fng¹³* (Irvine and Wieschaus, 1994; Correia et al., 2003). To create CG31235-nuclear green fluorescent protein (nGFP) and CG31235-Gal4, we PCR amplified 3004 base pairs (bp) of regulatory sequences located immediately upstream of CG31235 using the primers 5'-CATAGTGCATTGGTGGAGGTGT-3' and 5'-TACTGGGTGCGCGTTAGGTC-3'. This fragment was inserted into pCR2.1-TOPO and, for CG31235-nGFP, subsequently excised (XbaI/KpnI) and cloned into XbaI/KpnI-digested pH-Stinger (Barolo et al., 2000). For CG31235-Gal4, the fragment was cloned into a modified version of pH-Stinger in which the sequences encoding GFP were replaced by GAL4. Transgenic flies were generated in a *w⁻* background by standard microinjection procedures (BestGene).

Immunohistochemistry. Embryos and larvae were dissected and fixed according to standard procedures. Monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank included the following: mouse anti-Prospero (dilution 1:25); mouse anti-Fas2 (1:10); mouse anti-Even-skipped (Eve) (dilution 1:10); and mouse anti-Repo (1:50). Other antibodies used were as follows: rabbit anti-GFP (1:1000, Invitrogen); rabbit anti- β -gal (1:1000, MP Biomedicals); mouse anti-glutamine synthetase 2 (Gs2) (1:100, Millipore Bioscience Research Reagents); rabbit anti-cleaved caspase-3 (1:100, Cell Signaling Technology); rabbit anti-VGluT (1:400) (Mahr and Aberle, 2006); and rabbit anti-Nazgul (Naz) (1:500) (von Hilchen et al., 2010). Secondary antibodies used were as follows: Alexa Fluor 488-conjugated goat anti-rabbit and goat anti-mouse (1:300, Invitrogen); Alexa Fluor 647-conjugated goat anti-mouse (1:300, Invitrogen); and Rhodamine Red-X-conjugated goat anti-rabbit and goat

anti-mouse (1:300, Jackson ImmunoResearch). Fluorescence-labeled embryos were mounted in Prolong Gold anti-fade medium (Invitrogen). Staining for apoptosis with acridine orange was done as described previously (Arama and Steller, 2006).

In situ hybridization. A 1.7 kb probe was synthesized from a HindIII-digested *Eaat1* cDNA (RE20434), using a digoxigenin (Dig)-labeling kit (Roche) and T3 RNA polymerase. Prepared embryos (4–20 h after egg laying) were hybridized overnight at 55°C using standard procedures, and visualized using anti-Dig-AP (1:1000, Roche). For probe detection in fluorescence *in situ* hybridization, we used either Fast Red chromogen (Roche) with anti-Dig-AP, or Cy-3-conjugated tyramide reagent (1:50, PerkinElmer) with anti-Dig-POD (1:100, Roche); then, embryos were processed for immunohistochemistry as described above.

Microscopy and imaging. Differential interference contrast microscopy was performed on a Zeiss Axioskop-2 microscope equipped with Nomarski optics. Images were acquired with an AxioCam HRC digital camera and AxioVision software (Zeiss). Confocal microscopy was performed using a Yokogawa spinning disk confocal system (PerkinElmer) and a Nikon Eclipse TE2000-U inverted microscope. Z-series images were collected using MetaMorph software (Molecular Devices) and compiled into figures with Adobe Photoshop and Illustrator.

Creation of *Eaat1* mutants. Mutations in *Eaat1* were generated by imprecise excision of the P-element *P{EPgy2}Eaat1^{EY20741}*, situated 472 bp upstream of the *Eaat1* start codon. A total of 130 *w⁻* excision lines were screened via PCR (primers: 5'-TTACCAGCATCAAGCTCTCGCTTC-3'; 5'-CGCATTCTCCAATGGTGGTACCT-3'). Two imprecise excisions were identified by amplification of fragments <2.166 kb, and these fragments were sequenced to determine the extent of the induced deletion and to confirm removal of the *Eaat1* start codon. A precise excision line was also isolated for use as a control.

Reverse transcriptase-PCR. Reverse transcriptase (RT)-PCR was performed on total RNA isolated from first-instar (L1) control *w¹¹¹⁸* and *Eaat1^{SM2/SM2}* mutant larvae (RNEasy Mini Kit and One-Step RT-PCR Kit, Qiagen). The *Eaat1* primer set was designed to amplify a band of 448 bp from control *w¹¹¹⁸* larvae but not *Eaat1^{SM2/SM2}* mutants (5'-ATTGGTGGACTC-GACCTGAG-3'; 5'-CGCTCAGTGTGGTGAAGAAA-3'); the amplicon bridges an intron–exon junction to distinguish cDNA from genomic DNA templates. An *Actin5C* fragment of 586 bp was amplified as a control (5'-GAGCGCGGTTACTCTTTCAC-3'; 5'-ATCCCGATCCTGATCCTCTT-3').

Semi-quantitative RT-PCR was used to compare *Eaat1* expression in *fng^{13/L73}* mutants to heterozygous controls (genotype: *fng¹³/TM3, twi-GAL4, UAS-GFP*). Briefly, total RNA was isolated from pools of ~80 L1 larvae (RNEasy Plus Mini Kit, Qiagen), then 1 μ g was used as a template for cDNA synthesis with M-MuLV RT (New England Biolabs), oligo-dT primers, and random hexamers. PCR was then performed with Platinum Taq (Invitrogen) and either the *Eaat1* or *Actin5C* primers above, using 1 μ l of cDNA as a template in 25 μ l reactions. Paired samples from controls and mutants were removed from the thermal cycler at each cycle from 21 through 27, and immediately incubated at 72°C for 10 min. Samples were run on a 1% agar gel stained with ethidium bromide, and the measured intensity of the *Eaat1* PCR product was normalized to that of *Actin5C* for each of cycles 21 through 27, using ImageJ software to analyze non-saturated images.

Behavior assays. Studies of mechanosensory performance and larval crawling were done as described previously (Kernan et al., 1994; Carhan et al., 2004) on L1 larvae selected 0–2 h posthatching and placed on 2.3% agar plates (room temperature). For each assay, the animals were allowed to acclimatize for 5 min. Before and after crawling, each animal was touched lightly to ensure they could respond to mechanical stimulation. For each trial, the number of full-body peristaltic contractions (forward or backward) was counted during a 3 min period. Three consecutive trials were performed for each larva, and these were averaged to produce a single data point. For each genotype, 15–40 larvae were examined. Heterozygous controls were generated by crossing *Eaat1* alleles to *w¹¹¹⁸* flies.

To assess the developmental or acute requirement for *Eaat1* in larval locomotion, a temperature-sensitive *Gal80* allele (*Gal80^{ts}*) was used to repress Gal4-mediated rescue of *Eaat1^{SM2/SM2}* mutants at larval stages (McGuire et al., 2003). *Gal80^{ts}* binds and represses Gal4 activity at 18°C,

but at 29°C it is nonfunctional. The broadly expressed *tubulin-Gal80^{ts}* (*tubP-Gal80ts*) was added into the background of a rescue (genotype: *Eaat1^{SM2}, CG31235-Gal4; UAS-Eaat1::GFP/tub-Gal80^{ts}*). Embryos were collected and raised at 29°C to inactivate Gal80^{ts} and allow sufficient expression of the *UAS-Eaat1::GFP* transgene to rescue throughout embryogenesis. Upon hatching (0 h), larvae were assayed for full-body peristaltic contraction rates as described above, then shifted to 18°C to repress *UAS-Eaat1::GFP* expression for the duration of the experiment. Larval contraction rates were again assessed at 30, 40, 44, and 48 h following the temperature shift to 18°C. Control animals were of the same genotype but lacked *tubP-Gal80ts*. To examine levels of *Eaat1::GFP* expression over the course of this experiment, larvae at 0 and 48 h were assessed by anti-GFP immunohistochemistry.

Electrophysiology. Whole-cell voltage-clamp recordings were performed at room temperature (22–24°C) in dissected L1 larvae, 1–4 h after hatching, using thick-walled borosilicate glass electrodes (GC100F-10; Harvard Apparatus) fire polished to resistances of 15–20 MΩ. External saline composition was as follows: 135 mM NaCl, 5 mM KCl, 4 mM MgCl₂·6H₂O, 2 mM CaCl₂, 5 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid and 36 mM sucrose, pH 7.15. The internal patch solution was as follows: 140 mM KCH₃SO₃, 2 mM MgCl₂, 2 mM EGTA, 2 mM KCl, and 20 mM HEPES, pH 7.4. Central neurons were visualized and accessed for electrophysiology using an Olympus BX51W1 microscope and 60× water-immersion lens combined with Nomarski optics, (Baines and Bate, 1998). RP2 and aCC motor neurons were initially identified by their size and location; after recording, their identity was confirmed (on the basis of morphology and axon trajectory) by labeling with 0.1% Alexa Fluor 488 hydrazide (Invitrogen), which was included in the internal patch solution. Recordings were made using a Multiclamp 700B amplifier controlled by pClamp 9.2 via a Digidata 1322A analog-to-digital converter [Molecular Devices (MDS Analytical Technologies)]. Only cells with input resistance >1 GΩ were accepted for analysis. After breakthrough, currents were measured for 3 min. If no currents were recorded in this period, the cell was termed “silent.” Traces were sampled at 20 kHz and filtered at 2 kHz low pass. Amplitudes of spontaneous rhythmic currents (SRCs) were measured using Minianalysis 6.0.3 (Synaptosoft). Currents shown were normalized for cell capacitance (determined by integration of the area under the capacitive transients for the average of 10 steps from –60 to –90 mV). SRCs showing multiple peaks were quantified as a single event; the amplitude was taken from the largest peak, which was the first one in nearly every instance. For pharmacology experiments, a 100 mM stock solution of DL-TBOA (Tocris Bioscience) was used to prepare a working solution at 300 μM in external saline. Saline bathing the preparation during larval dissection was exchanged for this working solution of TBOA before recording.

Statistics. When comparing two groups, data were analyzed using either a paired or unpaired *t* test (two-tailed, Mann–Whitney) as appropriate. When comparing more than two groups, we used one-way ANOVA. For ANOVA, the Kruskal–Wallis test with Dunn’s *post hoc* comparisons was used if the data in one or more of the groups were not normally distributed, as determined by the Shapiro–Wilk test. If data in all groups were normally distributed, one-way ANOVA with Dunnett’s test was used to compare the groups. Values shown are mean ± SEM.

Results

Eaat1 expression in *Drosophila* CNS

The major nerve tracts of the *Drosophila* ventral nerve cord (VNC), called commissures and longitudinal connectives, mark a dense neuropil of axon projections, dendrites, and synapses within the segmentally repeated embryonic and larval CNS (Fig. 1*A, B*). A subset of CNS glial cells expresses the gene *CG31235* (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), including the nine longitudinal glia (LG) found in each VNC hemi-segment. LG lie just dorsal to the longitudinal connectives and ensheath the neuropil (Jacobs et al., 1989; Stacey et al., 2007). In building genetic tools to study these glia *in vivo*, we found that a 3 kb promoter/enhancer of *CG31235* can direct

the expression of *Gal4* (Fig. 1*B'*) or nuclear GFP (nGFP) (Fig. 1*C'*) transgenes to the nine LG (Beckervordersandforth et al., 2008), plus five additional glial cells in each VNC hemi-segment (supplemental Figs. S2, S3, available at www.jneurosci.org as supplemental material). We used *in situ* hybridization to examine the expression of *Eaat1* transcripts in the VNC of *CG31235-nGFP* animals (Fig. 1*C–C'*) and noted that *Eaat1* was expressed in glial cells, including a subset of LG (Fig. 1*D*). Onset of *Eaat1* transcript expression occurred rather late in embryogenesis (stages 15–16), which is consistent with previous reports (Besson et al., 1999; Soustelle et al., 2002), and only narrowly precedes the initiation of spontaneous and uncoordinated muscle contractions (Crisp et al., 2008).

Using *Eaat1-Gal4* to mark *Eaat1*-expressing cells in the VNC (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), we found that virtually all of them also expressed *CG31235-nGFP* (423/430 cells counted). The nine LG in each hemi-segment can be subdivided further because the anterior-most six of these cells express the transcription factor Prospero (Pros) (Thomas and van Meyel, 2007). We found that 84% (173/205) of *Eaat1-Gal4* cells are also Pros positive (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), indicating that a large majority of *Eaat1*-expressing cells are of the anterior LG subtype. This subtype also expresses *Gs2* (Freeman et al., 2003; Thomas and van Meyel, 2007). Glutamine synthetases convert glutamate to glutamine, which is synaptically inert and can be safely recycled back to neurons (Hertz et al., 1999). Coexpression of *Gs2* and *Eaat1* in the anterior LG strongly suggests that this subtype of glial cell is well equipped for the uptake and metabolism of glutamate from CNS synapses in *Drosophila*, and could potentially modulate glutamatergic neurotransmission. Consistent with this, the presynaptic vesicular glutamate transporter VGlut (Mahr and Aberle, 2006; Daniels et al., 2008), and the postsynaptic glutamate receptor KaiRIA (GluRIID) (Featherstone et al., 2005) are both expressed in the dorsal neuropil of the VNC of embryos and larvae, near the cell bodies of LG. To determine whether *Eaat1*-expressing LG infiltrate the neuropil and express *Eaat1* near putative glutamatergic synapses in first instar (L1) larvae, we used *Eaat1-Gal4* to drive expression of an *Eaat1::GFP* fusion protein (*UAS-Eaat1::GFP*) (Fig. 1*E*; supplemental Fig. S4, available at www.jneurosci.org as supplemental material), and colabeled with either the membrane-targeted reporter mCD8-red fluorescent protein (RFP) (supplemental Fig. S4, available at www.jneurosci.org as supplemental material) or anti-VGlut (Fig. 1*E'*) to mark potential sites of glutamatergic presynaptic terminals in first instar larvae. *Eaat1::GFP* was broadly expressed among the RFP-labeled glial membranes and, relative to RFP, appeared to be enriched in glial membranes that had infiltrated the CNS neuropil (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). VGlut-positive puncta were located dorsally within the VNC neuropil (Fig. 1*E''*), similar to the pattern observed previously in third instar larvae (Daniels et al., 2008). Optical sections through the neuropil revealed extensive *Eaat1::GFP* labeling in close proximity to VGlut-positive puncta (Fig. 1*F, G*), consistent with the idea that glutamatergic transmission at CNS synapses in *Drosophila* could be influenced by the Pros-positive anterior LG subtype that express both the glutamate transporter *Eaat1* and the glutamine synthetase *Gs2*.

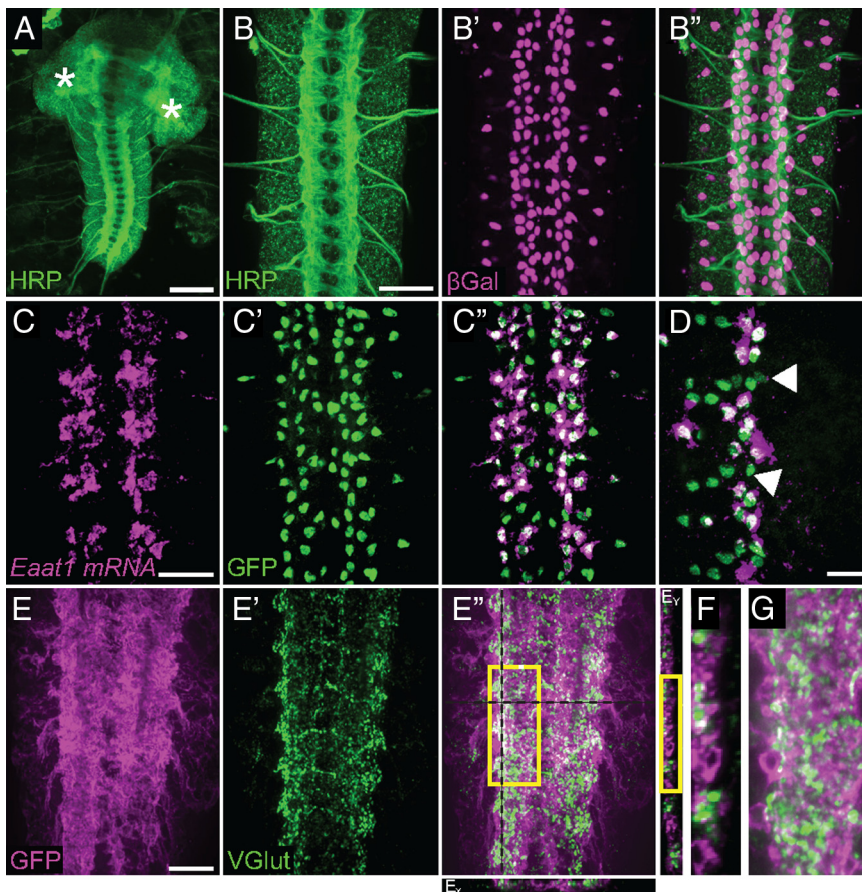


Figure 1. *Eaat1* is expressed in a subset of CNS glia. **A–B'**, Dorsal view of the CNS of a fillet embryo at stages 16–17 (genotype: *CG31235-Gal4/UAS-nuclear-lacZ; CG31235-Gal4/+*). **A**, Anti-HRP (green) labels all neurons, and reveals the brain lobes (asterisks) and the major axon tracts of the VNC. Scale bar, 50 μ m. **B–B'**, Higher magnification of the same genotype to highlight five segments of the VNC. **B**, Anti-HRP (green) reveals the ladder-like organization of commissures and longitudinal connectives. Scale bar, 20 μ m. **B'**, *CG31235-Gal4*-expressing glia revealed with anti- β -galactosidase staining (magenta). **B''**, Overlay of **B** and **B'**. Based on cell number, position, and colabeling with other markers (supplemental Figs. S2, S3, available at www.jneurosci.org as supplemental material), the *CG31235-Gal4*-positive subpopulation of glia in the VNC is likely composed of all nine LG, all three cell body glia (MM-CBG, M-CBG, and L-CBG), plus two intersegmental nerve glia (M-ISNG, L-ISNG). **C–C'**, Five segments of the VNC of a *CG31235-nuclear-GFP* embryo at stage 16. **C**, Whole-mount fluorescence *in situ* hybridization for *Eaat1* (magenta). **C'**, *CG31235-nGFP* glia (green). Scale bar, 20 μ m. **C''**, Overlay of **C** and **C'**. A subset of LG coexpress *Eaat1* and *CG31235-nGFP*. **D**, Embryo of same genotype and age at higher magnification, showing only three hemi-segments. Arrowheads indicate several *CG31235*-positive glia that do not express *Eaat1*. Scale bar, 10 μ m. **E–G**, A single optical slice showing five segments of the VNC of a L1 larva 0–2 h posthatching (genotype: *Eaat1-Gal4/+; UAS-Eaat1::GFP/+*). **E**, *Eaat1::GFP* (magenta) is extensively localized to *Eaat1* glial membrane projections within the VNC. Scale bar, 20 μ m. **E'**, Staining with anti-VGluT (green), a marker for presynaptic glutamatergic terminals, to reveal potential sites of glutamatergic synapses. VGluT staining is punctate and segmentally repeated. **E''**, Overlay of **E** and **E'**. **E_y**, Longitudinal section of VNC at position indicated by black dotted vertical black line. *Eaat1::GFP* (magenta) is observed immediately adjacent to VGluT (green) puncta. **E_x**, Cross section of VNC at position indicated by dotted horizontal black line. **F**, Close-up view of yellow boxed area in **E_y**. **G**, Close-up view of yellow boxed area in **E_x**. Anterior is at the top in all panels of all figures.

Regulation of *Eaat1* by Fringe-mediated Notch signaling

Activated Notch signaling in the Pros-expressing anterior LG of wild-type (WT) embryos can be visualized directly with the Notch-sensitive reporter *Su(H)-lacZ* (Fig. 2*A–A'*) (Griffiths et al., 2007). We have found previously that the glycosyltransferase Fng is expressed in the anterior LG subtype, where it sensitizes the Notch receptor to signaling induced by its neuron-derived ligand Delta and thereby promotes the expression of Pros (Thomas and van Meyel, 2007). To determine whether *Eaat1* expression in LG was also regulated by Fng-mediated Notch signaling, we performed two experiments; first, we examined *fng*^{13/L73} mutants (Fig. 2*C*), and second, we overexpressed the Notch antagonist Hairless (Fig. 2*D*), a transcrip-

tional repressor specific for the Notch pathway (Morel et al., 2001). We have shown previously that LG are neither missing nor improperly specified under either of these experimental conditions (Thomas and van Meyel, 2007). We observed that *Eaat1* expression in the anterior LG subtype is severely reduced in *fng* mutant embryos; this is likely due to a failure to elevate Notch signaling in these cells, since overexpression of Hairless had the same effect.

We also used semi-quantitative RT-PCR to estimate the relative level of *Eaat1* expression between *fng* mutants and controls in newly hatched L1 larvae. Normalized *Eaat1* levels in *fng*^{13/L73} mutants were reduced to 27–64% of *fng*^{13/+} heterozygous controls (Fig. 2*E*). Thus, by controlling the expression of *Eaat1*, Fng promotes terminal differentiation of a glial cell subtype that can take up glutamate and perhaps modulate neurotransmission.

To investigate whether Fng acts via Pros to promote the expression of *Eaat1* in anterior LG, we used RNAi interference and found that *Eaat1* transcript levels remained present in LG lacking detectable levels of Pros (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). This result suggests that Pros is unlikely to promote the expression of *Eaat1* at embryonic stages, and that Fng is likely to regulate *Eaat1* and Pros independently. Second, we examined wild-type animals and found that Pros is normally exported from anterior LG and degraded at late stages of embryogenesis (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). Therefore, Pros is unlikely to be important for the maintenance of *Eaat1* in the anterior LG in L1 larvae.

Creation of *Eaat1* mutations in *Drosophila*

To observe the consequences of removing *Eaat1* *in vivo*, we created *Eaat1* null mutations by imprecise excision of a P element (*P{EPgy2}Eaat1^{EY20741}*) inserted 472 bp upstream of the *Eaat1* start codon (Fig.

3*A*). Using PCR and sequencing, two novel deletion mutations were identified and confirmed to remove the start codon of *Eaat1* (Fig. 3*A–C*). The allele *Eaat1*^{SM1} was found to remove 841 bp of the *Eaat1* gene, while *Eaat1*^{SM2} removed 1293 bp (Fig. 3*B*). If translated, both mutations would fail to encode the first two transmembrane domains of the *Eaat1* transporter, with a portion of the third transmembrane domain also missing in *Eaat1*^{SM2}. Since both mutations remove the start codon and multiple transmembrane domains, they are predicted to severely disrupt the properties of *Eaat1*. To determine whether *Eaat1* mRNA is lost due to *Eaat1*^{SM2} mutation, we used RT-PCR to compare *Eaat1* transcripts between control (WT) and *Eaat1*^{SM2/SM2} larvae at L1

(Fig. 3C). Robust amplification of an *Eaat1*-specific RT-PCR product was observed in WT larvae but not *Eaat1*^{SM2/SM2} mutants, confirming loss of *Eaat1* expression.

Severe locomotor dysfunction in *Eaat1* mutants

Mutant L1 larvae hatched at a frequency of 26% from an intercross of balanced, heterozygous parents, but they were 1–2 h slower to emerge from the eggshell and had severely compromised motor function. *Drosophila* larvae normally move in a forward direction by peristaltic crawling, with rhythmic and coordinated waves of muscle contraction passing along the body wall segments in a posterior-to-anterior direction. Larvae homozygous for either *Eaat1*^{SM1} or *Eaat1*^{SM2} seldom made full-body peristaltic contractions (Fig. 3D). They had long periods of inactivity that were only occasionally interrupted by attempted contractions that were often slow and incomplete, and left the animal contorted (supplemental Movies 1, 2, available at www.jneurosci.org as supplemental material).

We quantified the number of full-body peristaltic contractions made during 3 min trials and found that *Eaat1*^{SM2/+} heterozygous controls made an average of 89.1 ± 6.0 contractions (mean ± SEM) (Fig. 3D). In contrast, the contraction rates in *Eaat1*^{SM1/SM1} and *Eaat1*^{SM2/SM2} mutants, as well as *Eaat1*^{SM2/Df} hemizygotes, were dramatically reduced, as follows: 1.8 ± 0.3, 0.7 ± 0.5, and 1.7 ± 0.4 contractions per 3 min, respectively. *Eaat1*^{SM2/+} heterozygotes had twice the rate of contractions as larvae heterozygous for a precise excision of *P{EPgy2}Eaat1*^{EY20741} (45.2 ± 3.0), but this was not significant in the nonparametric ANOVA used to analyze our complete dataset. Importantly, the severe crawling impairment that we quantified in the mutants supports the idea that both *Eaat1*^{SM1} and *Eaat1*^{SM2} are null alleles; the effects of *Eaat1*^{SM1/SM1} and *Eaat1*^{SM2/SM2} were similar, and the effects in *Eaat1*^{SM2/SM2} are as severe as *Eaat1*^{SM2/Df}.

We then measured the behavior of mutant larvae relative to heterozygous controls in an established mechanosensation assay where withdrawal, turning, and evasive crawling are scored in response to light touch (Kernan et al., 1994). Notably, all mutant larvae were sensitive to touch and withdrew, as did controls, indicating that they did not have a generalized paralysis. However, their score in this assay was reduced because they could not reliably perform the reverse or forward peristaltic contractions that typically allow escape (Fig. 3E; supplemental Movies 1, 2, available at www.jneurosci.org as supplemental material).

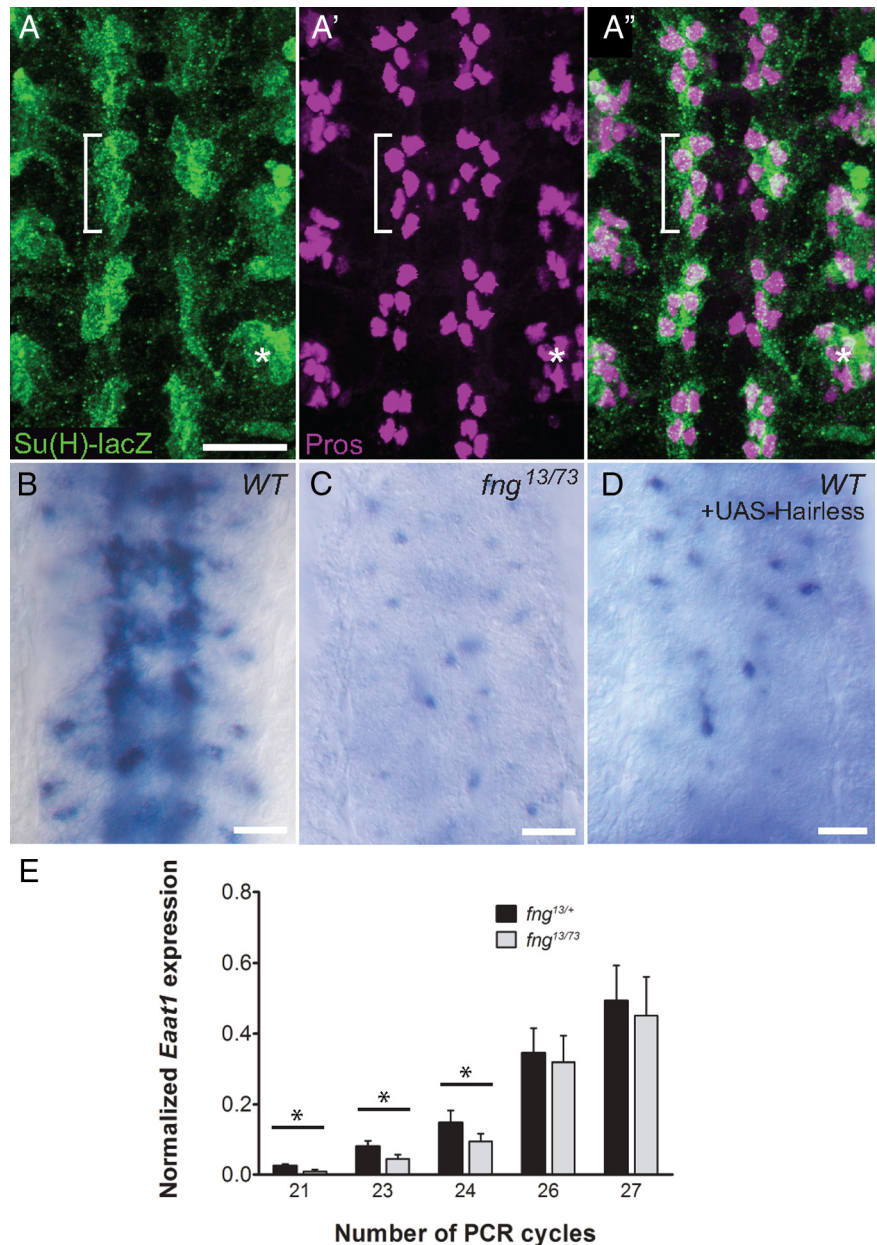


Figure 2. *Fng* is required for *Eaat1* expression in the anterior LG. **A–A'**, Four segments of the VNC of stage 16 embryo expressing *Su(H)-lacZ*, a Notch-responsive reporter. A white bracket notes where anti- β -galactosidase staining for *Su(H)-lacZ* (A, green) overlaps with the *Pros*-expressing anterior LG (A', magenta) in one hemi-segment. The white asterisk notes one cluster of *Pros*-positive neurons positioned laterally in each hemi-segment. Scale bar, 10 μ m. **A''**, Overlay of A and A'. **B–D**, All panels show whole-mount *in situ* hybridization staining for *Eaat1* in the VNC at embryonic stages 16–17. Scale bars, 10 μ m. **B**, *Eaat1* transcript is strongly expressed in a subset of LG in WT and control embryos (genotype shown: *htl-Gal4/+*). **C**, *fng*¹³/*fng*⁷³ mutants have dramatically reduced *Eaat1* levels. **D**, Expression of the Notch antagonist *Hairless* in LG (genotype: *htl-Gal4/UAS-Hairless*) also reduces *Eaat1* expression. Anterior is at the top in all panels of all figures. **E, F**, Plot of semi-quantitative RT-PCR comparing *Eaat1* expression (normalized to *Actin-5C*) between *fng*¹³/*TM3* heterozygous controls and *fng*^{13/73} mutants. Each bar represents the mean ± SEM of four distinct trials. At 21, 23, and 24 cycles (when PCR amplification still appears to be linear), the mutants had an average of 27, 55, and 64% the level of controls, respectively. Asterisks indicate significant differences at $p < 0.05$ (paired *t* test) for comparisons of controls and *fng* mutants. At cycles 26 and 27, the difference between the two groups was not significant, likely due to nonlinear amplification.

The locomotor defects of *Eaat1*^{SM2/SM2} mutants were fully rescued with a *UAS-Eaat1* transgene driven by two copies of *CG31235-Gal4*. Both the manner and frequency of the full-body contractions were restored to control levels (74.9 ± 10.3 per 3 min) (Fig. 3D). This demonstrates the specificity of the phenotype for *Eaat1* and refines the requirement for *Eaat1* in larval

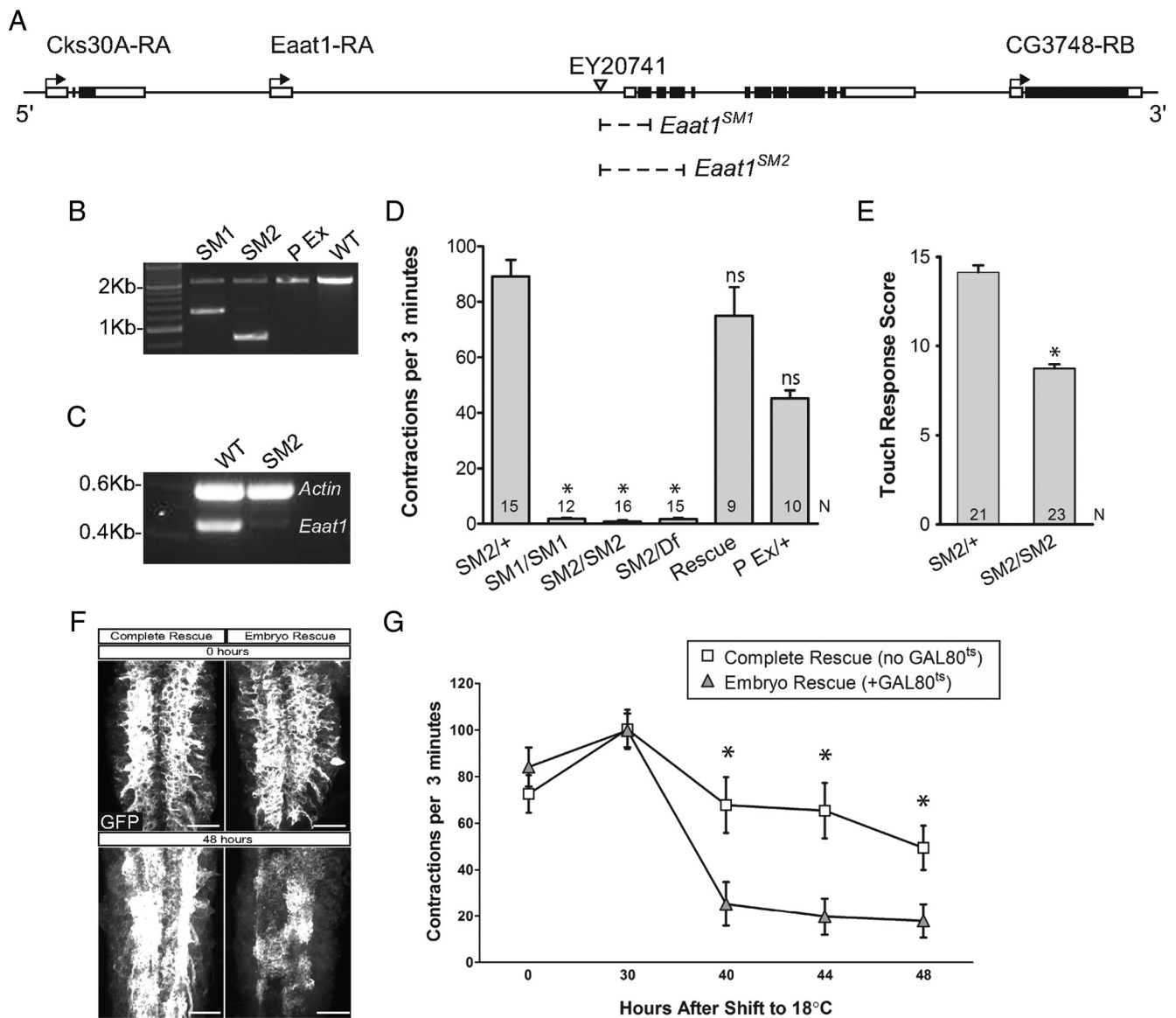


Figure 3. Generation of *Eaat1* mutants and characterization of crawling defects. **A**, The *Eaat1* locus and neighboring genes *Cks30A* and *CG3748*, and the extent of the induced deletion mutations *Eaat1*^{SM1} and *Eaat1*^{SM2} generated by imprecise excision of *EY20741* (inverted triangle). *Eaat1-RA* (FlyBase) encodes full-length *Eaat1* corresponding to EST RE20434. Coding exons are in black, noncoding exons are in white. **B**, *Eaat1*^{SM1} and *Eaat1*^{SM2} were identified via PCR screening. WT animals and precise excisions (P Ex) of *EY20741* generated a 2.166 kb PCR fragment, while imprecise excisions were identified by amplification of shorter fragments with the same primers. DNA sequencing of these shorter fragments revealed that *Eaat1*^{SM1} deleted 841 bp and *Eaat1*^{SM2} removed 1293 bp, both to the right of the original insertion site, as indicated by dotted lines in **A**. **C**, RT-PCR of WT and *Eaat1*^{SM2} mutants shows a strong *Actin5C* control band in both genotypes. A strong *Eaat1* band is present in the WT but negligible in *Eaat1*^{SM2}. **D**, *Eaat1* mutant larvae (L1, 0–2 h posthatching) show dramatically reduced numbers of peristaltic contractions. Bars show mean ± SEM for *Eaat1* heterozygotes (*Eaat1*^{SM2/+}), homozygotes (*Eaat1*^{SM1} and *Eaat1*^{SM2}), hemizygotes (*Eaat1*^{SM2/Df}), rescued animals (*CG31235-Gal4*, *Eaat1*^{SM2}; *UAS-Eaat1/+*), and controls heterozygous for the precise excision of *EY20741* (P Ex/+). Expression of *UAS-Eaat1* with *CG31235-Gal4* is sufficient to rescue *Eaat1*^{SM2} larval peristaltic contraction rates to control levels. Asterisks indicate significance at $p < 0.05$ compared with *Eaat1*^{SM2/+} heterozygotes (one-way ANOVA, Kruskal–Wallis test, Dunn’s *post hoc* comparisons). ns, Not significant. **E**, Touch response scores of *Eaat1*^{SM2/+} control and *Eaat1*^{SM2/SM2} larvae (Kernan et al., 1994). Bars show mean ± SEM. Each larva was tested four times, giving a score of 16. The number (N) of larvae tested is indicated for each genotype. Asterisk indicates significance at $p < 0.0001$ compared with *Eaat1*^{SM2/+} control (*t* test, two-tailed, Mann–Whitney). **F**, **G**, *Gal80^{ts}*-dependent withdrawal of *Eaat1::GFP* rescue following completion of embryogenesis. **F**, GFP expression in larval VNCs at hatching and 48 h following the shift to 18°C. At hatching, the levels of *Eaat1::GFP* are similar between the Complete Rescue (genotype: *Eaat1*^{SM2}, *CG31235-Gal4*; *UAS-Eaat1::GFP/+*) and Embryo Rescue (genotype: *Eaat1*^{SM2}, *CG31235-Gal4*; *UAS-Eaat1::GFP/tub-Gal80^{ts}*) groups. At 48 h, *Eaat1::GFP* expression is markedly decreased in the Embryo Rescue group. Scale bars, 20 μm. **G**, Plot of contraction rates (mean ± SEM) for Complete Rescue (white squares) and Embryo Rescue (gray triangles). Fifteen or more larvae were examined for each plotted mean. Asterisks indicate significant differences at $p < 0.01$ (*t* test, two-tailed, Mann–Whitney) for comparisons between Complete Rescue and Embryo Rescue at each time point.

locomotion to the subset of CNS glia that expresses *CG31235-Gal4*. Two additional glial-specific Gal4 drivers were also tested for their ability to rescue *Eaat1*^{SM2/SM2} mutants: *Eaat1-Gal4* (Rival et al., 2004) and *Repo-Gal4* (Sepp and Auld, 2003), which is expressed more broadly in all lateral glia. Like *CG31235-Gal4*, these drivers also rescued larval contractions (data not shown). In additional observations, we noted that *Eaat1*^{SM1} and *Eaat1*^{SM2}

mutations caused lethality before pupation, whether homozygous, hemizygous over *Eaat1*^{Df}, or in transallelic combination with one another. Mutant larvae all died by 48 h posthatching. Though both *CG31235-Gal4* and *Eaat1-Gal4* rescued larval contractions, neither rescued the viability of mutant animals beyond third instar. However, *Repo-Gal4* rescued larval contractions, and these animals survived to adulthood, suggesting

that expression of *Eaat1* in a broader number of glial cells is required for the animals to progress through pupation and eclosion.

The locomotor defect in *Eaat1* mutants could be secondary to developmental defects arising during embryogenesis or could result from acute lack of *Eaat1* function during larval locomotion. To address this directly, we rescued *Eaat1* function in *Eaat1* mutants through embryonic stages (with *CG31235-Gal4* and *UAS-Eaat1::GFP*), then used a temperature-sensitive repressor of Gal4 (*Gal80^{ts}*) to inhibit the expression of the *UAS-Eaat1::GFP* transgene and thereby discontinue the rescue in newly hatched L1 larvae (McGuire et al., 2003). To do this, we collected control and experimental embryos at 29°C, but switched them to 18°C immediately after hatching and raised them at 18°C thereafter. The control group (hereafter called *Complete Rescue*) was *Eaat1::GFP*-rescued mutants lacking *tubP-Gal80^{ts}* (genotype: *Eaat1^{SM2/SM2}, CG31235-Gal4; UAS-Eaat1::GFP/+*). The genotype of the experimental group (*Embryo Rescue*) was *Eaat1^{SM2/SM2}, CG31235-Gal4; UAS-Eaat1::GFP/tubP-Gal80^{ts}*. We used GFP fluorescence to measure the expression of *Eaat1::GFP* in the *Complete Rescue* control and the *Embryo Rescue* groups. At hatching, both groups had similar levels of GFP expression in the VNC (Fig. 3F). However, by 48 h at 18°C, there was a marked decrease in GFP expression in the *Embryo Rescue* larvae compared with the *Complete Rescue* larvae (Fig. 3F), likely reflecting the degradation of *Eaat1::GFP* produced at embryonic stages, and reduced synthesis of new *Eaat1::GFP* in *Gal80^{ts}*-expressing larvae.

Before proceeding, we studied whether simply raising larvae at 18°C shift influenced crawling behavior by measuring contraction rates among cohorts of control *Eaat1^{SM2/+}* heterozygotes at 0, 30, and 48 h following the shift to 18°C; these measured 110.6 ± 8.1 ($N = 17$), 110.9 ± 8.8 ($N = 17$), and 102.7 ± 9.7 ($N = 15$), respectively, and confirmed that there was no effect of the temperature shift alone.

We then quantified larval contraction rates in the *Complete Rescue* versus *Embryo Rescue* groups at hatching (0 h) and up to 48 h after the shift to 18°C (Fig. 3G). Average contraction rates for *Complete Rescue* controls (Fig. 3G, white squares) and *Embryo Rescue* animals (Fig. 3G, gray triangles) were similar at 0 and 30 h. However, at 40, 44, and 48 h, the mean contraction rate of the *Embryo Rescue* group was significantly reduced relative to the *Complete Rescue* group. The vast majority of *Complete Rescue* larvae was able to make full contractions at every time point in this experiment, but few larvae in the *Embryo Rescue* group made contractions at 40–48 h, though all could respond to touch (see Materials and Methods). These data demonstrate a postembryonic requirement for *Eaat1* during larval locomotion.

CNS development and apoptosis in *Eaat1* mutants

To investigate the effects of *Eaat1* mutations on CNS development, we examined the integrity of well known CNS markers in late-stage embryos with immunohistochemistry, including one for a subset of VNC axons (Fas2), one for all VNC axons (HRP), and another to detect all lateral glia (Repo) (supplemental Fig. S6, available at www.jneurosci.org as supplemental material). We also examined CNS markers in L1 larvae, including Fas2 (Fig. 4A, B), the LG marker Naz (Fig. 4C, D), and the neuronal marker Eve (Fig. 4E, F). For all of these markers, there were no abnormalities observed in *Eaat1^{SM2/SM2}* mutants relative to heterozygous controls, suggesting that the CNS of *Eaat1* mutants develops normally.

In the mammalian CNS, elevated levels of extracellular glutamate can lead to neuronal cell death from excitotoxicity (Sattler

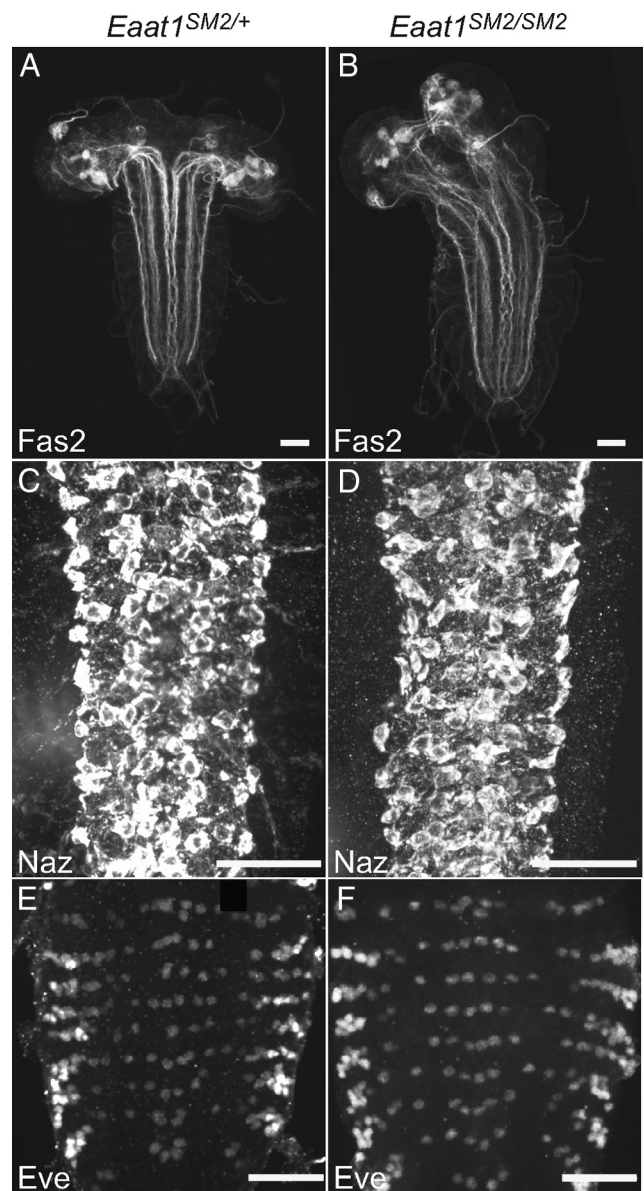


Figure 4. CNS markers are expressed normally in *Eaat1* mutant L1 larvae. *Eaat1^{SM2/+}* heterozygous controls and *Eaat1^{SM2/SM2}* L1 mutants were dissected within 2 h of hatching and stained with immunohistochemistry for CNS markers. All panels show maximum projections of confocal images (Z-stacks) of the larval VNC. **A, B**, The axonal marker anti-Fas2. **C, D**, LG marker anti-Naz. **E, F**, Neuronal marker anti-Eve. No differences were observed between *Eaat1^{SM2/+}* controls and *Eaat1^{SM2/SM2}* mutants, suggesting that the loss of *Eaat1* does not cause gross developmental defects of the CNS and does not lead to excitotoxicity-induced cell death. Scale bars, 20 μ m.

and Tymianski, 2001). Similarly, loss of *Eaat1* in *Drosophila* could lead to neurotoxicity and neuronal degeneration that could perhaps explain the larval crawling deficits. To examine this possibility further, we used immunostaining for cleaved caspase 3 and staining with acridine orange as two distinct methods to examine the occurrence of cell death in the CNS in *Eaat1* mutants versus controls (Denton et al., 2008). However, neither approach revealed any observable increase in cells undergoing apoptosis (supplemental Fig. S7, available at www.jneurosci.org as supplemental material). In addition, we counted the number of Eve-positive neurons per abdominal segment of the VNC (Fig. 4E, F). *Eaat1* mutants had the same number of Eve-positive neurons per segment (28.4 ± 0.6 , $N = 50$ segments) as heterozygous controls

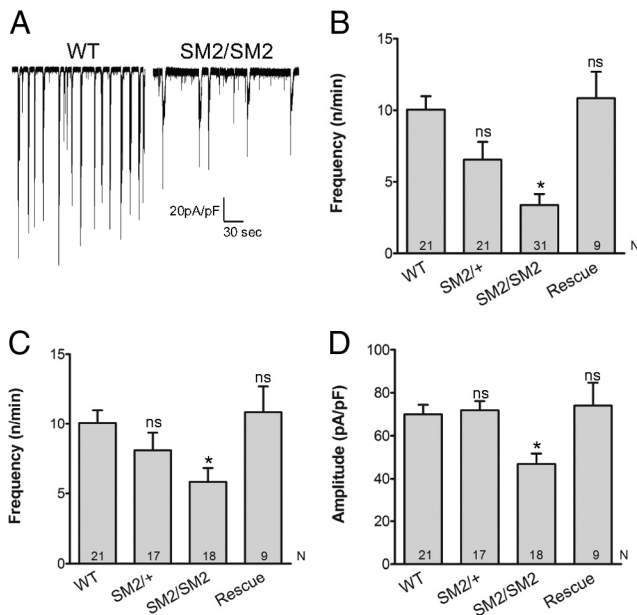


Figure 5. Excitation of motor neurons is reduced in *Eaat1* mutants. **A**, Representative whole-cell voltage-clamp recordings of SRCs in motor neurons of WT and *Eaat1*^{SM2/SM2}. **B**, **C**, SRC frequency is significantly decreased in *Eaat1*^{SM2/SM2} mutants relative to WT controls, whether “silent” cells are included (**B**) or excluded (**C**). Values shown are mean \pm SEM. Asterisk indicates significance at $p < 0.05$ (one-way ANOVA, Kruskal–Wallis test, Dunn’s *post hoc* comparisons). **D**, SRC peak amplitude is also decreased in *Eaat1*^{SM2/SM2} mutants relative to WT controls. Asterisk indicates significance at $p < 0.01$ (one-way ANOVA, Dunnett’s *post hoc* test). Examples of individual peaks used to measure SRC amplitude are indicated in Figure 6A. Both SRC frequency and amplitude were rescued to WT levels by expression of GFP-tagged *Eaat1* (genotype for rescue: *Eaat1*^{SM2}, *CG31235-Gal4*; *UAS-Eaat1::GFP*). ns, Not significant.

(29.0 ± 0.5 , $N = 65$ segments), providing additional evidence that the locomotor defects seen in newly hatched L1 larvae cannot be attributed to widespread neurotoxicity.

Synaptic excitation of motor neurons is reduced in *Eaat1* mutants

Within the *Drosophila* VNC, the dorsal motor neurons known as aCC and RP2 are situated near the LG, and their electrophysiological properties can be measured in whole-cell patch-clamp recordings from acutely exposed cells in dissected first-instar larvae. These motor neurons exhibit large excitatory synaptic currents, termed SRCs (Rohrbough and Broadie, 2002), between 10 and 15 times every minute (Fig. 5A, B) (Baines et al., 2002). These currents, which result from endogenous activity of the locomotor central pattern generator circuit, evoke bursts of action potentials in motor neurons and underlie the patterned motor activity driving locomotion (Baines et al., 2001). SRCs arise from direct cholinergic input to these motor neurons although the identity of the premotor interneurons remains unknown (Baines et al., 1999). It is likely that glutamatergic neurotransmission modulates motor activity, since glutamate receptor *GluR-III* null mutants exhibit a decrease in SRC frequency, associated with paralysis and death at late embryonic stages (Featherstone et al., 2005).

To determine whether *Eaat1* influences the electrophysiological properties of aCC and RP2 motor neurons, we measured SRC frequency and amplitude in WT and *Eaat1*^{SM2/SM2} mutant larvae within 1–4 h after hatching (Fig. 5A). In WT larvae, these two neurons exhibit nearly identical properties; therefore, the data have been pooled from both. We found that both SRC frequency (Fig. 5B) and amplitude (Fig. 5D) were significantly decreased in the *Eaat1*^{SM2/SM2} mutants, with frequency being most severely

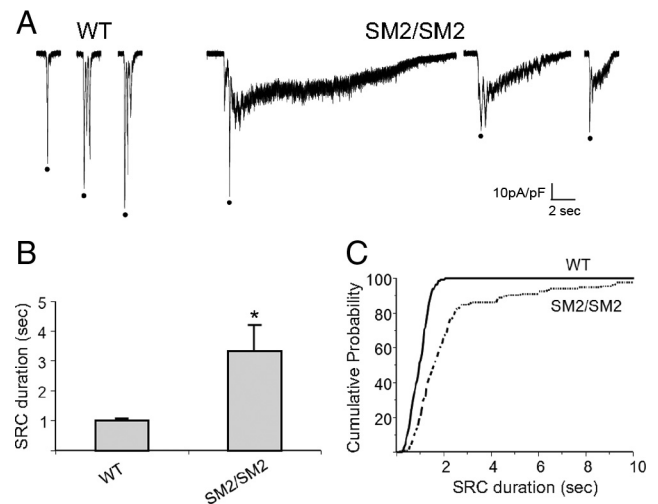


Figure 6. SRC duration is increased in *Eaat1* mutants. **A**, Representative SRC recordings in WT and *Eaat1*^{SM2/SM2}. The individual peaks used to measure SRC amplitude are indicated by the dots. **B**, SRC duration is significantly increased in *Eaat1*^{SM2/SM2}. Asterisk indicates significance at $p < 0.01$ (two-tailed *t* test, Mann–Whitney). **C**, Cumulative probability distribution of SRC duration in WT and *Eaat1*^{SM2/SM2} mutants.

affected. In fact, we observed the total absence of SRCs (during a 3 min recording period) in 42% of motor neurons (13/31). By comparison, the equivalent number of “silent” neurons in WT larvae was 0% ($N = 21$). We consider silent cells to be extreme instances of reduced SRC frequency for the following reasons: first, some nonsilent cells had extremely low SRC frequency in *Eaat1*^{SM2/SM2} mutants; and second, silent and nonsilent cells were occasionally recorded from the same *Eaat1*^{SM2/SM2} mutant larva. When silent cells were excluded from the analysis, SRCs in *Eaat1*^{SM2/SM2} mutants remained significantly less frequent than controls (Fig. 5C). For peak amplitudes of SRCs observed in *Eaat1*^{SM2/SM2} mutants, the mean was reduced to 67 and 65%, respectively, of WT and heterozygous controls (Fig. 5D).

In addition to reductions of frequency and amplitude, the kinetic properties of SRCs were also altered in *Eaat1* mutants. In WT, single SRCs often exhibit multiple components, a finding that is consistent with asynchronous release from multiple premotor interneurons that provide synaptic drive to motor neurons (Baines et al., 2002). These component currents exhibit a fast onset and relatively fast offset, producing sharp events (Fig. 6A). However, SRCs recorded in *Eaat1*^{SM2/SM2} mutant larvae often had an abnormally long decay (Fig. 6A), which increased mean SRC duration to 3.4 ± 0.8 s from 1.0 ± 0.1 s in wild-type controls (Fig. 6B). A plot of cumulative probability indicated that $\sim 40\%$ of SRCs recorded in *Eaat1*^{SM2/SM2} were longer than the maximum duration seen in wild-type controls (Fig. 6C). Thus, in addition to regulating both amplitude and frequency of motor neuron synaptic drive, *Eaat1* also contributes to the kinetics of SRCs.

The frequency and amplitude of SRCs in motor neurons of *Eaat1*^{SM2/SM2} mutants were fully rescued by *CG31235-Gal4*-driven expression of a GFP-tagged *Eaat1* (Fig. 5B–D). Since *CG31235-Gal4* is restricted to glia that reside in the CNS, and since dramatic SRC defects are readily recorded from the cell bodies of central motor neurons, we propose that the larval crawling defects observed in *Eaat1* mutants are due, at least in part, to synaptic signaling defects within the CNS.

To determine whether the electrophysiological defects observed in *Eaat1* mutants were consistent with a failure to trans-

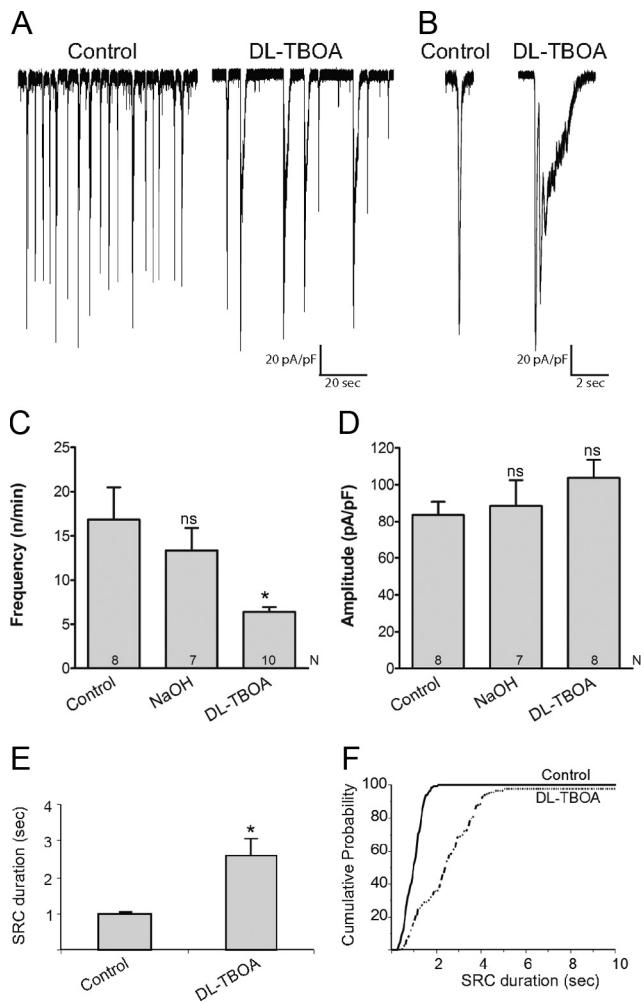


Figure 7. SRC frequency is reduced by the competitive EAAT antagonist DL-TBOA. **A, B**, Representative SRC recordings from WT motor neurons in the absence (Control or NaOH vehicle) or presence of bath-applied DL-TBOA. **C, D**, DL-TBOA reduces SRC frequency but does not influence amplitude. Values shown are mean \pm SEM. Asterisk indicates significance at $p < 0.05$ (one-way ANOVA, Dunnett's *post hoc* test). **E**, SRC duration is increased in the presence of DL-TBOA treatment. Asterisk indicates significance at $p < 0.01$ (two-tailed *t* test, Mann–Whitney). **F**, Cumulative probability distribution of SRC duration in WT in the absence (Control) or the presence of DL-TBOA. ns, Not significant.

port glutamate, we bath applied DL-TBOA, a competitive antagonist for EAATs (Shimamoto et al., 1998) (Fig. 7*A, B*) onto wild-type larvae. As in *Eaat1* mutants, the mean SRC frequency was reduced; it reached only 48% of controls at the concentration tested (300 μ M) (Fig. 7*C*). There was no effect on amplitude (Fig. 7*D*), but, as in *Eaat1* mutants, the mean SRC duration was increased in the presence of DL-TBOA, from 1.0 ± 0.1 s in controls to 2.6 ± 0.5 s (Fig. 7*E*). More than 50% of SRCs recorded in the presence of DL-TBOA were longer than the maximum duration seen in controls (Fig. 7*F*).

Together, our results show that CNS glial expression of the glutamate transporter *Eaat1* has a role in modulating patterned activity of motor neurons and support the idea that *Eaat1* expression in the anterior LG modulates glutamate neurotransmission.

Discussion

The role of the glutamate transporter *Eaat1* in the *Drosophila* CNS

We have created the first mutations in *Drosophila Eaat1* as a means to better understand the importance of glutamate uptake

for CNS development and function. Previous approaches using RNAi had uncovered no function for *Eaat1* in larvae (Rival et al., 2004; Rival et al., 2006); this is likely due to insufficient knock-down at larval stages since here we found *Eaat1* mutants have a severe crawling deficit. We narrowed the requirement for this glutamate transporter to a subpopulation of CNS glia and found that the crawling defect could be induced by conditional inactivation of *Eaat1* after embryogenesis. Consistent with our observations that several immunohistochemical markers of neurons and glia were unaffected in *Eaat1* mutants, these results indicate that the crawling deficit is not secondary to developmental defects. Furthermore, we found no evidence for widespread cell death in *Eaat1* mutant larvae, suggesting that the crawling deficit is not a consequence of neurotoxic damage induced by excess glutamate.

Interestingly, *Eaat1* mutant larvae withdrew and turned their heads normally in response to mechanical stimulus, raising the possibility that CNS neural circuits controlling rhythmic contractions required for larval crawling were specifically affected. The importance of glutamate-mediated neurotransmission in the CNS of *Drosophila* larvae has not been well characterized, though it has a well studied and essential role at the neuromuscular junction (DiAntonio, 2006). We have not specifically tested the function of neuromuscular synaptic transmission in *Eaat1* mutants, but several lines of evidence indicate the crawling deficit is primarily due to a requirement for *Eaat1* within the CNS. First, electrophysiological recordings from motor neuron cell bodies within the CNS showed that *Eaat1* mutants displayed decreased frequency of synaptic drive onto motor neurons. This mimics the effects we observed with acute pharmacological manipulations of glutamate transport, and resembles a published report on mutants of the AMPA-type glutamate receptor KaiRIA (also known as GluR-IIID or *brec*) (Featherstone et al., 2005). Second, *Eaat1* has been reported to be absent from neuromuscular junctions in larvae (Rival et al., 2006). Third, crawling was rescued with a Gal4 driver expressed exclusively in CNS glia. Together, our results lead us to propose that the crawling deficit in *Eaat1* mutants is primarily due to a failure of specific glial cells in the CNS to efficiently remove excess glutamate from central synapses, leading to perturbed glutamatergic neurotransmission and reduced motor output. Since *Drosophila* motor neurons receive direct cholinergic input from premotor interneurons, we currently favor the idea that *Eaat1* acts to influence the patterning of rhythmic motor neuron activity by modulating glutamatergic synapses onto these premotor interneurons, or perhaps synapses further upstream in the circuitry (Baines et al., 2001; Featherstone et al., 2005).

Previous research has demonstrated that proteins essential for glutamatergic neurotransmission are expressed in the embryonic and larval CNS, such as VGlut (Mahr and Aberle, 2006; Daniels et al., 2008), and the ionotropic glutamate receptor subunits KaiRIA, GluR-IA, GluR-IB, Nmdar1, and Nmdar2 (Ultsch et al., 1992; Volkner et al., 2000; Featherstone et al., 2005). As noted above, AMPA-like receptors have already been implicated since KaiRIA (*brec*) mutants are paralyzed and have reduced SRC frequency (Featherstone et al., 2005). In addition, noncompetitive NMDA receptor antagonists have also been shown to inhibit rhythmic locomotor activity in larvae (Cattaert and Birman, 2001), but the role of these NMDA-like receptors in CNS motor circuitry remains to be tested by genetic means. The metabotropic glutamate receptor mGluRA is unlikely to be involved since null mutants are viable and have only mild defects of synaptic plasticity and morphology at neuromuscular syn-

apses (Parmentier et al., 1996; Ramaekers et al., 2001; Bogdanik et al., 2004; Devaud et al., 2008).

One might predict that loss of glial glutamate uptake in *Eaat1* mutants would elevate extracellular glutamate levels and lead to hyperactivity of glutamate receptors. However, SRC frequency is reduced by loss-of-function mutations of either *Eaat1* or the KaiRIA (*brec*) glutamate receptor subunit, implying that *Eaat1* mutants have reduced glutamatergic signaling instead. Perhaps this is due to receptor desensitization in the presence of excess glutamate. *Eaat1* mutant larvae remain capable of some movement, while *brec* null mutants are completely paralyzed (Featherstone et al., 2005). This difference may reflect reduced glutamate neurotransmission resulting from excess receptor desensitization, versus the complete loss of neurotransmission in animals lacking an essential receptor subunit.

Currently, we cannot explain why pharmacological manipulations of glutamate transport affect SRC frequency and duration, while *Eaat1* mutations also affect SRC amplitude. It could reflect differences between acute, short-term treatment with DL-TBOA versus the genetic approach, in which loss of *Eaat1* function may lead to additional compensatory changes induced by prolonged increases of extracellular glutamate; it is conceivable that reduced SRC frequency is an acute and proximate effect of *Eaat1* mutations, and that the changes we observed in SRC peak amplitude are induced secondarily.

Our findings complement genetic studies in mice that are complicated by functional redundancy of the glial glutamate transporters GLAST and GLT-1. Mice singly mutant for GLAST or GLT-1 are viable (Tanaka et al., 1997; Watase et al., 1998), but double-knock-out mice die *in utero* and exhibit multiple proliferation and migration defects of stem cells and/or neurons in cortex, hippocampus, and olfactory bulb (Matsugami et al., 2006). As in *Drosophila*, glutamate neurotoxicity was not readily apparent in double-mutant mice, but the neuroanatomical defects observed in mice may reflect added importance for glutamate- and activity-dependent processes in the development of mammalian nervous systems. Knock-out mice for only GLAST fail complex motor tasks (Watase et al., 1998). This might reflect a role for this transporter in Bergmann glia of the cerebellum, where loss of GLAST is associated with inappropriate innervation and neurotransmission at glutamatergic synapses onto Purkinje neurons (Watase et al., 1998; Marcaggi et al., 2003; Takayasu et al., 2005, 2006). The acute role in crawling that we uncovered by conditional inactivation of *Eaat1* after embryogenesis supports the idea that, in *Drosophila* as in mice, glutamate transport strongly influences neurotransmission controlling motor function *in vivo*.

Differentiation of functionally distinct glial cell subtypes

We have found that the requirement for *Eaat1* in locomotor behavior is limited to a subpopulation of glia marked by the CNS-specific driver CG31235-Gal4. At present, the tools available cannot distinguish the relative importance of glial cells located in the VNC versus the brain lobes. Nonetheless, *Eaat1* is expressed in a limited subset of neuropil-associated glia in the VNC, including the anterior LG subtype, where it is coexpressed with the glutamate recycling enzyme Gs2 (Freeman et al., 2003; Thomas and van Meyel, 2007) and its expression is regulated by the glycosyltransferase Fng. Fng sensitizes the Notch receptor on the anterior LG to stimulation from developing axons bearing the Delta ligand and thereby promotes neuron-to-glia signaling during embryogenesis (Thomas and van Meyel, 2007). Anterior and posterior LG are derived from a common glioblast (Schmidt et

al., 1997; Beckervordersandforth et al., 2008), and so, as a consequence of this interplay between neurons and glia, Fng provides a mechanism for the selective expression of *Eaat1* in the anterior LG subtype. Thus, Fng promotes the emergence of a functionally distinct glial cell subtype that can take up glutamate and has the potential to modulate neurotransmission at central synapses.

In vitro studies using cocultures of mammalian neurons and astrocytes have shown that factors secreted from neurons, and direct neuron–glial contact, can promote the expression of GLAST and/or GLT-1 in astrocytes (Swanson et al., 1997; Schlag et al., 1998; Munir et al., 2000; Zelenia et al., 2000; Filosa et al., 2009; Yang et al., 2009). Studies *in vivo* provide evidence that the expression of glutamate transporters in the mammalian CNS is regulated by neuron–glial communication during development and also at mature stages. In the developing rodent cerebellum, for example, neuron–glial signaling through Notch and its ligand DNER regulates the maturation of Bergmann glia and promotes GLAST expression in these specialized astrocytes (Eiraku et al., 2005). Interestingly, the Fng ortholog Lunatic Fringe is expressed in Bergmann glia (Gong et al., 2003), but its involvement in neuron–glial interactions there remains unknown. In the mature hippocampus, direct neuron–glial contact and EphA4-ephrinA3 signaling regulate synaptic plasticity by controlling the expression of GLAST and GLT-1 and thereby regulating glutamate transport (Carmona et al., 2009; Filosa et al., 2009). Thus, mammals and insects both use neuron–glial communication to regulate glial glutamate transporter expression. The mechanism of regulation by Fng-mediated Notch signaling that we have discovered in flies may be conserved in the mammalian CNS, and the *Eaat1* mutants described in this study provide an important model to study the molecular pathogenesis of CNS diseases in humans that result from dysregulation of glutamate transport.

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