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ORIGINAL ARTICLE

Taurine Transporter dEAAT2 is Required for Auditory Transduction in *Drosophila*

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Abstract *Drosophila* dEAAT2, a member of the excitatory amino-acid transporter (EAAT) family, has been described as mediating the high-affinity transport of taurine, which is a free amino-acid abundant in both insects and mammals. However, the role of taurine and its transporter in hearing is not clear. Here, we report that dEAAT2 is required for the larval startle response to sound stimuli. dEAAT2 was found to be enriched in the distal region of chordotonal neurons where sound transduction occurs. The Ca^{2+} imaging and electrophysiological results showed that disrupted dEAAT2 expression significantly reduced the response of chordotonal neurons to sound. More importantly, expressing dEAAT2 in the chordotonal neurons rescued these mutant phenotypes. Taken together, these findings indicate a critical role for *Drosophila* dEAAT2 in sound transduction by chordotonal neurons.

Keywords *Drosophila* dEAAT2 · Taurine · Chordotonal neurons · Sound transduction

Introduction

Taurine (2-aminoethane-sulfonic acid) is one of the most abundant free amino-acids in mammals [1]. Although it is not incorporated into proteins, taurine has various physiological functions, such as osmoregulation, neuroprotection, Ca^{2+} modulation, antioxidant defense, and membrane stabilization [1–3]. It also plays a role in neural development [4, 5]. Several studies have shown that taurine plays an important role in the functional development of the auditory system [6, 7]. Taurine supplementation in the diet has an effect on hearing development in preterm infants [8–11]. In addition, taurine acts as a neuromodulator. In the central auditory pathway, it reduces neuronal excitability and depresses synaptic transmission in the inferior colliculus by activating glycine receptors [12, 13]. Moreover, it acts as a neuromodulator to strengthen glycinergic and GABAergic neurotransmission in rat anteroventral cochlear nucleus neurons [14]. In the cochlea, taurine is abundant in supporting cells [15–17], but its role in the peripheral auditory pathway remains unclear.

In mammals, a high intracellular taurine level is maintained by a Na^+- and Cl^--dependent taurine transporter (TauT/SLC6A). Taurine transporter knockout ($taut^{-/-}$) mice show a strongly reduced taurine level [18] and this occurs in various diseases, including retinal degeneration, reduced olfactory function, unspecific hepatitis, and liver fibrosis [19]. However, the function of the TauT in hearing remains poorly understood.

Excitatory amino-acid transporters (EAATs) are membrane proteins that mediate the clearance of glutamate,

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aspartate, and other molecules released during synaptic transmission in the nervous system [20, 21]. Previous studies have shown that the glutamate/aspartate transporter (GLAST; EAAT1), which occurs in the mammalian organ of Corti [22, 23], is the main glutamate transporter involved in the modulation of auditory transmission between inner hair cells and spiral ganglion neurons [24]. To date, two EAAT members have been identified in *Drosophila*: dEAAT1 and dEAAT2 [25]. dEAAT1 is a glutamate transporter and plays an essential role in regulating locomotion in *Drosophila* [26, 27]. dEAAT2 is the only taurine transporter identified in *Drosophila* [28]. A previous study has shown that disrupted dEAAT2 expression significantly reduces the taurine level [29]. However, the role of dEAAT2 in hearing remains elusive. The fruit fly *D. melanogaster* is a prominent model system in neuroscience [30–32]. The chordotonal organs are specialized for hearing in most insects including *Drosophila* [33]. Studies have shown that the chordotonal organs share transduction mechanisms similar to vertebrate hair cells [34, 35]. In adult *Drosophila*, the chordotonal neurons of Johnston's organ in the second antennal segment mediate hearing as well as sensing gravity and wind [36, 37]. We previously reported that larval chordotonal neurons, like their counterparts in adult *Drosophila*, are required for sound transduction [38]. Thus, *Drosophila* is a suitable model in which to study the function of dEAAT2 in hearing.

Therefore, in the present study we explored the role of *Drosophila* dEAAT2 in larval chordotonal neurons in sound transduction.

Materials and Methods

Drosophila Stocks

All flies were raised on a standard cornmeal medium at 25 °C with 60% relative humidity under a 12 h light/dark cycle. The following strains were used: w^{1118}, *IAV-Gal4*, *NOMPC-Gal4*, *UAS-GFP*, *UAS-CD8-GFP*, *UAS-GCaMP6*, *UAS-CD4-tdTomato*, $dEaat2^{MI}$ (stock #36215), *dEAAT2-Gal4*, and *UAS-dEAAT2-EGFP*. All the fly lines, except for *dEAAT2-Gal4*, *UAS-dEAAT2-EGFP*, *UAS-GCaMP6,* and *UAS-CD4-tdTomato*, were from the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN). The *UAS-GCaMP6* and *UAS-CD4-tdTomato* strains were gifts from Yuh-Nung Jan (University of California, San Francisco, CA) [39]. The *dEAAT2-Gal4* and *UAS-dEAAT2-EGFP* transgenic lines were constructed in our laboratory.

Generation of Transgenic Flies and Confirmation of the $dEaat2^{MI}$ Mutation

The *dEAAT2-Gal4* was generated by amplifying the *Drosophila* dEAAT2 promoter region from the genomic DNA of w^{1118} via the polymerase chain reaction (PCR). The following primers with specific restriction sites were used: forward 5′-GAATTCGGCAACCTTTGGCG-GACTCCCCATCAA-3′; reverse 5′-GGATCCTGTC-CAAATGTCCAAGCTTCCGGGTG-3′.

And then the PCR fragment was inserted into the EcoR III/BamH I sites located before the GAL4 sequence in the pCaSpeR-GAL4 vector.

To generate the *UAS-dEAAT2-EGFP* construct, dEAAT2 cDNA was prepared by RT-PCR. Then the dEAAT2-coding sequence was amplified by PCR using the following primers with specific restriction sites: forward 5′-GAATTCATGGGTCCCCCCACCTCAACTG-3′; reverse 5′-GGTACCGACCCTGCGGTTACAATGCTCCT-3′.

The reverse primer was designed to remove the stop codon from the dEAAT2 sequence. The purified PCR products were cloned into a pUAST-EGFP vector at the EcoR I and Kpn I sites. The UAS-dEAAT2-EGFP and dEAAT2-Gal4 recombinant plasmids were then injected into w^{1118} embryos. Transgenic flies were obtained by P-element-mediated germ-line transformations.

We confirmed the presence of the Minos element insertion in $dEaat2^{MI}$ strain DNA by PCR using the following primers: Primer 1: 5′-GTTCGTCAGCAT-CAGCTCTATAGTCCC-3′; Primer 2: 5′-TAG-GATCCGTTGACCTGCAGGTCGA-3′; Primer 3: 5′-GTATTCAGCGTATGCGTCCGCGTAC-3′.

We also examined the transcript levels of dEAAT2 in the $dEaat2^{MI}$ strain using quantitative reverse-transcription PCR. Total RNA was extracted from 10 larvae using TRIzol reagent (Thermo Fisher Scientific, Shanghai, China). The cDNA was synthesized using HiScript® Reverse Transcriptase (Vazyme, Nanjing, China) following the manufacturer's instructions. After an 8-fold dilution of cDNA, we performed real-time quantitative PCR using ChamQ™ SYBR® qPCR Master Mix (Vazyme, Nanjing, China) and the CFX96 Real-time System (Bio-Rad, Hercules, CA). Actin 5C (Primers: 5′-TGTGACGAA-GAAGTTGCTGC and 5′-CTCATCACCCACGTAC-GAGT) was used as an internal control. Relative quantification was calculated using the $2^{-\Delta Ct}$ formula. The primers for *dEaat2* were as follows: Primer 4: 5′-AAGCTCGATTGTGATGGGGT-3′; Primer 5: 5′-CTCATCACCCACGTACGAGT-3′.

Behavioral Tests

Startle Response Assay

Each third instar larva was carefully picked up and washed twice with phosphate-buffered saline (PBS). Then groups of 20 larvae were gently transferred to a 2% agar plate, which was placed on top of a loudspeaker. For the startle assay, custom software was written to use MATLAB to generate a sine tone of 500 Hz. The sound signal was amplified by the loudspeaker. The sound intensity was measured with a CEL-63x Series Sound Level Meter (Casella CEL, Kempston, UK) following the manufacturer's instructions. At the same sound intensity, the freely-moving larvae were stimulated with a 2-s sound pulse repeated 3 times. When exposed to a sound, they exhibited startle behaviors: pausing, turning, mouthhook retraction, and backward locomotion. To evaluate the sound response, a larva was counted as responsive when it exhibited any of the above behaviors more than once in response to sound stimuli. The ratio of larvae that had startle responses was calculated for different sound intensities.

Touch Sensitivity Assay

All flies were raised at 25 °C in an incubator. Groups of 20 third-instar larvae were carefully picked up and washed with PBS. A single larva was then gently transferred to a 2% agar plate. During linear locomotion on the plate, the larva was gently touched with an eyelash on the anterior thoracic segment, and this was repeated 4 times. When touched, the larva stopped crawling, contracted the head, and then turned to either side or retracted. The behavioral response was scored as 0 for those with no response, 1 for stopping and hesitation, 2 for retracting the head or turning, and 3 for a wave of retraction. The sum of responses in 4 trials served as the touch response score, and wild-type (WT) larvae had a score of $\sim$ 6.

In Vivo Ca^{2+} Imaging

Functional Ca^{2+} imaging can be used to study neurons in the peripheral nervous system [40, 41]. *In vivo* Ca^{2+} imaging of larval chordotonal neurons was performed as described previously [38]. In brief, a freely-moving third-

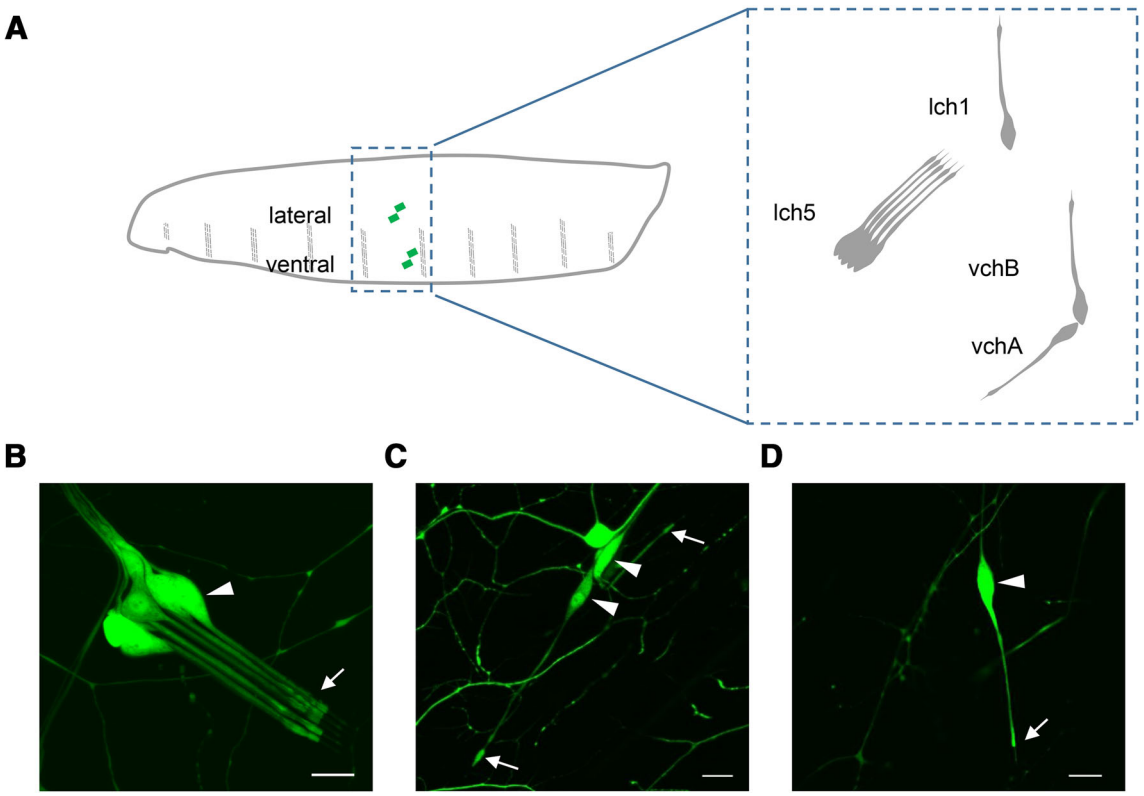


Fig. 1 dEAAT2 is expressed in chordotonal neurons. **A** Schematic of chordotonal neurons in an abdominal hemisegment of the larval body wall. Green diamonds mark the chordotonal neurons (left). The chordotonal neurons include three singlet neurons (vchA, vchB, and lch1) and a lateral cluster of five neurons (lch5) (right). **B–D** All four chordotonal neurons were labeled by *dEAAT2-Gal4*: lch5 (**B**), vchA and vchB (**C**), and lch1 (**D**). Arrowheads, cell bodies; arrows, terminal dendritic structures. Green indicates GFP signals. Scale bars, 10 μm.

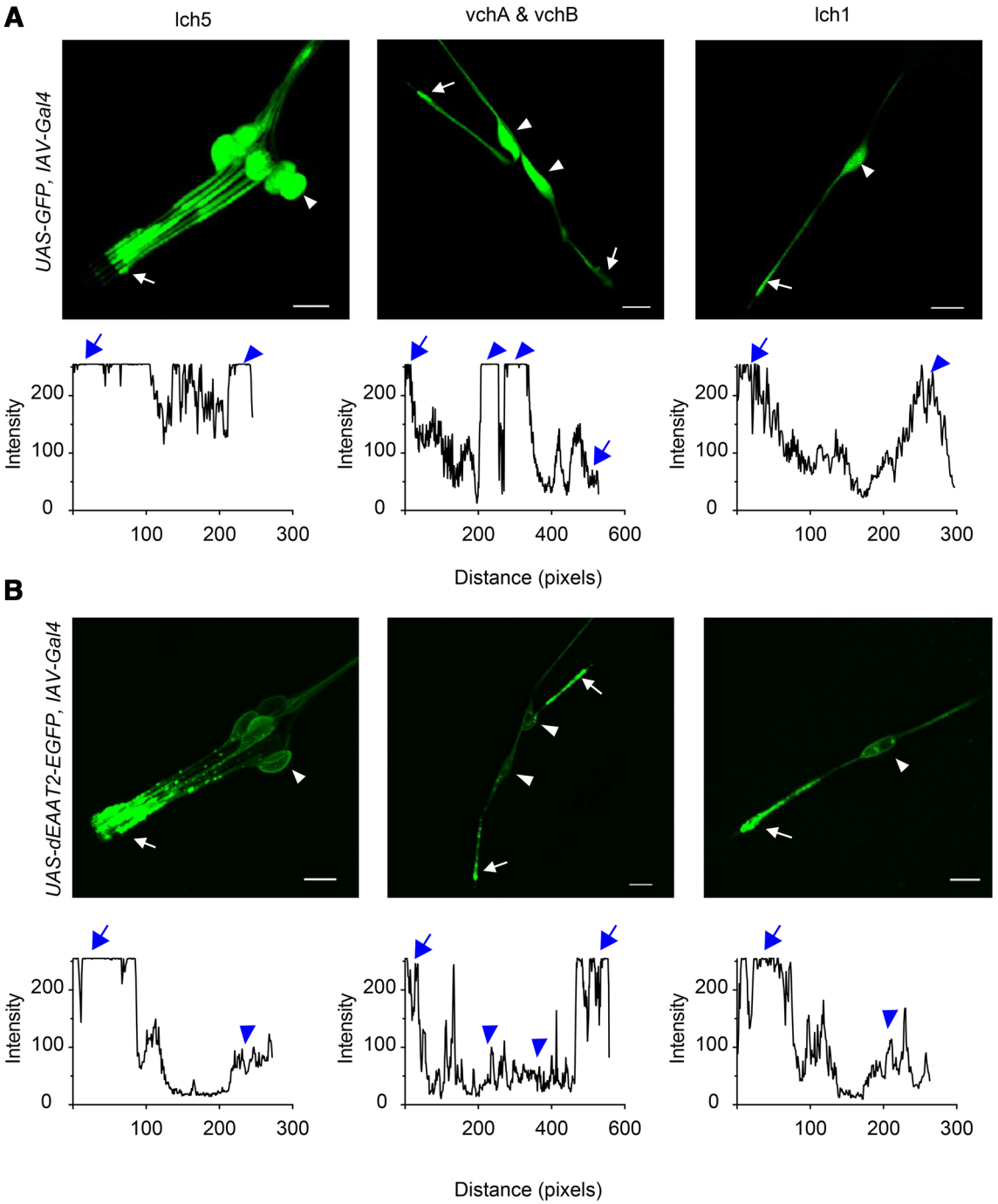


Fig. 2 Subcellular localization of dEAAT2 protein in chordotonal neurons. **A** Upper panels: GFP fluorescence signals are uniformly distributed in chordotonal neurons when expressing GFP alone. Lower panels: pixel intensity of GFP along chordotonal neurons plotted against distance. **B** Upper panels: confocal images showing that dEAAT2::EGFP protein is enriched in the distal dendritic region of chordotonal neurons. Lower panels: pixel intensity of dEAAT2::EGFP along chordotonal neurons plotted against distance. Green, dEAAT2::EGFP protein signals; arrowheads, cell bodies; arrows, terminal dendritic structures. Scale bars, 10 μm.

instar larva was picked up and rinsed with PBS at room temperature (25 °C). The larva was then pressed between two coverslips with the body lateral side up. The imaging data were acquired on a Zeiss LSM510 confocal microscope (Zeiss, Jena, Germany) with a 20× objective lens. The genetically-coded Ca^{2+} indicator GCaMP6 was used to measure the Ca^{2+} signal. GCaMP6 and red fluorescent proteins were excited respectively by a 488-nm and a 543-nm laser, and the fluorescence signals were collected. The soma was selected to measure GCaMP6 fluorescence

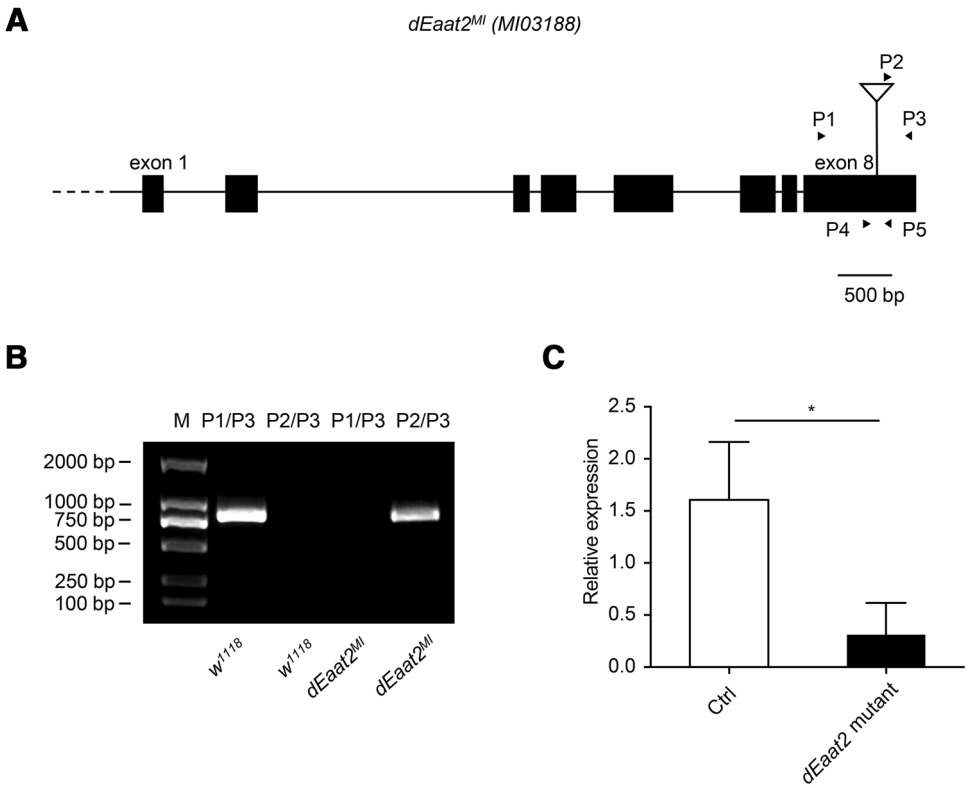


Fig. 3 Confirmation of the *dEaat2*^{MI} mutation. **A** Boxes represent exons; straight lines are introns. The location of the Minos insertion (*dEaat2*^{MI}) is indicated by a triangle. Arrows indicate the positions of the primers used for mutant verification. P1-5 means primer 1-5. **B** Genomic PCR confirming the presence of the Minos insertion in the *dEaat2* gene, using primers as indicated in **A**.A 750 bp genomic fragment of w^{1118} was amplified using P1 and P3; a 800 bp genomic fragment containing Minos element sequence of *dEaat2*^{MI} was amplified using P2 and P3. **C** dEAAT2 mRNA levels assessed by quantitative reverse-transcription (q-RT) PCR (*$P < 0.05$, unpaired *t*-test).

intensity. All recordings were from a lateral cluster of five (lch5) chordotonal neurons which are in the fifth abdominal segments (A5) of larvae. The average GCaMP6 signal from the first 5 s before the sound stimulus was taken as F_0, and $\Delta F/F_0$ was calculated for each data point. GCaMP6 signals from the soma were analyzed.

Electrophysiological Recording

Extracellular recordings were made as previously described [38]. The third-instar larvae were dissected in a modified hemolymph-like saline (in mmol/L: NaCl 103, KCl 3, HEPES 5, trehalose 10, glucose 10, sucrose 7, $NaHCO_3$ 26, NaH_2PO_4 1, $MgCl_2$ 4, pH 7.25). Before use, 2 mmol/L Ca^{2+} (in the form of $CaCl_2$) was added to the saline. Major muscles covering the chordotonal neurons were carefully removed under microscopy. Glass electrodes with a tip diameter of 10 μm for electrophysiological recordings were pulled with a P-97 puller (Sutter Instruments, Novato, CA) and filled with external solution. Recordings were made from vchA chordotonal neurons stimulated with a 1-s sound stimulus. Signals were amplified using an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA) recorded at a sample rate of 10 kHz and low-pass filtered at 1 kHz. Data were acquired with a Digidata 1550B (Molecular Devices) acquisition system, and analyzed off-line using pClamp 10.4 software (Molecular Devices).

The extracellularly-recorded action potentials (APs) of vchA chordotonal neurons were detected by a threshold-based search of the single-unit recordings. A time-window of fixed length (1 s) was used before and during sound stimulation. The numbers of APs in the two time windows were calculated. To obtain the adaptation rate of chordotonal neurons, the post-stimulus time histograms for 6 neurons were averaged and fitted to a curve.

Statistical Analyses

All data were analyzed using Prism6 (GraphPad) and are presented as mean ± SEM. For behavioral data, the χ^2 and two-tailed Mann–Whitney tests were used for significance testing. The two-tailed unpaired Student's *t* test was used for significance testing in the Ca^{2+}-imaging experiments. Two-tailed Mann–Whitney tests were used for the electrophysiological recordings. Statistical significance was defined as $P < 0.05$. All experiments were repeated three times.

Results

Drosophila dEAAT2 is Expressed in Larval Chordotonal Neurons

A previous study had shown that *Drosophila* dEAAT2 is expressed in larval peripheral sensory neurons, including a

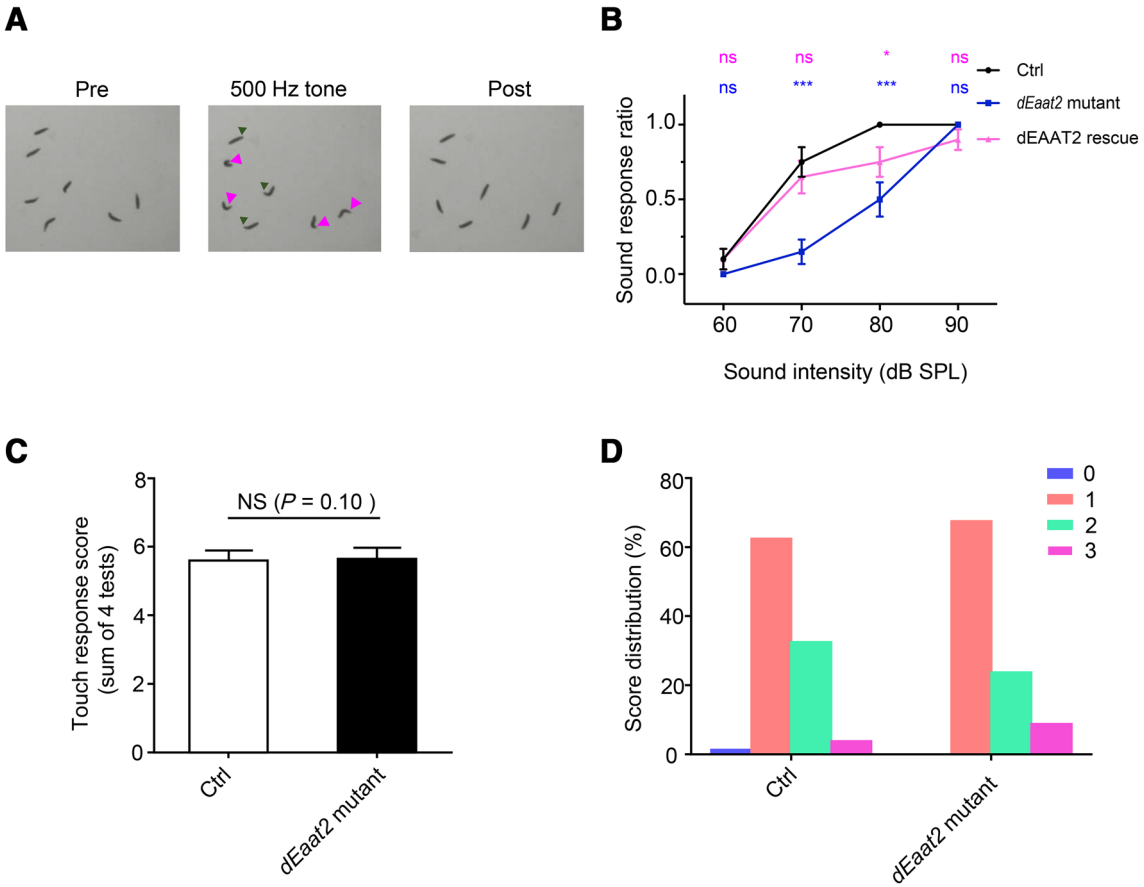


Fig. 4 dEAAT2 is required for the larval startle response to sound stimuli. **A** Startle response behaviors to sound stimuli in control larvae. Left, freely-moving larvae; center, larvae exhibiting a series of startle responses to sound (pause and contraction indicated by green arrowheads; turning, by magenta arrowheads); right, larvae recovered 5 s after the removal of the sound stimulus. **B** Larval startle responses to a 500-Hz sound of increasing intensity. The *dEaat2* mutant showed a reduced response, which was rescued by expressing dEAAT2 in chordotonal neurons. n = 20/group. Two-tailed Mann–Whitney tests of data sets against the WT background control were performed at each sound intensity. P values are indicated above each data point colored according to the genotype. **C** The *dEaat2* mutant showed touch sensitivity similar to control larvae. The histogram shows the responses in 4 trials of individual larvae of different genotypes. n = 20/group. The χ^2 test was used to test for the statistical significance of differences between control and *dEaat2* mutant. **D** Score distribution of control and *dEaat2* mutant larvae. 0, no response; 1, pause; 2, recoil; 3, reverse contraction. Ctrl: w^{1118}. *dEaat2* mutant: $dEaat2^{MI}/dEaat2^{MI}$; +/+. dEAAT2 rescue: $dEaat2^{MI}/dEaat2^{MI}$; *NOMPC-Gal4/USA-dEAAT2-EGFP*. NS, not significant; $^*P < 0.05$. Error bars represent ± SEM.

lateral cluster of five chordotonal neurons (lch5) and multidendritic neurons [29]. We previously reported that chordotonal neurons are required for the startle response of larvae to sound [38]. Larval chordotonal neurons contain three singlet chordotonal neurons (vchA, vchB, and lch1) and lch5 (Fig. 1A), and these neurons respond to sound with increased AP firing [38]. To explore the potential role of dEAAT2 in hearing, we first investigated whether it is expressed in larval auditory sensory organs by using the Gal4/UAS system. We generated a dEAAT2-Gal4 transgene containing a 4.4-kb genomic fragment upstream of the dEAAT2 translation initiation codon. The *dEAAT2-Gal4* transgenic line was then used to drive the expression of green fluorescent protein (GFP). Similar to the previous study [29], GFP signals were detected in third-instar larval chordotonal and multidendritic neurons, including lch5 (Fig. 1B), the three singlet chordotonal neurons (vchA, vchB, and lch1) (Fig. 1C, D) and class I neurons (Fig. S1B).

To further identify the subcellular localization of dEAAT2 in larval chordotonal neurons, we constructed a *UAS-dEAAT2-EGFP* transgenic line in which the enhanced GFP (EGFP) coding sequence was inserted into the C-terminal region of dEAAT2. *IAV-Gal4* was then used to drive the expression of GFP or the dEAAT2::EGFP fusion protein in the larval chordotonal neurons. When expressing GFP alone, we observed a uniform distribution of GFP fluorescence in the chordotonal neurons (Fig. 2A). In contrast, when expressing the dEAAT2::EGFP fusion protein, we found that EGFP labeling was present in all

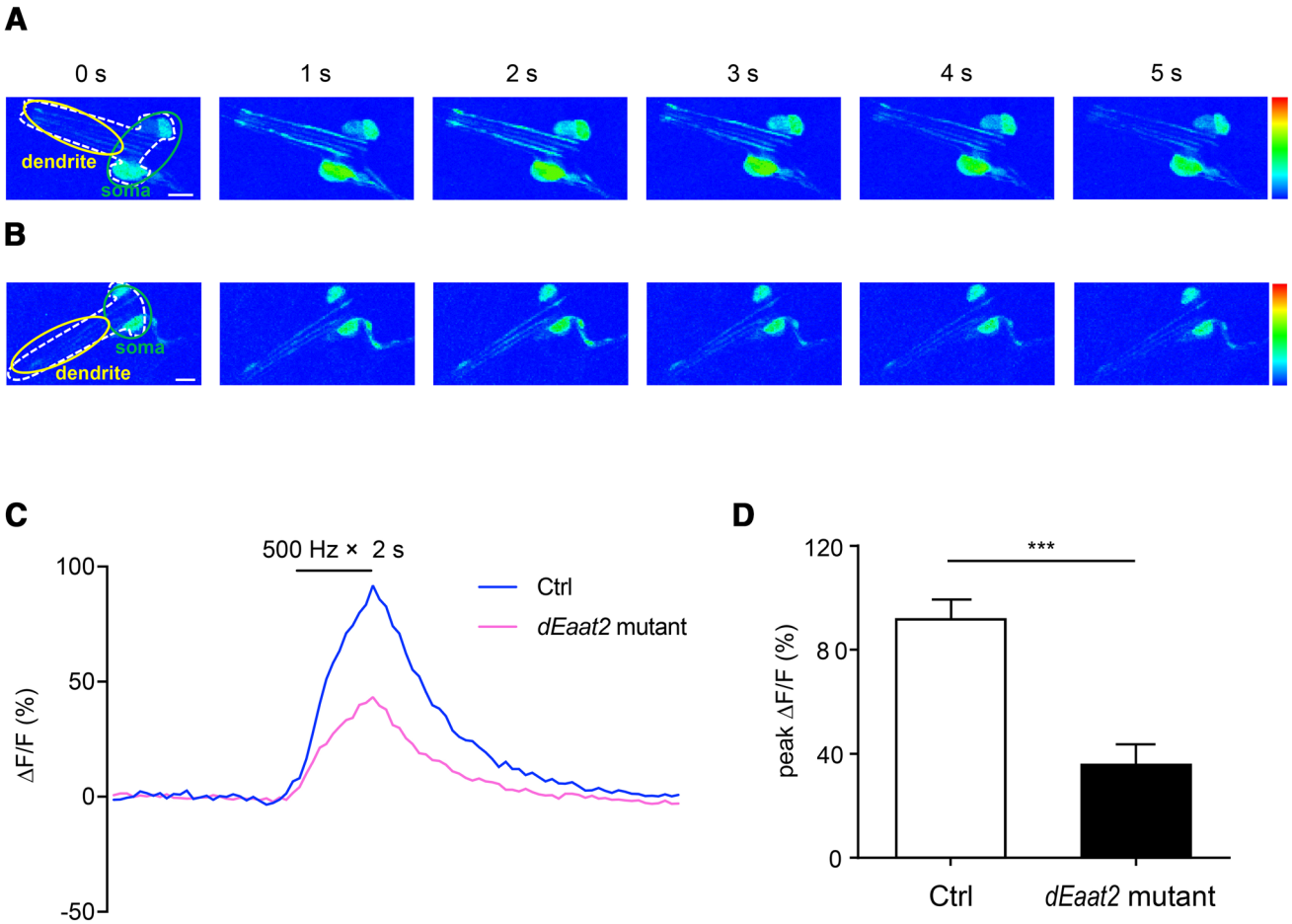


Fig. 5 The Ca^{2+} response of lch5 chordotonal neurons to sound. **A** and **B** Sound-evoked Ca^{2+} signals in control (**A**) and the *dEaat2* mutant (**B**). White dashed lines, border of lch5 chordotonal neurons; green outline, somata of lch5 chordotonal neurons; yellow outline, dendrites (rainbow color range: 0–255; scale bars, 10 μm). **C** Average Ca^{2+} responses to a 500-Hz tone of 90 dB in the cell bodies of lch5 chordotonal neurons. Black bar, sound stimulation. $n = 5$ in each case. **D** Statistical analysis of the Ca^{2+} responses in control and *dEaat2* mutant larvae. The *dEaat2* mutant showed a reduced Ca^{2+} response to a 500-Hz sound. Ctrl: *+/+; NOMPC-Gal4/UAS-GCamP6, UAS-CD4-tdTomato*. *dEaat2* mutant: $dEaat2^{MI}/dEaat2^{MI}$; *NOMPC-Gal4/UAS-GCamP6, UAS-CD4-tdTomato* ($n = 10$/group; two-tailed unpaired Student's *t*-test; $^{***}P < 0.001$; error bars represent ± SEM).

regions of the chordotonal neurons, including somata, dendrites, and axon. The dEAAT2::EGFP fusion protein was enriched in the distal dendritic region of chordotonal neurons (Fig. 2B). Thus, it seems likely that dEAAT2 is localized to dendritic tips. In *Drosophila* larvae, the ciliated dendrite is bathed in K^+-enriched receptor lymph secreted by the supporting cell [42, 43] and plays an important role in mechanotransduction [44]. These results indicate that dEAAT2 is involved in the sound sensation of larvae.

dEAAT2 is Required for Sensing Sound in Larvae

To investigate the function of *Drosophila* dEAAT2 in hearing transduction, we obtained a Minos element insertion in *dEaat2* exon 8 ($dEaat2^{MI}$) (Fig. 3A), the presence of which was confirmed by PCR (Fig. 3B). The transcript levels of dEAAT2 were reduced in the $dEaat2^{MI}$ line (Fig. 3C). We then examined the startle behavior of dEAAT2 mutant larvae in response to sound. When exposed to a 500-Hz sound (2 s duration at 8-s intervals), the WT larvae exhibited a series of startle behaviors, including pausing, turning, mouthhook retraction, and/or backward locomotion (Fig. 4A, Movie S1). To quantify this behavior, the number of larvae that showed responses was calculated. Neither the WT nor the dEAAT2 mutant larvae had startle-freeze reactions at a low sound intensity (60 dB) (Fig. 4B). Compared with the WT larvae, the responses of the dEAAT2 mutant were significantly reduced at 70 dB and 80 dB (Fig. 4B). At 90 dB, both the WT and dEAAT2 mutant larvae showed strong startle responses (Fig. 4B). *NOMPC-Gal4* was used to drive the expression of the *UAS-dEAAT2-EGFP* rescuing transgene in the $dEaat2^{MI}$ background. We found that this mutant phenotype was rescued by expressing dEAAT2 in

chordotonal neurons (Fig. 4B). These results revealed an important role of dEAAT2 in the sound response of chordotonal neurons.

In addition to chordotonal neurons, class III dendritic arborization (da) neurons were also labeled by *dEAAT2-Gal4* (Fig. S1A). As class III neurons contribute to gentle touch sensation [45], we next tested the touch sensitivity of dEAAT2 mutant larvae using a previously-established behavioral assay [45]. Both the WT and dEAAT2 mutant larvae showed various behavioral responses to gentle touch, including pause, recoil, and reverse contraction (Fig. 4D), with no difference in the scores between the WT and the dEAAT2 mutant (Fig. 4C). Taken together, the results suggested that dEAAT2 is required for sound perception, but not for tactile sensation.

Reduced Calcium Response of lch5 Chordotonal Neurons in the *dEaat2* Mutant

As there was no evident morphological defect in the lch5 chordotonal neurons of the *dEaat2* mutant (data not shown), dEAAT2 might be involved in sound transduction rather than neural development. To determine the role of dEAAT2 in sound transduction, we first performed *in vivo* Ca^{2+} imaging of the lch5 chordotonal neurons of larvae with or without the dEAAT2 mutation. In a previous study, we showed that the lch5 neurons are a cluster of five chordotonal neurons, and less affected by movements. Moreover, the Ca^{2+} response of lch5 neurons is sensitive and stable [38]. *NOMPC-Gal4* was used to drive the expression of the Ca^{2+} indicator GCamP6 in chordotonal neurons. When exposed to a 500-Hz sound at 90 dB, the Ca^{2+} level in the cell bodies of lch5 chordotonal neurons increased by 91.7% in WT larvae, and only increased by 35.7% in *dEaat2* mutant larvae (Fig. 5D). In addition, the Ca^{2+} signal in dendrites was also elicited by sound stimuli (Fig. 5A). However, we did not find any difference in behavioral responses at the same intensity (90 dB) compared to WT larvae (Fig. 4B). This discrepancy can be explained by the possibility that the defective activity of chordotonal neurons at 90 dB might be compensated for in the central nervous system, or the behavioral assay using the startle response was not sensitive enough to detect a difference.

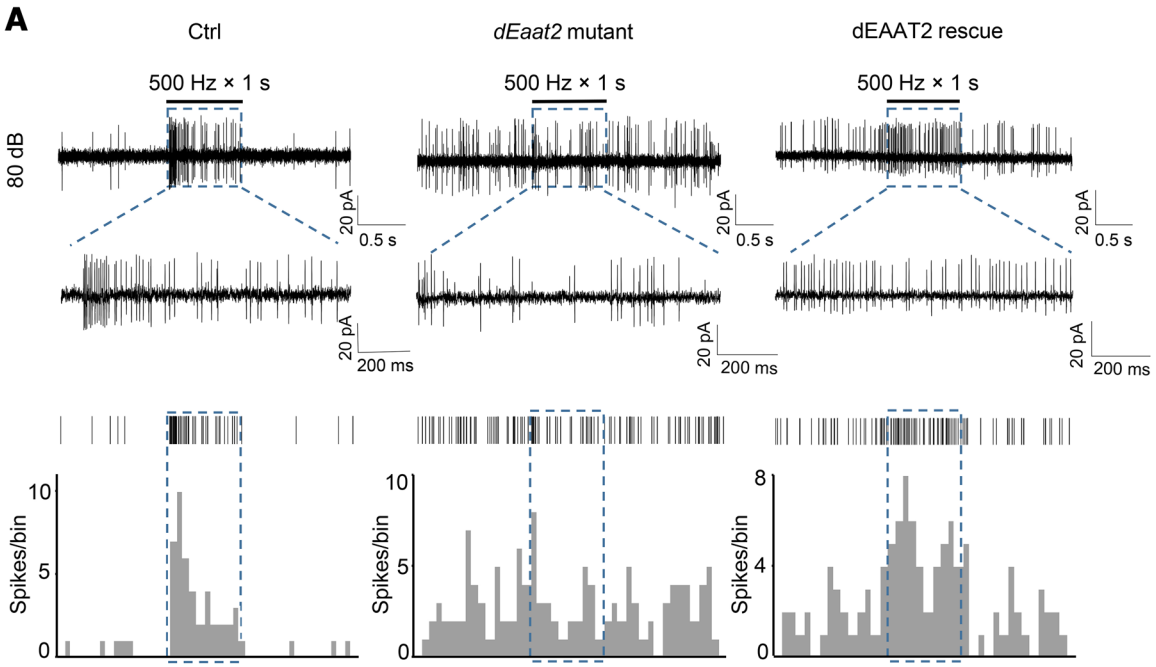
Decreased Action Potential Firing Responses of vchA Chordotonal Neurons in the *dEaat2* Mutant

To further examine the response of chordotonal neurons in *dEaat2* mutant larvae at 60 dB–90 dB, the AP firing rates of chordotonal neurons were recorded. We recorded extracellularly from vchA chordotonal neurons. In the absence of sound stimulation, these neurons fired spontaneously in both the WT and *dEaat2* mutant larvae (Fig. 6A, Fig. S2–3), and there was no difference in this spontaneous firing rate (Fig. 6B). When exposed to a 500-Hz sound at 70 dB, the WT responded with increased AP firing (Fig. S2B). At 80 (Fig. 6A) and 90 dB (Fig. S3), greater numbers of APs were elicited from the vchA chordotonal neuron in WT larvae. In contrast, the *dEaat2* mutant had significantly reduced responses at 80 dB and 90 dB (Fig. 6C).

We found a decreased startle response in *dEaat2* mutant larvae at 70 dB (Fig. 4B), but did not find any difference in the AP firing response at the same intensity compared to WT larvae (Fig. 6C). It is possible that our electrophysiological recording at low sound intensity was not sensitive enough to detect the difference. Consistent with the results of Ca^{2+} imaging, chordotonal neurons of the *dEaat2* mutant showed a decreased response to sound at 90 dB (Fig. 6C).

The vchA chordotonal neurons of WT larvae adapted quickly to a prolonged sound stimulus of 90 dB with a τ of 125 ms (Fig. 6D). The *dEaat2* mutant had an adaptation rate similar to WT larvae (Fig. 6D). We found that the defect in firing response was rescued by dEAAT2 expression (Fig. 6C). Interestingly, dEAAT2 rescue larvae showed an adaptation rate different from the WT (τ = 698 ms) (Fig. 6D). Together, the findings indicate that dEAAT2 is required for sound transduction in larval chordotonal neurons.

Fig. 6 *dEaat2* mutant has a decreased AP firing response of vchA chordotonal neurons to sound. **A** AP firing responses of vchA chordotonal neurons to a 500-Hz sound at 80 dB in control (left), *dEaat2* mutant (center), and dEAAT2 rescue (right) larvae. Upper panels: original traces; middle panels: raster plots of spikes; lower panels: post-stimulus time histograms of spikes (bin width, 100 ms). Black bars indicate sound stimulation; blue boxes and dashed lines indicate expanded views of spiking activity during delivery of a prolonged 500 Hz sound. **B** Statistical analysis of the rate of spontaneous firing in control (n = 6), *dEaat2* mutant (n = 5), and dEAAT2 rescue larvae (n = 6; two-tailed Mann–Whitney tests). **C** Summary of AP firing response of vchA chordotonal neurons in the control, *dEaat2* mutant, and dEAAT2 rescue larvae. Δno. APs: increase of the number of APs in 1 s after sound onset compared to 1 s before sound stimulus onset. The *dEaat2* mutant exhibited dramatically reduced firing responses at 80 dB and 90 dB, and this was rescued by expressing dEAAT2 in the chordotonal neurons (n = 6/group; two-tailed Mann–Whitney tests; P values are indicated above each data point colored according to the genotype). **D** Exponential decay curve of response at 90 dB in a 100-ms bin. A 1-s sound stimulus was used (n = 6/group; τ, time constant of adaptation rate). vchA chordotonal neurons of control larvae adapted quickly to sound with a τ of 125 ms; the *dEaat2* mutant showed an adaptation rate similar to control (τ = 198 ms). dEAAT2 rescue exhibited an adaptation rate different from control larvae (τ = 698 ms). Ctrl: +/+; *NOMPC-Gal4/UAS-CD8-GFP*. *dEaat2* mutant: $dEaat2^{MI}$/$dEaat2^{MI}$; *NOMPC-Gal4/UAS-CD8-GFP*. dEAAT2 rescue: $dEaat2^{MI}$/$dEaat2^{MI}$; *NOMPC-Gal4/UAS-dEAAT2-EGFP*. NS, not significant; $^{**}P < 0.01$; error bars represent ± SEM.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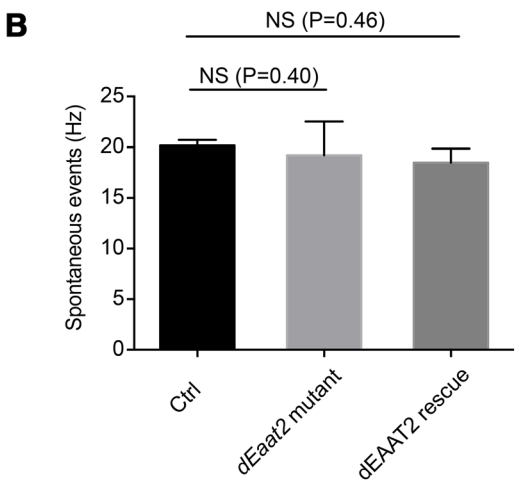
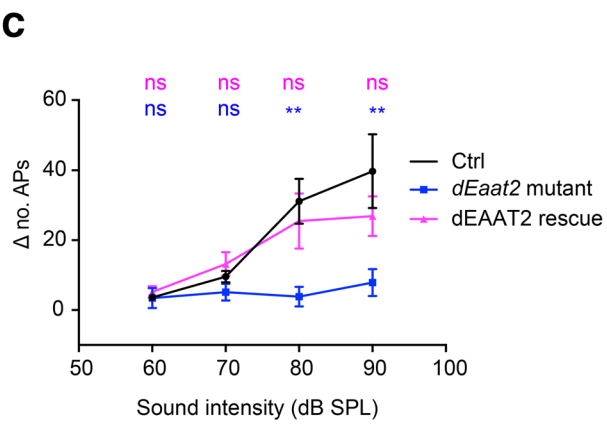
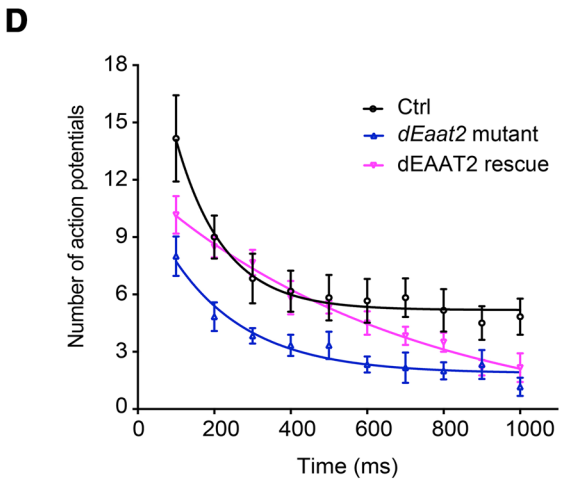

B
NS (P=0.46)
NS (P=0.40)
Spontaneous events (Hz)
Ctrl
dEaat2 mutant
dEAAT2 rescue


C
ns
**
Δ no. APs
Sound intensity (dB SPL)
Ctrl
dEaat2 mutant
dEAAT2 rescue


D
Number of action potentials
Time (ms)
Ctrl
dEaat2 mutant
dEAAT2 rescue

Discussion

In the current study, multiple lines of evidence were provided to support the hypothesis that dEAAT2, a taurine/aspartate transporter, is critical for sound transduction in *Drosophila* larvae. First, we found that dEAAT2 was required for the larval startle response to sound stimuli. Second, dEAAT2 was enriched in the distal regions of chordotonal neurons. Third, the sound-induced Ca^{2+} response and the AP firing response of chordotonal neurons in the dEAAT2 mutant were dramatically reduced. Moreover, the mutant phenotype was rescued by expressing dEAAT2 in chordotonal neurons, indicating that dEAAT2 functions in *Drosophila* chordotonal neurons for sound transduction.

In *D. melanogaster*, the chordotonal neurons of Johnston's organ in the second antennal segment mediate hearing as well as sensing gravity and wind [36, 37]. The ciliated dendrites of chordotonal neurons play an important role in sound transduction [44]. Genes required for Johnston's organ function in *Drosophila* have been identified, such as *ato* (*atonal*), *cut*, *ck* (*crinkled*), *nompA* (*no mechanoreceptor potential A*), *nompB* (*no mechanoreceptor potential B*), *btv* (*beethoven*), *rempA* (*reduced mechanoreceptor potential A*), *tilB* (*touch insensitive larva B*), *nompC* (*no mechanoreceptor potential C*), *iav* (*Inactive*), *nan* (*Nanchung*), and *unc* (*uncoordinated*) [35, 46, 47]. Three of these genes, *ato*, *ck*, and *cut*, are required for Johnston's organ development [48, 49]. Ion channels of the transient receptor potential family encoded by *Nompc*, *nan*, and *iav,* are important for the mechanotransduction of hearing in *Drosophila* [50, 51].

Unlike other EAAT orthologues, dEAAT2 has been reported to mediate the high-affinity transport of aspartate, an excitatory amino-acid, and taurine. Taurine is found at high concentrations in mammals and has been associated with a variety of physiological functions in the central nervous system, from development to cytoprotection [4, 5]. Particularly, it has long been known that taurine is highly concentrated in the mammalian retina [52, 53]. In the rat, taurine is critical for the development and maintenance of retinal function and promotes the development of rod photoreceptors [54]. Taurine is critical for photoreceptor function both as an antioxidant and as an osmolyte [55, 56]. Moreover, a previous study showed that taurine is involved in the activation of cyclic GMP-gated channels, an important step in the phototransduction process [57]. It also acts in various ways to regulate synaptic signal transmission in retinal ganglion cells [57, 58]. In addition, the olfactory bulb contains a high level of taurine. In slices of rat olfactory bulb, the selective inhibitory effects of taurine by activating $GABA_A$ receptors suggest that taurine plays a role in olfaction [59]. Considering its broad distribution and its functional significance, taurine deserves detailed study. Many of its functions rely on its intracellular concentration, which is maintained by a high-affinity TauT transporter that takes up taurine from the extracellular compartment [60]. A decrease of taurine levels in a variety of tissues has been reported in $taut^{-/-}$ mice, and these taurine-deficient mice show severe retinal degeneration [61] and reduced olfactory function [19].

In insects, taurine is the second most abundant amino-acid after glutamate, but little is known about its function(s) in the nervous system. Taurine has been detected in *Drosophila* mushroom bodies, an integrative structure implicated in learning and memory [62] and dEAAT2 is a taurine transporter in *Drosophila*. Here, we found that dEAAT2 is involved in sound transduction in *Drosophila*.

In conclusion, we have investigated the expression and functional role of *Drosophila* dEAAT2 in the peripheral auditory system. These findings show that dEAAT2 is required for auditory transduction in *Drosophila*. Analyses of *dEaat2* gene functions in *Drosophila* hearing add to an appreciation of the importance of taurine in the auditory pathway.

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Compliance with ethical standards

Conflict of interest All authors claim that there are no conflicts of interest.

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