Activity-dependent regulation of astrocyte GAT levels during synaptogenesis

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Astrocytic uptake of GABA through GABA transporters (GATs) is an important mechanism regulating excitatory/inhibitory balance in the nervous system; however, mechanisms by which astrocytes regulate GAT levels are undefined. We found that at mid-pupal stages the *Drosophila* melanogaster CNS neuropil was devoid of astrocyte membranes and synapses. Astrocyte membranes subsequently infiltrated the neuropil coordinately with synaptogenesis, and astrocyte ablation reduced synapse numbers by half, indicating that *Drosophila* astrocytes are pro-synaptogenic. Shortly after synapses formed in earnest, GAT was upregulated in astrocytes. Ablation or silencing of GABAergic neurons or disruption of metabotropic GABA receptor 1 and 2 (*GABA*B*R1/2*) signaling in astrocytes led to a decrease in astrocytic GAT. Notably, developmental depletion of astrocytic GABA*B* R1/2 signaling suppressed mechanosensory-induced seizure activity in mutants with hyperexcitable neurons. These data reveal that astrocytes actively modulate GAT expression via metabotropic GABA receptor signaling and highlight the importance of precise regulation of astrocytic GAT in modulation of seizure activity.

Brain circuits comprise complex ensembles of excitatory and inhibitory neurons and glial cells. Neurons and glia are intimately associated from very early developmental stages, and the proper assembly of functional neural circuits is thought to require extensive neuron-glia signaling1-6. Defining precisely how neurons and glia communicate during development to ensure proper neural circuit assembly remains a major challenge for the field. Astrocytes have emerged as critical regulators of neuronal development, particularly with respect to promoting synapse formation1,3,5.

Reciprocal mechanisms by which synapses might signal to astrocytes to regulate their development remain more mysterious, even though astrocytes regulate key aspects of neural circuit function, including the balance of excitatory and inhibitory neurotransmission. Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and can be rapidly cleared by astrocytes via uptake through excitatory amino acid transporters (EAATs)7,8. The principal inhibitory neurotransmitter GABA serves as a break to dampen excitatory neurotransmission when appropriate. GABA inhibitory activity is mediated by GABA receptors (GABA-Rs) either locally at synapses, where it hyperpolarizes the postsynaptic cell, or at extrasynaptic sites, where it provides widespread tonic inhibition of neuronal firing9,10. Debilitating diseases are caused by imbalances in excitatory and inhibitory firing. For instance, in epilepsy, misregulated GABA levels are believed to lead to hyperexcitability and ultimately to seizure11-13.

GABAergic signaling can be fine-tuned at several levels, including changes in GABA-R density or alterations in GABA-R subtype composition14. However, uptake of GABA by astrocytic GABA transporters (GATs) is also an important regulatory mechanism. GABA removal and degradation at synapses is critical for proper termination of GABAergic signaling, while uptake at nonsynaptic sites can influence GABA tone across larger areas in the brain15,16. Reduced GAT-3 levels in mouse hippocampal astrocytes results in increased tonic inhibitory currents and reduced IPSC amplitudes in hippocampal interneurons, likely owing to elevated GABA levels and subsequent desensitization of GABA-Rs17. Similarly, blockade of astrocytic GAT-3 in the rat hippocampus contributes to increased extracellular GABA concentrations and increased tonic GABA-R-mediated currents in dentate granule cells18.

Despite the importance of astrocytic GATs in modulating GABAergic signaling, surprisingly little is known about how GAT levels are set during development or dynamically regulated in the mature brain. Rat astrocytic GAT-3 immunoreactivity is detectable at birth, but it does not appear to take on adult patterns of expression until postnatal week 3 in the cerebral cortex19. Notably, the timing of these postnatal changes coincides with periods of astrocyte morphogenesis and synaptic refinement20. This raises the possibility that initial GABAergic synaptic activity might contribute to shaping the spatiotemporal pattern of astrocytic GAT expression.

GABAergic signaling is also a major component of neural circuit activity in the *Drosophila* nervous system21-23. There is a single *Drosophila* ortholog of the mammalian GABA transporters belonging to the SLC6 family, termed GAT, which is expressed in CNS astrocytes but not neurons, suggesting that astrocytes are the primary cell type responsible for GABA clearance23,24. Consistent with this notion, GAT depletion from astrocytes causes profound defects in animal behavior24.

We explored synapse-astrocyte interactions that underlie GAT activation and modulation in *Drosophila* astrocytes and roles for GAT in balancing excitatory and inhibitory signaling in vivo. We found that during late metamorphosis, as the adult brain is forming, astrocyte development was tightly coupled with synaptogenesis and that *Drosophila* synapse formation depended on astrocytes. Coincident

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with synaptogenesis, astrocytes exhibited an increase in GAT, which we found to be modulated by GABAergic neuronal activity and astrocytic GABA\(_{\text{A}}\)R1/2 receptor signaling, suggesting that astrocytes regulate GAT levels by direct measurement of extracellular GABA. Finally, we demonstrated that inhibiting astrocytic GABA\(_{\text{A}}\)R1/2 signaling strongly suppressed seizure activity in bang-sensitive mutants, which have hyperexcitable neurons, arguing that this pathway is critical for modulating excitatory/inhibitory balance in vivo.

**RESULTS**

**Astrocytes invade the neuropil coordinately with synaptogenesis**

During *Drosophila* metamorphosis, the larval nervous system is dismantled and adult neural circuitry is constructed. Most pruning of larval neurites is complete by ~48 h after puparium formation (APF)\(^{25-27}\). We assayed astrocyte morphology and synapse formation in the central brain at 48, 60, 72, 84 and 96 h APF and at the adult stage. Astrocyte membranes were labeled using the astrocyte driver *alm-GAL4* with UAS-\(mCD8^{\text{::GFP}}\) and co-stained for the presynaptic active zone marker Bruchpilot (nc82 antibody) to label the neuropil (Fig. 1a). We focused primarily on the antennal lobe (AL) region of the brain, which appears, according to our analysis, characteristic of astrocyte infiltration and neuropil development throughout the brain (Supplementary Fig. 1). Astrocyte cell bodies were present at the interface of the neuropil and cortex 48 h APF, although astrocyte membranes were not observed invading the neuropil. By 60 h APF, short, thick astrocytic membrane processes infiltrated neuropil regions, and this increased dramatically by 72 h APF, when astrocyte processes were found throughout the neuropil. The fine branching that characterizes the bushy, tufted morphology of mature astrocytes was not observed 72 h APF (Fig. 1a and Supplementary Fig. 2). However, astrocytes 84 h APF densely infiltrated neuropil areas of the brain and displayed fine branching and tufted morphology (Fig. 1a and Supplementary Fig. 2). A comparable morphology was observed 96 h APF and in adults. Thus, the initial phases of astrocyte infiltration into the neuropil occurred between 60 and 84 h APF (Fig. 1a and Supplementary Fig. 2).

We next sought to determine when synaptic structures formed within the neuropil and whether this was coordinate with astrocyte infiltration. Because of the lack of reliable postsynaptic markers for CNS synapses in *Drosophila*, we turned to transmission electron microscopy (TEM) as a means to definitively identify synapses in the developing neuropil by ultrastructural criteria: we scored for the presence of a postsynaptic density in opposition to clusters of presynaptic vesicles and for the presence of T-bars. We focused our analysis on the AL, mushroom body (MB) and superior posterior slope (SPSL) neuropil regions of the brain at 48, 60, 72, 84 and 96 h APF and in the adult.

The progressive infiltration of the neuropil with astrocyte membranes coincided with the formation of morphologically identifiable synapses in the *Drosophila* pupal brain (Fig. 1b,c). At 48 h APF the neuropil was devoid of structures resembling synapses. At 60 h APF we observed the widespread appearance of immature synaptic structures, which were characterized by poorly defined postsynaptic densities that lacked presynaptic vesicles. By 72 h APF, mature synapses were frequently observed, and they continued to increase in number until 84 h APF, after which synaptic density and morphology remained largely unchanged. We conclude that the main wave of

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**Figure 1** Astrocyte infiltration and synaptogenesis are temporally correlated during late metamorphosis. (a) Confocal section through the AL region showing astrocyte infiltration at several time points during metamorphosis. Astrocyte membranes are labeled by UAS-\(mCD8^{\text{::GFP}}\) expression using the *alm-GAL4* driver (green; Astrocytes>GFP) and neuropil is labeled by nc82 antibody staining (red). Scale bar, 10 \(\mu\text{m}\). (b) Ultrastructure of AL neuropil at several time points during metamorphosis, highlighting the progression in synapse development. Arrows point to presynaptic sites and asterisks mark postsynaptic structures. Synaptic structures are prominent starting at 72 h APF. Scale bar, 0.5 \(\mu\text{m}\). (c) Quantification of the number of synaptic structures in the AL (\(n \geq 20\) sections for each time point), MB (\(n \geq 6\) sections for each time point) and SPSL (\(n \geq 9\) sections for each time point) during late metamorphosis. Error bars, s.e.m.
synaptogenesis occurs in the Drosophila brain between 60 and 84 h APF, and the infiltration of astrocyte membranes into the neuropil and synaptogenesis are temporally coordinated: that is, both occur between 60 and 84 h APF.

**Astrocyte compensatory growth and requirement in the adult CNS**

The above analysis raised the possibility that Drosophila pupal astrocytes might regulate synapse formation in a manner similar to that of mammalian astrocytes. To address this possibility, we ablated astrocytes during late metamorphosis and assayed for changes in synaptic numbers. To ablate astrocytes, we used conditional expression of the proapoptotic gene head involution defective (hid) in astrocytes specifically during late metamorphosis. Varying degrees of GAL80 activity and UAS-hid expression were achieved by varying the incubation temperature during late metamorphosis. Low-level Hid expression was achieved at 25 °C and maximal Hid expression was achieved at 30 °C. (b) Confocal slice of central brain and ventral nerve cord of adult animals immunostained with anti-GAT to visualize astrocytes. Astrocyte staining is moderately reduced following Hid expression in astrocytes (Astro-Hid) at 25 °C and robustly reduced after Hid expression in astrocytes at 30 °C, indicating moderate and severe ablation conditions, respectively. Scale bar, 10 μm.

(c) Confocal sections through AL of adult animals in which astrocytes are labeled by anti-GAT staining (green) and neuropil is labeled by anti-HRP staining (red). Astrocyte processes were able to fully cover neuropil space when ablations were performed at 25 °C. Large regions of neuropil were left unoccupied by astrocyte processes when ablations were performed at 30 °C. Scale bar, 10 μm.

(d) Number of astrocytes remaining in the AL, MB, SOG and TGab of the adult CNS following moderate (25 °C) astrocyte ablations (n = 5 brains for control and Astro-Hid AL, MB, SOG and TGab). (e) Number of astrocytes remaining in the AL, MB, SOG and TGab of the adult CNS following severe (30 °C) astrocyte ablations (n = 7 brains, control AL; n = 8 brains, Astro-Hid AL; n = 5 brains, control and Astro-Hid MB; n = 5 brains, control and Astro-Hid SOG; n = 5 brains, control TGab; n = 7 brains, Astro-Hid TGab). Fewer astrocytes remained when ablations were performed at 30 °C than at 25 °C; demonstrating the varying degrees of astrocyte ablation achieved by the two temperature conditions. (f) Fates of animals undergoing ablation procedure (n ≥ 30 flies for each condition). The majority of animals struggled to eclose when undergoing severe astrocyte ablations (30 °C).

Astrocytes were identified using immunofluorescence for GAT, an astrocyte-specific marker. To assess the degree of ablation under each condition, cells that were positive for both GAT and Repo (a pan-glial nuclear marker) immunofluorescence were counted in several CNS regions, including the AL, MB, subesophageal ganglion (SOG) and abdominal segments of the thoracic ganglion (TGab), and the percentage total cell numbers in the CNS, 100% of the pupae shifted to 25 °C survived to adulthood and did not display any obvious behavioral defects (Fig. 2f). This observation argues that astrocytes are generated in sufficiently high numbers to accommodate substantial reductions in...
the astrocyte population without in turn causing dramatic changes in development, animal survival or overt behavior.

After a shift to 30 °C (severe ablation), astrocyte numbers decreased more dramatically: from 24.75 ± 0.82 (n = 8 brains) to 4.5 ± 0.62 (n = 8 brains) in the AL, from 10 ± 0.32 (n = 5 brains) to 4.2 ± 0.58 (n = 5 brains) in the MB, from 32.4 ± 3.04 (n = 5 brains) to 7.2 ± 1.3 (n = 5 brains) in the SOG, and from 24.67 ± 1.7 (n = 5 brains) to 3.4 ± 0.57 (n = 7 brains) in the TGab (Fig. 2b,e). Under these conditions, the remaining astrocyte processes appeared sparsely distributed and were unable to fully cover the neuropil space (Fig. 2c). Moreover, 41% of pupae that underwent severe ablations failed to eclose (that is, animals died while still fully encased by the puparium), 32% died while emerging from their pupal cases, and only 27% survived as adults (Fig. 2f). This last collection of animals were highly uncoordinated, could not walk or fly, and died within a few days (Supplementary Videos 1–3). Thus, reducing the number of astrocytes in the CNS by ~50% during synaptogenesis did not noticeably affect animal survival or overt behavior, while ablation of ~75% or more of Drosophila astrocytes greatly reduced survival and resulted in severe defects in motor activity and, ultimately, death.

*Fig. 3 Synapse numbers, but not gross neural architecture, are altered when astrocytes are ablated during late metamorphosis. (a) Ultrastructure of synapses in the AL in adult and 84 h APF following 30 °C astrocyte ablation. Morphology of mature synaptic structures is unaltered by ablation. Arrows point to presynaptic sites and asterisks mark postsynaptic structures. Scale bar, 0.5 µm. (b) Quantification of postsynaptic density (PSD) length in AL, MB and SPSL regions of the adult brain after 30 °C astrocyte ablation (Astro>Hid) (n = 80 PSDs from 12 sections, AL control; n = 81 PSDs from 12 sections, AL Astro>Hid; n = 47 PSDs from 7 sections, MB control; n = 50 PSDs from 7 sections, MB Astro>Hid; n = 58 PSDs from 7 sections, SPSL control; n = 63 PSDs from 7 sections, SPSL Astro>Hid). (c) Quantification of the percentage of synapses with T-bar morphology in the AL, MB and SPSL regions of the adult brain following astrocyte ablation (n = 23 sections, AL control; n = 24 sections, AL Astro>Hid; n = 7 sections, MB control; n = 11 sections, MB Astro>Hid; n = 8 sections, SPSL control; n = 8 sections, SPSL Astro>Hid). (d) Quantification of the number of synaptic structures in the AL, MB and SPSL regions of adult and 84 h APF animals following 30 °C astrocyte ablation (n = 17 sections, AL adult control; n = 22 sections, AL adult Astro>Hid; n = 19 sections, AL 84 h APF control; n = 20 sections, AL 84 h APF Astro>Hid; n = 17 sections, MB adult control; n = 22 sections, MB adult Astro>Hid; n = 19 sections, MB 84 h APF control; n = 20 sections, MB 84 h APF Astro>Hid; n = 17 sections, SPSL adult control; n = 22 sections, SPSL adult Astro>Hid; n = 19 sections, SPSL 84 h APF control; n = 20 sections, SPSL 84 h APF Astro>Hid). (e) Brain architecture was grossly unaltered following astrocyte ablations performed at 30 °C. Projected confocal z-stacks showing (i) glomeruli structure in brains stained with nc82 antibody (red), (ii) morphology of projection neurons marked by GH146-QF/QUAS-mCD8:GFP, (iii) morphology of axonal projections and arborizations of projection neurons, (iv) morphology of PDF neuron axonal projections in the central brain, (v) morphology of PSD neuron dendritic arborizations in the optic lobe. Scale bars, 10 µm for i and iii, 50 µm for ii, 25 µm for iv and v. (f) Quantification of the number of projection neurons (PNs) per hemisphere (n = 6 brains, control and Astro>Hid) and (g) PDF* neurons per hemisphere (n = 6 brains, control; n = 5 brains, Astro>Hid) following 30 °C astrocyte ablation. **P < 0.01, ***P < 0.001, unpaired Student’s t-test for b–d,f,g. P = 0.0001, adult and 84 h APF AL; P = 0.0036, adult MB; P = 0.0003, 84 h APF MB; P = 0.0007, adult SPSL; P = 0.0003, SPSL 84 h APF for d. Error bars, s.e.m.

Drosophila astrocytes are required for synaptogenesis in vivo

We next sought to determine whether elimination of astrocytes would lead to changes in synaptogenesis. For these studies we used severe ablation conditions only. Gross synapse morphology as defined above by TEM did not appear altered in astrocyte-ablated animals (Fig. 3a). Postsynaptic density length was unchanged after astrocyte ablation (Fig. 3b), and the percentage of synapses with T-bars was unaltered (Fig. 3c). In contrast, in astrocyte-ablated animals synaptic density was reduced 32% in the AL, from 37.2 ± 1.4 (n = 23 sections) to 25.3 ± 0.88 (n = 27 sections) synapses per 100 µm²; 36% in the MB, from 48.3 ± 3.8 (n = 7 sections) to 30.1 ± 3.5 (n = 11 sections) synapses per 100 µm²; and 47% in the SPSL, from 30.6 ± 1.4 (n = 8 sections) to 16.1 ± 3.0 (n = 8 sections) synapses per 100 µm² (Fig. 3b). Astrocyte-ablated
tissue was also marked by an increased frequency of ruptured mitochondria (Supplementary Fig. 4), which might result from a prolonged absence of astrocytes, but we are unable to definitively state whether this is a direct or indirect result of astrocyte loss.

We quantified synapse numbers 84 h APF, the time point at which total synapse numbers reached ~90% in control animals (Fig. 1c). Even at this developmental time point, synapse numbers were significantly reduced in the absence of astrocytes (Fig. 3a,d and Supplementary Figs. 4 and 5). Of note, we saw several immature synaptic structures, similar in morphology to those normally observed 60 h APF, in astrocyte-ablated animals. Whether these structures are delayed in development and eventually mature or whether they fail to mature is unclear (Fig. 3a and Supplementary Fig. 6). We also examined whether neuronal architecture or survival were grossly affected in these animals. Surprisingly, gross morphology of the adult Drosophila brain appeared unaffected by severe astrocyte depletion. For example, after nc82 staining of the adult brain, AL glomerular organization appeared normal at the light microscopy level (Fig. 3e).

Figure 4 GAT is exclusively expressed in astrocytes and activated during synaptogenesis. (a) UAS-gat RNAi was expressed in astrocytes using the alrm-GAL4 driver. Western blots performed on larval CNS and adult brain lysates were probed with anti-GAT antibody to confirm specific knockdown of GAT in astrocytes. GAT migrates at approximately 50 kDa. (b) Quantification of GAT levels from western blot analysis shown in a (n = 3 experiments, third-instar larva; n = 3 experiments, adult). (c) The alrm-GAL4 driver was used to coexpress UAS-mCD8::GFP and UAS-gat RNAi or to express UAS-mCD8::GFP alone. Adult brains were stained with anti-GAT. Confocal section through AL shows that GAT (red) localizes specifically to astrocyte membranes (green). Higher magnification images of outlined regions are shown in bottom panels. Scale bars, 10 μm. (d) Western blot performed on wild-type brain lysates from several stages of metamorphosis, probed with anti-GAT. (e) Quantification of GAT levels from western blot analysis in d (n = 3 experiments). (f) Adult brains expressing UAS-syt::eGFP using the gad-GAL4 driver were stained with anti-GAT. GABAergic presynaptic sites (GABAergic neuron-Syt::eGFP) and GAT protein are present throughout the central brain. High magnification images (right) of the outlined AL and MB regions show that GABAergic presynaptic sites and GAT proteins are in close association. Scale bars, 20 μm. *P ≤ 0.05, ***P ≤ 0.001, unpaired Student’s t-test for b and one-way ANOVA (P < 0.0001) with Tukey’s post hoc test for e; *P = 0.0001, third-instar larva; P = 0.0254, adult for b. Error bars, s.e.m. Full-length blots are presented in Supplementary Figure 12.

Neuronal projections of second-order antennal lobe projection neurons as well as pigment dispersing factor (PDF) neurons, representing another population of higher order neurons, were morphologically normal (Fig. 3e). The numbers of projection neurons were found to be comparable in control and astrocyte-ablated animals (Fig. 3f) as were numbers of PDF neurons (Fig. 3g). Finally, the membranes of cortex and enshrouding glia in the adult brain exhibited normal morphology on the basis of anti-Draper immunofluorescence (Supplementary Fig. 7). Thus, ablation of astrocytes during late metamorphosis did not interfere in obvious ways with gross brain architecture, neurite morphology or neuronal survival, arguing for a more direct involvement of Drosophila astrocytes in synapse formation.

GAT levels are regulated by GABAergic neuron signaling

Astrocytic uptake of GABA through GATs is thought to be a key mechanism used to balance excitation and inhibition in the CNS15,16,29. Drosophila larval astrocytes express the sole Drosophila ortholog of the Na+– and Cl–-dependent GAT of the SLC6 family23, and its depletion results in uncoordinated animals that exhibit severely reduced motility in larvae and adults24, which argues that Drosophila astrocytes are important in modulating GABA CNS tone. Intrinsic and extrinsic mechanisms that regulate how astrocytes acquire the appropriate molecular and morphological phenotypes remain poorly defined. Given that glutamatergic neuronal signaling regulates levels of the astrocyte glutamate transporter GLT-1 and, thereby, astrocyte control of glutamate tone30–33, we explored potential roles for GABAergic signaling in regulating levels of astrocytic GATs.

We first confirmed the specificity of our GAT antibody by performing western blot analysis following knockdown of gat in astrocytes using UAS-gat RNA interference. In control animals we observed an ~50 kDa band corresponding to GAT, which was eliminated in gat knockdown animals (Fig. 4a,b). Immunofluorescent stains using this antibody also showed robust localization of GAT to adult astrocyte membranes (Fig. 4c). GAT immunofluorescence was dramatically reduced after gat knockdown in astrocytes, but unchanged following gat knockdown in neurons (Fig. 4c and Supplementary Fig. 8). To determine the time course of GAT expression during development of the adult nervous system, we performed western blot analysis of GAT expression in dissected pupal brains during late metamorphosis (Fig. 4d,e). GAT levels increased during late stages of metamorphosis and displayed the most notable increase around 84 h APF, a time point at which most CNS
synapses had formed (for example, Fig. 1c). To explore the spatial relationship between astrocyte GAT proteins and GABAergic synapses we examined GAT immunostaining while labeling GABAergic presynaptic sites using the GABA-neuron-specific driver gad-GAL4 to express UAS-syt::eGFP. As expected, the two markers did not colocalize but were in close association throughout the central brain (Fig. 4f).

To determine whether GABAergic neurons regulate astrocytic GAT levels, we used several approaches. First, we ablated GABA neurons during metamorphosis and examined GAT expression. The gad-GAL4 driver was used to express UAS-mCD8::mcherry and UAS-hid in a conditional manner with tub-GAL80p. GABAergic neurons were ablated specifically during metamorphosis by shifting animals to 30 °C (thereby activating Gal4/UAS) 0 h APF (Supplementary Fig. 9). Brains were then dissected and analyzed 84 h APF. Ablation of GABA neurons was confirmed by the reduction in the number of mCD8::mcherry-expressing cell bodies (Fig. 5a,b). The number of GABA neurons in the medial-ventral region of the brain decreased from 64.8 ± 1.2 (n = 5 brains) to 8.60 ± 2.4 (n = 5 brains) following Hid induction. Under these conditions, we observed a significant reduction in GAT expression throughout the brain by immunofluorescent stains (Fig. 5a,c). For instance, GAT levels were reduced by 33% and 37% in the AL and SOG, respectively. Quantification of astrocyte numbers revealed that ablation of GABA neurons did not result in astrocyte death (Fig. 5d), arguing against the notion that decreases in GAT were the result of astrocyte loss. We confirmed CNS-wide decreases in GAT levels in GABA neuron-ablated animals by western blot analysis performed on 84-h APF brains (Fig. 6b,c). Finally, we found gat transcript levels to be unaltered after GABA neurons were ablated using Hid (Fig. 6d), suggesting that regulation of GAT levels by GABAergic neurons may be post-transcriptional.

We next asked whether astrocytic GAT levels were regulated by GABAergic neuronal activity by using genetic tools to silence or activate GABAergic neurons. Synaptic vesicle release was blocked in GABA neurons by either expressing temperature-induced dominant negative Shibire (ShiP), or tetanus toxin light chain (TNT), which blocks synaptic release by cleaving Synaptobrevin. Inhibition of GABA neuron activity by ShiP specifically during late metamorphosis resulted in a 33% decrease in GAT expression (Fig. 6a–c). Astrocyte morphology as well as the distribution of GABA release sites appeared grossly unaffected (Supplementary Fig. 10). Similarly, expression of TNT resulted in a 33% decrease in GAT (Fig. 6b,c). As was the case with GABAergic neuronal ablation, gat mRNA levels were unchanged in response to expression of TNT in GABA neurons (Fig. 6d). We next assayed GAT expression after blocking action potential conduction by expressing the human inwardly rectifying K+ channel Kir2.1. This resulted in a 24% decrease in GAT (Fig. 6a–c). We next explored whether increased GABAergic neuronal activity could increase astrocytic GAT using the temperature-sensitive cationic channel TrpA1, which enables temperature-induced activation of neuronal depolarization. Despite activation of TrpA1 in GABAergic neurons, we did not observe significant changes in astrocytic GAT levels (Fig. 6a–c).

Sensitivity of astrocyte GAT levels to GABAergic signaling could be specific to development during the main wave of CNS synaptogenesis. Alternatively, GABAergic neuronal activity might serve as a mechanism to regulate astrocyte GAT levels throughout animal life. To discriminate between these possibilities, we inhibited GABA neuron activity either by blocking synaptic vesicle release with ShiP or by expressing Kir2.1 (using tub-GAL80p), during adult stages only. Under these conditions, we did not see changes in GAT levels (Fig. 6e,f). This suggests that GAT expression can be fine-tuned in response to GABA release only during a specific developmental phase of neural circuit assembly, between ~60 and 84 h APF. From the above data, we conclude that astrocytic

**Figure 5** GAT expression is sensitive to GABA neurons. (a) Confocal sections showing anti-GAT immunostaining (green) in the central brain and AL region after ablation of GABA neurons. GABA neurons are labeled by UAS-mCD8::mcherry expression using the gad-GAL4 driver (red). Substantial reduction in GAT levels is seen throughout the central brain in correspondence with a reduction in GABA neurons. Localization of GAT appears unaltered by GABA neuron ablations, as seen in images from the AL. Flies were 84 h APF upon preparation. Scale bars, 20 µm. (b) Quantification of the number of GABA neurons in the ventral medial region of the central brain (n = 5 brains for control and GABA neuron>Hid). (c) Quantification of the number of astrocytes in the AL and SOG regions (n = 8 brains for control and GABA neuron>Hid for AL and SOG). (d) Quantification of the number of astrocytes in the AL and SOG regions (n = 4 brains, control AL; n = 5 brains, GABA neuron>Hid AL; n = 4 brains, control SOG; n = 5 brains, GABA neuron>Hid SOG). ***P ≤ 0.001, unpaired Student’s t-test for b and d, paired Student’s t-test for c: P = 0.0001 for ; P = 0.0001, SOG for c. Error bars, s.e.m.
GAT levels are upregulated in the CNS during the main wave of synaptogenesis and that GAT levels in astrocytes are sensitive to GABAergic neuronal signaling during development of the adult CNS.

Astrocite GABA<sub>γ</sub> receptors regulate GAT during development

Astrocytes are known to express a number of neurotransmitter receptors and can be directly sensitive to neurotransmitter release<sup>34-37</sup>. Given our observation that synaptic release from GABAergic neurons could regulate GAT levels in astrocytes, we explored the possibility that this might be regulated by GABA-Rs. Astrocyte-specific knockdown of each of the Drosophila ionotropic GABA-R subunits did not appear to have an effect on GAT levels (data not shown). However, knockdown of the metabotropic GABA-R subunit GABA<sub>γ</sub>R2 in astrocytes resulted in a 31% decrease in GAT expression 84 h APF (Fig. 7a,b).

**Figure 6** GAT expression is fine-tuned in response to GABA release specifically during synaptogenesis. (a) Temperature shift scheme for conditional manipulation of GABA neuron activity. The temperature-sensitive constructs, UAS-shi<sup>ts</sup> and UAS-trpA1, as well as UAS-Kir2.1 with tub-GAL80<sup>ts</sup>, were activated in a conditional manner using the gad-GAL4 driver. Dominant negative shi expression, Kir2.1 expression or TrpA1 activation was induced at 30 °C. (b) Western blots performed on brains 84 h APF following various neuronal manipulations using the gad-GAL4 driver. Blots were probed with anti-GAT. GABA neurons were inactivated with expression of UAS-hid, UAS-shi<sup>ts</sup>, UAS-TNT and UAS-Kir2.1. Alternatively, GABA neurons were activated using UAS-trpA1. (c) Quantification of GAT from western blot analysis in (n = 4 experiments, Hid; n = 3 experiments, Shi<sup>ts</sup>; n = 5 experiments, TNT; n = 3 experiments, Kir2.1; n = 3 experiments, TrpA1). GAT levels were significantly reduced when GABA neurons were inactivated. This is in contrast to unaltered GAT levels when GABA neurons were activated. (d) Relative gat mRNA levels are unaltered when GABA neuronal activity is silenced by expression of UAS-hid (n = 3 experiments) or UAS-TNT (n = 3 experiments) using the gad-GAL4 driver. (e) Western blots performed on adult brains following adult-specific silencing of GABA neuronal activity by expression of UAS-shi<sup>ts</sup> or UAS-kir2.1 using the gad-GAL4 driver. (f) Quantification of GAT from western blot analysis in e (n = 3 experiments, Shi<sup>ts</sup>; n = 3 experiments, Kir2.1). **P < 0.01, ***P < 0.001, paired Student’s t-test for c and f, unpaired Student’s t-test for d. P = 0.0054, Hid; P = 0.0005, Shi<sup>ts</sup>; P = 0.0035, TNT; P = 0.0022, Kir2.1; P = 0.1800, TrpA1 for c. Error bars, s.e.m. Full-length blots are presented in Supplementary Figure 12.

**Figure 7** GAT expression is modulated through astrocytic metabotropic GABA-Rs.

(a) Western blots performed on brains 84 h APF following inhibition of GABA<sub>γ</sub>R1/2 signaling in astrocytes using the arm-GAL4 driver. Blots were probed with anti-GAT. GABA<sub>γ</sub>R1/2 signaling was impaired by expressing UAS-GABA<sub>γ</sub>R1 RNAi, UAS-GABA<sub>γ</sub>R2 RNAi or UAS-ptx. (b) Quantification of GAT from western blot analysis shown in a (n = 5 experiments, GABA<sub>γ</sub>R1 RNAi; n = 3 experiments, GABA<sub>γ</sub>R2 RNAi; n = 3 experiments, Ptx). (c) Relative gat mRNA levels were unaltered when astrocytic GABA<sub>γ</sub>R1/2 signaling was perturbed by expressing UAS-GABA<sub>γ</sub>R2 RNAi (n = 3 experiments) or UAS-pox (n = 3 experiments) using the arm-GAL4 driver. (d) Western blots performed on adult brains following adult-specific inhibition of GABA<sub>γ</sub>R1/2 signaling in astrocytes. Blots were probed with anti-GAT. (e) Quantification of GAT from western blot analysis in d (n = 3 experiments). (f) Western blots performed on adult brains following inhibition of GABA<sub>γ</sub>R1/2 signaling throughout development in astrocytes using the arm-GAL4 driver. Blots were probed with anti-GAT. GABA<sub>γ</sub>R1/2 signaling was impaired by expressing UAS-GABA<sub>γ</sub>R1 RNAi or UAS-GABA<sub>γ</sub>R2 RNAi throughout development. (g) Quantification of GAT from western blot analysis in f (n = 3 experiments, GABA<sub>γ</sub>R1 RNAi; n = 4 experiments, GABA<sub>γ</sub>R2 RNAi). *P < 0.05, **P < 0.01, paired Student’s t-test for b and g, unpaired Student’s t-test for c and one-way ANOVA with Tukey’s post hoc test for e. P = 0.0025, GABA<sub>γ</sub>R1 RNAi; P = 0.0042, GABA<sub>γ</sub>R2 RNAi; P = 0.0169, ptx for b and P = 0.0122, GABA<sub>γ</sub>R1 RNAi; P = 0.0467, GABA<sub>γ</sub>R2 RNAi for g. Error bars, s.e.m. Full-length blots are presented in Supplementary Figure 12.
GABA<sub>R</sub> functions through obligate dimerization with the GABA<sub>B</sub>R1 subunit<sup>38–41</sup>. We therefore assayed the effects of GABA<sub>B</sub>R1 depletion from astrocytes by RNAi and also found a 28% decrease in GAT levels (Fig. 7a,b). GABA<sub>B</sub>R1 directly binds GABA, whereas GABA<sub>B</sub>R2 signals through the α subunit of a type o G protein (G<sub>α/o</sub>) in both Drosophila and mammals<sup>38,42</sup>. Consistent with GABA<sub>B</sub>R2 signaling through G<sub>α/o</sub> in astrocytes, we found that expression of pertussis toxin (Ptx), a specific enzymatic inhibitor of G<sub>α/o</sub> in Drosophila, resulted in a similar 30% decrease in GAT levels at 84 h APF (Fig. 7a,b). In accordance with our observation that GABAergic neuron activity–dependent regulation of astrocytic GAT was post-transcriptional, inhibition of GABA<sub>B</sub>R1/2 signaling via expression of GABA<sub>B</sub>R2 RNAi or Ptx in astrocytes did not affect the amount of GAT transcripts in the brain (Fig. 7c). Likewise, the requirement for GABA<sub>B</sub>R1/2 signaling appears limited to a developmental time window: when either GABA<sub>B</sub>R1 or GABA<sub>B</sub>R2 was knocked down in adult stages only, there was no change in GAT levels (Supplementary Fig. 11 and Fig. 7d,e), while developmental knockdown of GABA<sub>B</sub>R1 or GABA<sub>B</sub>R2 resulted in reduced GAT levels that persisted in adult animals (Fig. 7f,g). Thus, GAT expression is fine-tuned through GABA<sub>B</sub>R1/2 signaling during synaptogenesis, and this developmental resetting of GAT levels ultimately determines GAT levels in the mature CNS.

Decreasing astrocyte GAT suppresses seizure induction

A primary function for astrocytic GAT proteins is balancing excitatory and inhibitory neuronal signaling in the CNS. To explore whether decreasing astrocyte GAT levels by inhibition of the GABA<sub>B</sub>R1/2 signaling pathway is sufficient to modify the balance of excitation and inhibition in the Drosophila CNS, we turned to behavioral studies in bang-sensitive mutants, a common model used to study nervous system hyperactivity and seizure<sup>43</sup>. Bang-sensitive mutants undergo seizure activity followed by paralysis when stimulated by mechanical shock as a result of neuronal hyperexcitability<sup>44</sup>. Intriguingly, bang-sensitive mutants such as easily shocked (eas)<sup>45</sup> are rescued from seizure activity by application of antiepileptic drugs such as gabapentin<sup>46</sup> or valproate<sup>47</sup>, both of which have been implicated in increasing extracellular GABA<sup>48</sup>.

We reasoned that if impairment of astrocytic GABA<sub>B</sub>R1/2 signaling could reduce functional GAT levels, GABA would not be cleared as efficiently from the CNS, and this would in turn suppress bang sensitivity in the eas<sup>PC80</sup> mutant background. We therefore crossed RNAi constructs targeting GABA<sub>B</sub>R2 and the UAS-pxt construct into the eas<sup>PC80</sup> mutant background, subjected animals to mechanical shock for 10 s to induce seizures followed by paralysis, and then assayed recovery time for 200 s. Consistent with previous reports, we found that while control animals recovered within 10 s after treatment, eas<sup>PC80</sup> mutants underwent robust seizure activity and paralysis with a mean recovery time of ~80 s (Fig. 8a,b). Similar recovery times were observed for all driver- or UAS-alone controls in an eas<sup>PC80</sup> mutant background. In contrast, we found that eas<sup>PC80</sup> animals expressing astrocytic GABA<sub>B</sub>R2 RNAi or Ptx required significantly less time (~46 s mean recovery time) to recover from paralysis (Fig. 8a,b). Consistent with our previous results in control animals, we found that GAT levels were reduced when GABA<sub>B</sub>R1/2 signaling was inhibited in adult eas<sup>PC80</sup> mutant animals (Fig. 8c,d). These data provide direct functional support for the notion that GABA<sub>B</sub>R1/2 signaling regulates functional GAT on astrocyte membranes in response to GABAergic neuronal activity, which is in turn important for modulation of excitation and inhibition balance in the nervous system.

DISCUSSION

In this study we explored the nature of astrocyte-neuron communication during assembly of the Drosophila adult brain. We found that the major wave of synaptogenesis occurred during the second half of pupal development and that appropriate synapse formation depended on the presence of fly astrocytes. Furthermore, we found that neuronal activity could reciprocally regulate astrocyte development. Proper upregulation of the sole Drosophila GAT of the SLc6 family required GABAergic neural activity. Astrocytes appeared to sense changes in extracellular GABA through activation of the metabotropic GABA<sub>B</sub>R1/2. Alterations
Formation of *Drosophila* adult CNS synapses requires astrocytes

On the basis of the upregulation of selected presynaptic markers, previous studies have argued that synaptogenesis occurs during late metamorphosis\(^25\), but whether this reflects actual assembly of pre- and postsynaptic compartments and the appearance of mature synaptic structures was not known. We directly examined synapse formation by electron microscopy in several parts of the developing adult *Drosophila* central brain and found that most synaptic structures formed between 60 and 84 h APF. To our knowledge, this is the first detailed ultrastructural analysis of when mature synaptic structures form in the developing adult *Drosophila* CNS. Notably, infiltration of astrocyte processes into the adult brain was tightly coordinated with the formation of adult brain synapses, raising the possibility that *Drosophila* astrocytes and CNS synapses might be reciprocally interdependent for formation during development. Indeed, we found that genetic ablation of ~75% of an animal’s astrocytes during the synaptogenic window resulted in a 30–50% (depending on brain region) reduction in synapses throughout the pupal and adult brains. This loss of synapses was accompanied by dramatic defects in adult behavior, such as severe defects in motor function, although we cannot determine whether these result from deficits in synapses, astrocytes or both.

Why was there only a partial loss of synapses in the adult when astrocytes were ablated? One possibility is that many CNS synapses in *Drosophila* may form in the absence of input from astrocytes. Astrocyte-secreted factors in mammals are critical for both excitatory and inhibitory synapse formation *in vivo*; however, no mutants affecting pro-synaptogenic astrocyte-derived molecules have been described in which more than 35% of CNS synapses are eliminated *in vivo*\(^3,5,49\). It also remains possible that ablation of *Drosophila* astrocytes preferentially affects specific types of synapses (for example, cholinergic, glutamatergic or GABAergic), which could not be determined by our TEM analysis. Perhaps in the absence of astrocytes the majority of one subtype of synapse is completely eliminated. While it appears that other subtypes of *Drosophila* glia do not grow into neuropil regions and occupy the domains normally covered by astrocytes, other glial subtypes might functionally compensate for the loss of pro-synaptogenic astrocytic cues at a distance. Finally, our TEM analysis was performed on the few animals that survived to adult stages, and these probably retained more astrocytes than those animals that expired at earlier developmental stages. As such, the observation that only 30–50% of synapses were eliminated could be explained by our inability to remove all astrocytes from the CNS and still generate adult animals. It is possible that a small number of astrocytes can still have a pro-synaptogenic effect on their surroundings, especially since many astrocyte-secreted factors have been shown to potentiate promote synapse formation. Nevertheless, our *in vivo* demonstration of a requirement for *Drosophila* astrocytes in synaptogenesis is consistent with pro-synaptogenic roles for astrocytes in mammal\(^3,5,49\), and our data reveal that astrocytic control of synapse formation is a conserved feature of mammalian and invertebrate CNS development.

Astrocyte GAT is regulated by GABAergic neuronal activity

It remains unclear how astrocytes acquire their final morphological and molecular phenotypes and how much of this is governed by their environment. We provide mechanistic evidence for direct regulation of the astrocytic GABA transporter, GAT, in response to GABAergic neuronal activity. Western blot analysis of GAT expression during adult synaptogenesis revealed that GAT was strongly upregulated during the main wave of synapse formation and coordinately with astrocyte infiltration of the neuropil. Strong expression of GAT was observed 84 h APF, a developmental time point at which ~90% of synaptic structures have formed and astrocytes have densely infiltrated neuropil regions and taken on their mature tufted morphology.

Several lines of evidence argue that astrocytic GAT activation is regulated directly by GABA release by GABAergic neurons. In animals 84 h APF, ablation of GABA neurons reduced GAT throughout the brain. Likewise, blockade of synaptic vesicle release (using Shi\(^{19}\) and TNT) or action potential firing (using Kir2.1) in GABA neurons also resulted in reduced astrocytic GAT, indicating that GAT is modulated by GABA release. Interestingly, astrocytes are likely to be capable of directly measuring extracellular GABA through GABA\(_B\)R1/2 and adjusting GAT levels accordingly. Depletion of GABA\(_B\)R1 or GABA\(_B\)R2 by RNAi or inhibition of GABA\(_B\) signaling by PTx expression specifically in astrocytes resulted in reduced GAT at late pupal stages. Somewhat surprisingly, we saw this mechanism of GAT regulation only during development and coincident with synaptogenesis. Adult-specific manipulations of GABA neuron activity or GABA\(_B\)R1/2 signaling did not noticeably alter GAT expression. Nevertheless, fine tuning of GAT during development is important for establishing adult levels, as knockdown of GABA\(_B\)R1 or GABA\(_B\)R2 during development reduced GAT expression even in adult stages. Direct measurement of GABA by astrocytes through GABA\(_B\)R1/2 signaling would provide a simple mechanism for how astrocytes adjust their GAT expression (and therefore ability to clear extracellular GABA) in response to alterations in GABA release. Adult expression patterns of mammalian astrocytic GAT-3 are established during postnatal stages that also coincide with periods of neuronal circuit refinement and astrocyte maturation\(^19\). Furthermore, mammalian astrocytes have been found to express GABA\(_B\) receptors\(^34,50\). We therefore speculate that a similar astrocyte-synapse signaling event may therefore also modulate astrocytic GAT levels in mammals, but this awaits exploration.

Each of our manipulations resulting in block of GABAergic neuronal activity or GABA\(_B\)R1/2 signaling resulted in no more than an ~50% reduction in astrocytic GAT levels. On one hand, this could be explained by the nature of the manipulations: perhaps they did not result in a complete loss of GABA neurons, neuronal activity or GABA\(_B\)R1/2 signaling. Also, because our antibody against GAT does not distinguish between GAT that is inserted in the membrane and GAT that is retained in intracellular pools, the level of functional GAT at the membrane may in fact be less than what is observed in immunostainings. Finally, it is important to note that several mechanisms are likely to exist for modulating astrocytic GAT. It is possible that the initiation of GAT expression is hardwired at some level during development to establish a baseline for handling GABA, which might explain the consistent ~50% reduction in expression level we observed in all our manipulations of GABAergic neurons or astrocytic GABA\(_B\) receptor signaling. Subsequent modulation and fine-tuning of GAT levels may then be regulated by activity-dependent plasticity imparted by GABA\(_B\)R1/2 signaling or other physiological mechanisms. Indeed, recent studies by Shigetomi *et al.*\(^17\) revealed a noteworthy role for astrocyte TrpA1-dependent calcium events in regulating GAT-3 levels, demonstrating another mechanism by which astrocytic GAT levels are fine-tuned. Given the significance of GABA signaling in neural circuit function, it is not surprising that several pathways exist to modulate GAT levels.
Modulation of GAT levels through GABA receptor signaling does not appear to occur at the transcriptional level. Our real-time quantitative PCR data revealed that gat mRNA levels were unchanged in dissected brains when GABAergic activity was blocked or when GABA receptor signaling was inhibited. These data argue that post-transcriptional regulation is likely to be important for GABAergic activity-dependent changes in GAT. For example, translation of GAT mRNAs may be regulated locally, or GAT protein degradation may be regulated in response to GABA receptor signaling. Shigetomi et al. also found that mammalian astrocytic GAT-3 expression can be regulated by mechanisms involving dynamin-dependent endocytosis. Thus, post-transcriptional regulation of astrocytic GATs may be an important means by which astrocytes modulate GABA tone.

Regulation of GAT in CNS excitatory/inhibitory balance

Tight regulation of GABA transporter levels and activity is critical in establishing balance between excitatory and inhibitory signaling. We provide strong behavioral evidence in the bang-sensitive Drosophila mutant eas-POC0, a model for seizure activity, that GABA receptor signaling can modulate extracellular GABA by regulating GAT expression and suppress hyperexcitability. Neurons in the eas-POC0 mutant have an altered phospholipid profile. Stimulation of eas-POC0 mutant neurons by sensory input (mechanosensory) or high-frequency stimulation correlates with a seizure phase, which is then followed by conduction failure and paralysis that is resolved by a wait of ~2 min, whereas control animals are unaffected by these stimulations. We found that inhibition of GABA receptor signaling by Pttx or GABA receptor RNAi expression in astrocytes significantly ameliorated the effects of seizure activity. These data provide in vivo evidence supporting a role for astrocytic GABA receptor signaling in modulating neuronal circuit function. The simplest mechanistic interpretation of our results is that inhibition of GABA receptor signaling results in decreased GAT and increased extracellular GABA, which in turn suppresses the effects of neuronal hyperexcitability.

Deciphering the molecular pathways mediating GAT regulation in vivo will be critical to understanding how the balance between excitatory and inhibitory signals is maintained. Our demonstration that GABA receptor signaling can directly modulate GAT levels in vivo now provides insight into how astrocytes adjust GAT levels in response to GABA release.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.K.M. performed the experiments; T.S. generated GAT antibodies; M.R.F. supervised the project; A.K.M. and M.R.F. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Ullian, E.M., Sapperstein, S.K.S., Christopherson, K.S.K. & Barres, B.A.B. Control of seizure frequency by glia. Science 291, 657–661 (2001).
2. Schafe, D.P., et al. Microglia sculpt postnatal neural circuits in a activity and complement-dependent manner. Neuron 74, 691–705 (2012).
3. Allen, N.J., et al. Astrocyte glycans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptor signaling. Nature 460, 410–414 (2012).
4. Engjl, C., et al. Gabapentin receptor e2e-1 is a neuronal thombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139, 380–392 (2009).
5. Christopherson, K.S., et al. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120, 421–433 (2005).
6. Freeman, M.R. Specification and morphogenesis of astrocytes: unique molecular programs to engulf pruned neuronal debris from distinct subsets of neurons. Genes Dev. 28, 20–33 (2014).
7. Su, Z.-Z., et al. Insights into glutamate transport regulation in human astocytes: cloning of the promoter for excitatory amino acid transporter 2 (EAAT2). Proc. Natl. Acad. Sci. USA 100, 1955–1960 (2003).
8. Lehre, K.P.K., et al. Storm-Mathis, J.J., & Danbolt, N.C. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J. Comp. Neurol. 333, 1833–1853 (1995).
9. Isaacson, J.S., Solis, J.M. & Nicoll, R.A. Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 10, 165–175 (1993).
10. Rossi, D.J. & Hamann, M. Spinal cord excitotoxicity and in vivo injections: markers for spinal glutamatergic neurons. Neuron 20, 783–795 (1998).
11. Briggs, S.W. & Galanopoulou, A.S. Altered GABA signaling in early life epilepsies. Brain 115, 257605 (2011).
12. Dudek, F.E. & Staley, K.J. How does the balance of excitation and inhibition shift during epileptogenesis? Epilepsy Curr. 7, 86–98 (2007).
13. Cope, D.W. & et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
14. Jacob, T., et al. A functional role for astrocytic transporter trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343 (2008).
15. Schousboe, A. Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–352 (2003).
16. Sarup, A., et al. A.GABAR, O.M. Schousboe, A.GABA transporters and GABA-transaminase as drug targets. Curr. Drug Targets CNS Neurol. Disord. 2, 269–277 (2003).
17. Shigetomi, E., et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
18. Jacob, T., et al. A functional role for astrocytic transporter trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343 (2008).
19. Schousboe, A. Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–352 (2003).
20. Sarup, A., et al. A.GABAR, O.M. Schousboe, A.GABA transporters and GABA-transaminase as drug targets. Curr. Drug Targets CNS Neurol. Disord. 2, 269–277 (2003).
21. Shigetomi, E., et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
22. Jacob, T., et al. A functional role for astrocytic transporter trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343 (2008).
23. Schousboe, A., et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
24. Jacob, T., et al. A functional role for astrocytic transporter trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343 (2008).
25. Schousboe, A. Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–352 (2003).
26. Sarup, A., et al. A.GABAR, O.M. Schousboe, A.GABA transporters and GABA-transaminase as drug targets. Curr. Drug Targets CNS Neurol. Disord. 2, 269–277 (2003).
27. Shigetomi, E., et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
28. Jacob, T., et al. A functional role for astrocytic transporter trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343 (2008).
29. Schousboe, A., et al. Enhanced tonic GABA A inhibition in typical absence epilepsy. Neuron 15, 1392–1398 (1995).
30. Freeman, M.R. Specification and morphogenesis of astrocytes. Science 330, 774–778 (2010).
31. Enell, L., et al. The role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–352 (2003).
32. Kümper, S., et al. Molecular cloning, behavior, and physiology. Invert. Neurosci. 3, 279–294 (1998).
33. Stork, T., et al. Heartless FGF receptor signaling pathway mediates morphogenesis of Drosophila astrocytes. Neuron 83, 388–403 (2014).
25. Jefferis, G.S.X.E. et al. Developmental origin of wiring specificity in the olfactory system of Drosophila. Development 131, 117–130 (2004).
26. Marin, E.C., Watts, R.J., Tanaka, N.K., Ito, K. & Luo, L. Developmentally programmed remodeling of the Drosophila olfactory circuit. Development 132, 725–737 (2005).
27. Zhu, S., Chiang, A.-S. & Lee, T. Development of the Drosophila mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. Development 130, 2603–2610 (2003).
28. Hughes, E.G., Elmariah, S.B. & Balice-Gordon, R.J. Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. Mol. Cell. Neurosci. 43, 136–145 (2010).
29. Madsen, K.K., White, H.S. & Schousboe, A. Neuronal and non-neuronal GABA transporters as targets for antiepileptic drugs. Pharmacol. Ther. 125, 394–401 (2010).
30. Benediktsson, A.M. et al. Neuronal activity regulates glutamate transporter dynamics in developing astrocytes. Glia 60, 175–188 (2012).
31. Tanaka, K. et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276, 1699–1702 (1997).
32. Devaraju, P., Sun, M.-Y., Myers, T.L., Lauderdale, K. & Fiacco, T.A. Astrocytic group I mGluR dependent potentiation of astrocytic glutamate and potassium uptake. J. Neurophysiol. 109, 2404–2414 (2013).
33. Yang, Y. et al. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron 61, 880–894 (2009).
34. Charles, K.J., Calver, A.R., Jourdain, S. & Pangalos, M.N. Distribution of a GABA-like receptor protein in the rat central nervous system. J. Neurosci. 23, 135–146 (2003).
35. Juechue, N.M., Marx, M.-C., Cheey, Q. & Bilipur, B. Activation of glutamate transport evokes rapid glutamine release from perisynaptic astrocytes. J. Physiol. (Lond.) 590, 2317–2331 (2012).
36. Porter, J.T. & McCarthy, K.D. Astrocytic neurotransmitter receptors in situ and in vivo. Prog. Neurobiol. 51, 439–455 (1997).
37. Hamilton, N.B. & Atwell, D. Do astrocytes really exocytose neurotransmitters? Nat. Rev. Neurosci. 11, 227–238 (2010).
38. Padgett, C.L. & Slesinger, P.A. GABA<sub>B</sub> receptor coupling to G-proteins and ion channels. Adv. Pharmacol. 58, 123–147 (2010).
39. Mezler, M., Möller, T. & Raming, K. Cloning and functional expression of GABA<sub>B</sub> receptors from Drosophila. Eur. J. Neurosci. 13, 477–486 (2001).
40. Bettler, B., Kaupmann, K., Mosbacher, J. & Gassmann, M. Molecular structure and physiological functions of GABA<sub>B</sub> receptors. Physiol. Rev. 84, 835–867 (2004).
41. Kaupmann, K. et al. GABA<sub>B</sub>-receptor subtypes assemble into functional heteromeric complexes. Nature 396, 683–687 (1998).
42. Dahdal, D., Reeves, D.C., Ruben, M., Akabas, M.H. & Blau, J. Drosophila pacemaker neurons require G protein signaling and GABAergic inputs to generate twenty-four hour behavioral rhythms. Neuron 68, 964–977 (2010).
43. Parker, L., Howlett, I.C., Rusan, Z.M. & Tanouye, M.A. Seizure and epilepsy: studies of seizure disorders in Drosophila. Int. Rev. Neurobiol. 99, 1–21 (2011).
44. Pavlidis, P. & Tanouye, M.A. Seizures and failures in the giant fiber pathway of Drosophila bang-sensitive paralytic mutants. J. Neurosci. 15, 5810–5819 (1995).
45. Pavlidis, P., Ramaswami, M. & Tanouye, M.A. The Drosophila easily shocked gene: a mutation in a phospholipid synthetic pathway causes seizure, neuronal failure, and paralysis. Cell 79, 23–33 (1994).
46. Reynolds, E.R. et al. Treatment with the antiepileptic drugs phenytoin and gabapentin ameliorates seizure and paralysis of Drosophila bang-sensitive mutants. J. Neurobiol. 58, 503–513 (2004).
47. Kuebler, D. & Tanouye, M. Anticonvulsant valproate reduces seizure-susceptibility in mutant Drosophila. Brain Res. 958, 36–42 (2002).
48. Sills, G.J. & Brodie, M.J. Update on the mechanisms of action of antiepileptic drugs. Epileptic Disord. 3, 165–172 (2001).
49. Kucukdereli, H. et al. Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. Proc. Natl. Acad. Sci. USA 108, E440–E449 (2011).
50. Oka, M., Wada, M., Wu, Q., Yamamoto, A. & Fujita, T. Functional expression of metabotropic GABA<sub>B</sub> receptors in primary cultures of astrocytes from rat cerebral cortex. Biochem. Biophys. Res. Commun. 341, 874–881 (2006).
**ONLINE METHODS**

*Drosophila* strains. The following *Drosophila* strains were used: Canton S, w^{118} (Canton S, elav-GAL4 [ref. 51], gad-GAL4 [ref. 52] (gift from G. Miesenböck), elav-GAL4 [ref. 53], GH146-QF [ref. 54], UAS-mCD8::GFP [ref. 55], UAS-syt::eGFP [ref. 56], UAS-mCD8::mcherry, UAS-hid [ref. 57], UAS-shi [ref. 58], UAS-AGA-CTCTTACCTCAGCTTTCA-BHQ1; Rpl32 52-F primer, GGGCGCAAGATCG TGAAAGAGG; Rpl32 B primer, Rlpl32 B-primer, Rlpl32 B-primer, and inactive IMPMT-Va, gifts from J. Carlson and S. Waddell), UAS-Kir2.1 [ref. 60], UAS-TrapA1 [ref. 61], UAS-pts [ref. 62] (gift from V. Budnik), UAS-GABA4-R2RNAi (TRIPJar02989), UAS-GABA4-R2RNAi (VDRC transmam tandem 1784), UAS-GAT-RNAi (VDRC transmam tandem 13359), QUAS-mCD8::GFP [ref. 54], tub-GAL80p [ref. 63], cagP300 (gift from B. Ganetzky and M. Tanouye).

Temperature shift schemes. To assess control conditions, GAL4 driver lines were crossed to w^{118} Canton S flies. For all experiments, control and experimental animals underwent identical temperature shifts. Because there are variations in developmental speed at different temperatures, the following incubation periods were based on morphological features that correspond to the developmental stages of interest at 25 °C.

Astrocye ablations. elav-GAL4 flies were crossed to UAS-hid, tub-GAL80p flies. Crosses were set up at 18 °C, and 0 h APF pupae were collected and incubated at 18 °C for another 48 h. For studies conducted in the adult, the staged pupae were shifted to 25 °C or 30 °C until animals were 1 d old. For studies conducted on animals ~60 or ~84 h APF, the staged pupae were shifted to 30 °C for 24 h or 48 h respectively. To assess loss of astrocytes, brains were immunostained with anti-GAT and anti-Repo antibodies (Developmental Studies Hybridoma Bank: 8D12 anti-Repo). Repo-positive nuclei that belonged to GAT-positive cells were counted as astrocytes. When astrocytes were counted in the AL, SOG and TGab, these neuropil regions of interest were identified by anti-HRP staining. When astrocytes were counted in the MB, anti-Fasciclin II staining was used to identify the boundaries of the MB.

GABA neuron ablations. gat-GAL4, UAS-mCD8::mcherry flies were crossed to UAS-hid, tub-GAL80p flies. Crosses were raised at 18 °C. Animals were collected at 0 h APF and transferred to 30 °C for 72 h, at which point they displayed morphological features of 84 h APF. For adult-specific inhibition of GABA neuron activity, crosses were raised at 18 °C, and 1-d-old adults were shifted to 30 °C for 7 d for *shibire* experiments and for 3 d for *Kir2.1* experiments.

Real-time PCR. Pupal heads (84 h APF) were dissected in Jan’s saline (1.8 mM CaCl2) and immediately frozen on dry ice. Total RNA was extracted using Trizol reagent. RNA pellets were resuspended in diethylpyrocarbonate-treated water and RNA concentration was measured using a Nanodrop 2000c spectrometer (Thermo Scientific). RNA was DNase treated (DNase I, amplification grade, 101-fold range in concentration (gt, 101%; Rpl32 92%, 0.025 μg cDNA template was used per reaction. Statistical analysis was performed on 2−ΔΔCt values.

Immunohistochemistry. Pupal brains were dissected in PTX (0.3% Triton X-100, PBS) and fixed in 4% formaldehyde for 20 min. For adult brains and CNS preparations, heads or whole flies, respectively, were fixed in 4% formaldehyde for 17 min, washed four times for 3 min with PTX, dissected in PTX and then fixed in 4% formaldehyde again for 7 min. For all preparations, the final fixation was followed by five quick washes with PTX and then three quick washes with PBT (0.1% BSA, 0.3% Triton X-100, PBS). Tissues were then blocked in PBT for 30 min at room temperature and then probed with appropriate primary antibodies for two nights at 4 °C. Samples were washed six times for 10 min with PBT, followed by appropriate secondary antibodies overnight at 4 °C, washed six times for 10 min with PBT and then stored in Vectashield anti-fade reagent (Vector Laboratories). Antibodies were used at the following dilutions: 1:50 mouse nc82 (Developmental Studies Hybridoma Bank: nc82); 1:5000 rabbit anti-ATG24; 1:10 mouse anti-PDF (Developmental Studies Hybridoma Bank: PDF C7); 1:500 mouse anti-GFP (Chemicon MAB3580); 1:150 rat anti-Fasciclin II (Developmental Studies Hybridoma Bank: ID4 anti-Fasciclin II); 1:100 mouse anti-Elav (Developmental Studies Hybridoma Bank: Elav-98F8A9); 1:500 rabbit anti-Draper61; 1:500 goat Cy3-conjugated anti-HRP (Jackson ImmunoResearch 123-165-021), 1:200 donkey FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch 711-095-152), 1:200 donkey Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch 711-165-152), 1:1000 donkey FITC-conjugated anti–mouse IgG (Jackson ImmunoResearch 713-159-150), 1:2000 Cy3-conjugated anti–mouse IgG (Jackson ImmunoResearch 713-165-150).

Confocal microscopy. Tissues were mounted in Vectashield anti-fade reagent and imaged using a Zeiss upright confocal microscope. Whole brain images for Supplementary Figure 1 were taken using a Zeiss EC Plan-Neofluar 10× objective (NA 0.3). Whole brain images for Figure 4f were taken using a Plan-Apochromat 20× objective. All other images were taken using a Zeiss Plan-Apochromat 40× oil objective (NA 1.3). Images were stitched together when regions of interest did not fit in a single field of view (Fig. 2a).

Immunoblotting. *Drosophila* brains were dissected in PBS and collected by light centrifugation. Brains were homogenized in SDS loading buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue) and centrifuged at 16,000g for 10 min to clear the homogenate. Supernatants were then collected and boiled for 5 min. Samples were resolved by SDS-PAGE (Bio–Rad) and transferred to nitrocellulose membranes (Bio–Rad). Membranes were blocked in blocking buffer (5% non–fat dry milk, 0.01% Tween-20, PBS) and then immobilized with appropriate antibodies. All antibodies were diluted in blocking buffer. Primary antibody incubations were performed overnight at 4 °C, followed by five immobilized washes in wash buffer (0.01% Tween-20, PBS), appropriate HRP-conjugated secondary antibody incubation for 1.5 h at room temperature, five 10-min washes in wash buffer and then chemiluminescence detection (ECL Plus Amersham). Immunoblots were stripped by rocking in mild stripping buffer (0.2 M glycine, 0.1% SDS, 1% Tween, pH 2.2) at room temperature for 10 min, followed by two 5-min washes in PBS and then two 5-min washes in wash buffer. After this, immunoblots were blocked again and reprobed. Antibodies were used at the following dilutions: 1:10,000 rabbit anti-ATG24; 1:50,000 mouse anti-tubulin (Sigma T9026); 1:6,000 sheep HRP-conjugated anti–mouse IgG (Abcam ab6808); 1:6,000 goat HRP-conjugated anti-rabbit IgG (Abcam ab6721).

Chemiluminescence was detected using Fujifilm Luminescence Image Analyzer LAS-4000. Western blots were analyzed using Fujifilm MultiGauge Software.

Behavioral assays. Flies were aged 3–7 days before testing. 3–10 flies were transferred to fresh food vials and vortexted for 10 s to provide mechanical stimulus and induce paralysis in bang sensitive animals. The numbers of flies standing and remaining normal behavior were noted at 10 s intervals until all flies had recovered from paralysis. Mean recovery time was calculated as the average time taken by an individual fly to recover from paralysis (n > 100 flies for all genotypes).

Transmission electron microscopy. For all electron microscopy experiments, at least two brains were independently prepared and sectioned for every condition/time-point. *Drosophila* heads were incubated in fixation buffer (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2) for 1 h at 4 °C. Brains were then dissected out in fixation buffer and incubated in fixation buffer overnight at 4 °C. Samples were then processed and analyzed at the University of Massachusetts Medical School Electron Microscopy core facility according to standard procedures. Briefly, fixed samples were transferred to fresh fixation buffer and incubation
continued overnight at 4 °C. Samples were rinsed twice with fixation buffer and treated with 1% osmium tetroxide for 1 h at room temperature. Samples were washed twice with dH₂O for 5 min and then dehydrated through a graded ethanol series of 20% increments that ended with two changes in 100% ethanol. Samples were infiltrated with two changes of 100% propylene oxide and then with a 1:1 propylene oxide/SPI-Pon 812 resin mixture. The following day, the samples were infiltrated with three changes of fresh 100% SPI-Pon 812 resin, after which the samples were polymerized at 68 °C in plastic capsules. Thin sections were placed on copper support grids and contrasted with lead citrate and uranyl acetate. Sections were examined using the FEI Tecnai 12 BT electron microscope with 80 kV accelerating voltage, and images were captured using a Gatan TEM CCD camera.

Statistical analysis. GraphPad Prism was used to perform two-tailed unpaired Student's t-tests, two-tailed paired Student's t-tests, one-way ANOVA with Tukey's post hoc tests and repeated measures ANOVA with Tukey's post hoc tests. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. Also, data collection and analysis were not performed blind owing to the conditions of the experiments. Data were not collected and processed randomly. Animals were assigned to the various experimental groups on the basis of genotype. A Supplementary Methods Checklist is available.

51. Doherty, J., Logan, M.A., Tasdemir, O.E. & Freeman, M.R. Ensheathing glia function as phagocytes in the adult Drosophila brain. J. Neurosci. 29, 4768–4781 (2009).