Focal adhesion molecules regulate astrocyte morphology and glutamate transporters to suppress seizure-like behavior

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Astrocytes are important regulators of neural circuit function and behavior in the healthy and diseased nervous system. We screened for molecules in *Drosophila* astrocytes that modulate neuronal hyperexcitability and identified multiple components of focal adhesion complexes (FAs). Depletion of astrocytic Tensin, β-integrin, Talin, focal adhesion kinase (FAK), or matrix metalloproteinase 1 (Mmp1), resulted in enhanced behavioral recovery from genetic or pharmacologically induced seizure. Overexpression of Mmp1, predicted to activate FA signaling, led to a reciprocal enhancement of seizure severity. Blockade of FA-signaling molecules in astrocytes at basal levels of CNS excitability resulted in reduced astrocytic coverage of the synaptic neuropil and expression of the excitatory amino acid transporter EAAT1. However, induction of hyperexcitability after depletion of FA-signaling components resulted in enhanced astrocyte coverage and an approximately twofold increase in EAAT1 levels. Our work identifies FA-signaling molecules as important regulators of astrocyte outgrowth and EAAT1 expression under normal physiological conditions. Paradoxically, in the context of hyperexcitability, this pathway negatively regulates astrocytic process outgrowth and EAAT1 expression, and their blockade leading to enhanced recovery from seizure.

astrocyte | focal adhesions | *Drosophila* | glutamate transporters | hyperexcitability

Astrocytes are highly branched nonneuronal cells that tile with one another and densely infiltrate synapse-rich regions of the brain. The fine processes of astrocytes form close contacts with neuronal cell bodies and synapses, where they support and modulate neuronal activity. Astrocytes provide metabolic support to neurons to meet the high energy demands of neural activity and buffer ions and pH to maintain brain homeostasis (1). Through dynamic changes in calcium signaling, astrocytes act as important regulators of neural activity (2, 3) and neurovascular coupling (4). Despite our growing appreciation for the importance of astrocytes in neural circuit function and maintenance (5, 6), we know surprisingly little about the signaling pathways regulating astrocyte associations with synapses or how they are modulated by dynamic changes in activity.

A major mechanism by which astrocytes impact synaptic physiology is through the regulation of extracellular levels of excitatory and inhibitory neurotransmitters (NTs). Following neuronal release of glutamate or GABA, excitatory amino acid transporters (EAATs) or GABA transporters (GATs), respectively, on astrocyte membranes remove these NTs from the synaptic and extrasynaptic space (7). Efficient astrocyte clearance of NTs is important for proper termination of synaptic signaling to avoid chronic receptor desensitization at synapses (8), regulation of the spillover of neurotransmitters between synapses (7, 9), and setting proper basal excitatory/inhibitory (E/I) tone (10–12). Conditional elimination of EAATs from astrocytes in mice results in fatal epilepsy (13), and mutations in human EAATs cause episodic ataxia and seizure (14).

EAATs on astrocyte membranes appear to be subject to diverse modes of regulation. EAAT levels change in response to factors secreted from neurons including glutamate (15), neuronal contact (16), or neuronal activity (17). Pathological changes in EAATs have been associated with disease including epilepsy and seizure (10), neurodevelopmental disorders (18), ataxia (14, 19), and ALS (20), where dysregulation of EAATs is thought to drive changes in E/I balance. Treatments that up-regulate GLT1 (EAAT2 in humans) expression in vitro show neuroprotective effects (21), arguing that rebalancing EAAT levels may be a valuable therapeutic approach to reestablishing normal E/I balance. However, precision in tuning EAATs will be essential: for example, increasing GLT1 expression in vivo, through application of β-lactam, ceftriaxone, or interfering with neuroglia EphA signaling, had no effect on basal excitatory synaptic transmission, but resulted in impaired long-term plasticity and learning and memory performance, presumably because glutamate levels were not sufficiently high to drive synaptic strengthening (21–23).

Significance

Astrocytes are nonneuronal cells that closely associate with neuronal synapses to regulate synaptic function in brain circuits. A powerful mechanism by which astrocytes can modulate neurotransmission is through their uptake of synaptically released neurotransmitters (NTs). Imbalances in the uptake of the excitatory NT glutamate or inhibitory NT GABBA can change the balance of excitatory and inhibitory signaling in the brain and lead to hyperactivity or seizure. In this study, we explore how astrocytes modulate neuronal hyperactivity and show that focal adhesions, a class of cell-adhesion molecules, are key regulators of astrocyte associations with synapses and astrocyte expression of NT transporters. This work provides potential targets for therapeutic manipulation to suppress seizure activity or excess neuronal excitability.

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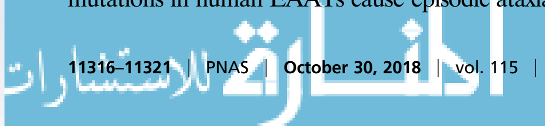

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The *Drosophila* brain houses astrocytes that are similar to mammalian astrocytes by morphological, molecular, and functional criteria (24). Fly astrocytes are found within the synaptic neuropil, and they exhibit a highly branched morphology and tile with one another to cover the entire synaptic space (25–28). Mature fly astrocytes express NT transporters for glutamate and GABA and enzymes that metabolize these NTs into intermediates used to resupply neurons (27, 29, 30). During development *Drosophila* astrocytes promote synapse formation (12) and mediate neuronal remodeling, including synaptic pruning (28, 31). In the mature nervous system, fly astrocytes exhibit multiple types of calcium transients, some of which are regulated by neuronal activity, and which are essential to exert the neuromodulatory effects of octopamine and tyramine (i.e., invertebrate norepinephrine) in neural circuit activity and complex behaviors (3). The striking conservation of many aspects of astrocyte biology among flies and mammals suggests that *Drosophila* will be a useful model to dissect genetic pathways mediating astrocyte function in vivo.

We wanted to identify novel pathways by which astrocytes regulate neuronal activity. In this study, we performed a comprehensive in vivo RNAi screen in *Drosophila* to identify astrocyte-expressed genes that could phenotypically modify a genetically induced seizure model. Unexpectedly, we found that astrocyte-specific depletion of components of focal adhesion complexes (FAs) and a matrix metalloproteinase suppressed seizure activity in both genetic and pharmacological models of seizure (i.e., picrotoxin exposure). We show that, under normal physiological conditions, FA molecules are required for proper establishment of astrocyte coverage of the synaptic neuropil and expression of excitatory amino acid transporter 1 (EAAT1), while the levels of the sole *Drosophila* GABA transporter, GAT, are unchanged. Interestingly, in the context of hyperexcitability, FA molecules negatively regulated EAAT1 levels and astrocyte coverage of the synaptic field, and their elimination led to enhanced EAAT1 expression, increased astrocyte coverage of the synaptic neuropil, and more rapid recovery from seizure-like behavior.

Results

An in Vivo RNAi Screen Identifies *Tensin* as an Astrocyte-Expressed Regulator of Seizure Activity. To identify astrocyte genes capable of modulating neural circuit activity, we performed an in vivo RNAi screen in adult *Drosophila* for genes that, when knocked down selectively in astrocytes, enhanced or suppressed seizure activity. We took advantage of the *easily shocked* (eas^{PC80}) mutant, a well-studied model that provides a sensitized genetic background in which neurons are hyperexcitable and seizure activity can be induced by a simple mechanical stimulus (32) (*SI Appendix*, Fig. S1*A*). While control animals resume normal locomotor behavior immediately after vortexing in culture vials for 10 s, eas^{PC80} mutants show severe seizure-like behaviors including paralysis, vigorous wing beating, and uncoordinated locomotion after the stimulus, and they recover over a few minutes (*SI Appendix*, Fig. S1*A*). We knocked down genes specifically in astrocytes (using the astrocyte-specific *alrm-Gal4* driver) in the *eas* mutant background to identify astrocyte-specific modifiers of seizure activity that would either enhance or suppress seizure phenotypes. We screened a collection of ∼2,000 UAS-RNAi lines that targeted genes encoding the majority of secreted, transmembrane, or membrane-associated molecules in the fly genome (*SI Appendix*, Fig. S1*B*). Animals were vortexed for 10 s, and the percentage of animals that righted themselves were scored at 1, 1.5, 2, 3, and 4 min (*SI Appendix*, Fig. S1*A*). Suppressors of seizure susceptibility led to 100% recovery within 1.5 min, and enhancers were those that delayed recovery until after 4 min. In total, we identified 100 RNAi lines that suppressed seizure activity and 86 lines that enhanced seizure activity when expressed in astrocytes (*SI Appendix*, Tables S1 and S2).

We found that astrocyte-specific knockdown of Tensin, a component of FAs and regulator of cell-extracellular matrix interactions, led to a 100% recovery from seizure-like behavior in less than 1 min (Fig. 1*A*). We confirmed this observation by repeating the eas^{PC80} seizure assay with an additional, nonoverlapping $tensin^{RNAi}$ line, in this case recording recovery rates at 10-s intervals. We observed a dramatic shift of recovery plot with astrocyte-specific depletion of *tensin* compared with controls (Fig. 1*A*) and a decrease in mean recovery time (Fig. 1*A′*). To determine whether Tensin was expressed in astrocytes and targeted by our RNAi lines, we visualized Tensin using an endogenously GFP-tagged *tensin* line (33). We labeled astrocyte membranes with anti-Gat antibodies (a highly specific marker for astrocyte membranes) in larval ventral nerve cords (Fig. 1*B*) and focused on the quantification of Tensin::GFP that fell within the domains of anti-Gat immunoreactivity. We found that Tensin::GFP was localized in punctate structures in astrocyte cell bodies and astrocyte processes as they projected into the neuropil area (Fig. 1*B*). Furthermore, we found that Tensin::GFP levels were decreased by 34% in $tensin^{RNAi}$ animals compared with its control (Fig. 1*B′*). We note that the efficiency of RNAi knockdown may be greater given that we focused only on the quantification of Tensin::GFP that fell within the domains of anti-Gat immunoreactivity, which labels only the cell membrane. Indeed, when we expressed *UAS-tensin*^{RNAi} under the control of *tubulin-GAL4*, Tensin::GFP puncta were not detectable (Fig. 1*C*). These data demonstrated that Tensin is expressed in astrocytes, localized to punctate structures within astrocytes, and that our

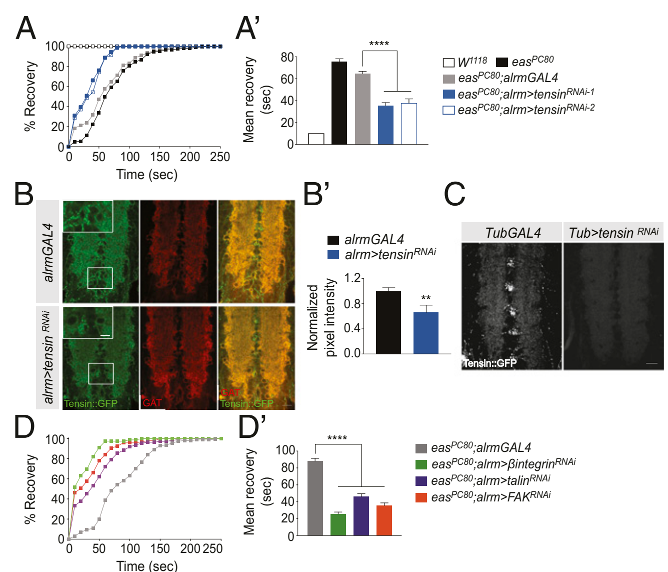

Fig. 1. Astrocyte focal adhesion molecules regulate seizure-like behavior in bang-sensitive mutant eas^{PC80}. (*A*) Percentage of recovery was measured every 10 s following 10 s of mechanical shock. Wild-type flies (W^{1118}) recovered to 100% within 10 s while eas^{PC80} displayed seizure-like behavior with delayed recovery. Astrocyte knockdown of *tensin* suppressed seizure-like behavior using two independent RNAi lines. (*A′*) Mean recovery time based on *A*. $n > 100$ flies quantified for all genotypes. (*B*) Tensin localization was visualized with Tensin-GFP in control and astrocyte tensin knockdown, and astrocyte membranes were labeled using α-Gat antibody. (*B′*) Knockdown efficiency was quantified by measuring the intensity of Tensin-GFP signal within astrocyte membrane. *alrmGAL4*, $n = 8$; *alrm* > $tensin^{RNAi}$, $n = 6$ animals quantified. (*C*) Expressing tensin RNAi with TubGAL4 driver removed Tensin-GFP puncta throughout the ventral nerve cord of third instar larvae. *TubGAL4*, $n = 8$; *Tub* > $tensin^{RNAi}$, $n = 7$ animals. (*D*) RNAi to multiple components of focal adhesions including *β-integrin*, *talin*, and *FAK* suppressed seizure-like behavior in eas^{PC80} mutant backgrounds. (*D′*) Quantification of mean recovery time from data in *D*. $n > 100$ flies quantified for all genotypes. $^{**}P < 0.01$; $^{****}P < 0.0001$. (Scale bars: *B*, *Inset*, 1 μm; *B*, 10 μm; *C*, 10 μm.)

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RNAi lines successfully targeted *tensin*. We conclude that Tensin is an astrocyte molecule that can modulate seizure activity in vivo.

Multiple Components of Focal Adhesion Complexes Function in Astrocytes to Modulate Seizure Activity. Tensin is a component of FAs, which are large multiprotein complexes observed in cultured mammalian cells, that include heterodimeric integrin receptors that interact with the extracellular matrix (ECM), and adaptor proteins that connect integrin receptors to the actin cytoskeleton (34) (*SI Appendix*, Fig. S2*A*). We sought to determine whether additional known components of FAs functioned in astrocytes to modulate hyperexcitability-induced seizure. Immunofluorescent labeling with anti–β-integrin antibodies revealed that β-integrin colocalized with Tensin::GFP$^+$ puncta within the ventral nerve cord (*SI Appendix*, Fig. S2*B*), which supports the notion that Tensin colocalizes with β-integrin in vivo. We next screened additional defined FA components in the adult eas^{PC80} seizure assay and found that astrocyte-specific RNAi knockdown of β-integrin, the adaptor protein Talin, or focal adhesion kinase (FAK), strongly suppressed seizure-like behavior at levels similar to that of $tensin^{RNAi}$ (Fig. 1 *D* and *D*′). These results demonstrate that known components of FAs function in astrocytes to regulate nervous system hyperexcitability and seizure.

Seizures Induced by Pharmacological Blockade of GABA-A Receptors Are Modulated by Focal Adhesion Molecules in Astrocytes. To complement our studies using the eas^{PC80} genetic model of seizure, we turned to pharmacological blockade of GABA-A receptor signaling, a common model for seizure. Exposure of larvae to food containing the GABA-A receptor inhibitor picrotoxin (PTX) results in widespread CNS disinhibition, an overall increase in neuronal activity, and larval nervous system hyperexcitability (35). We collected third instar larvae fed PTX-laced or control food for a period of 9 h and compared the locomotion speed of sham controls to animals treated with two different doses of PTX (0.5 or 1.5 mg/mL) using a tracking system, FIM (an FTRI-based imaging method) (36). We observed no changes in basal locomotion in animals that were sham-treated and no alterations in locomotion speed (mm/min) after astrocyte-specific knockdown of *tensin*, *β-integrin*, *talin*, or *FAK* (Fig. 2 *A* and *B*). Therefore, depletion of FA-signaling components in astrocytes did not appear to alter baseline motor behavior in locomotion assays. After exposure of animals to 0.5 mg/mL PTX, we observed a dose-dependent decrease in motility in controls, with crawling speed being decreased by 65% and further decreased by 83% at 1.5 mg/mL PTX (Fig. 2 *A* and *B*). PTX-induced decreases in larval locomotion were significantly suppressed at both doses in animals with astrocyte-specific RNAi depletion of *tensin*, *β-integrin*, *talin*, or *FAK* (Fig. 2 *A* and *B*). The strength of suppression was not further enhanced by simultaneous expression of RNAi constructs for *β-integrin* and *tensin* (Fig. 2*C*), which is consistent with the notion that these FA molecules signal in the same genetic pathway. To provide genetic evidence supporting a role for Tensin in astrocyte control of seizure, we utilized a *tensin* null animal (by^{33c}). The by^{33c} animals exhibited normal locomotion compared with controls and suppressed PTX-induced seizure at levels indistinguishable from astrocyte $tensin^{RNAi}$ (*SI Appendix*, Fig. S3*D*). We conclude that astrocyte FA molecules modulate behavioral changes in the neuronal excitation/inhibition balance at larval and adult stages and in response to both genetic and pharmacological manipulation of hyperexcitability.

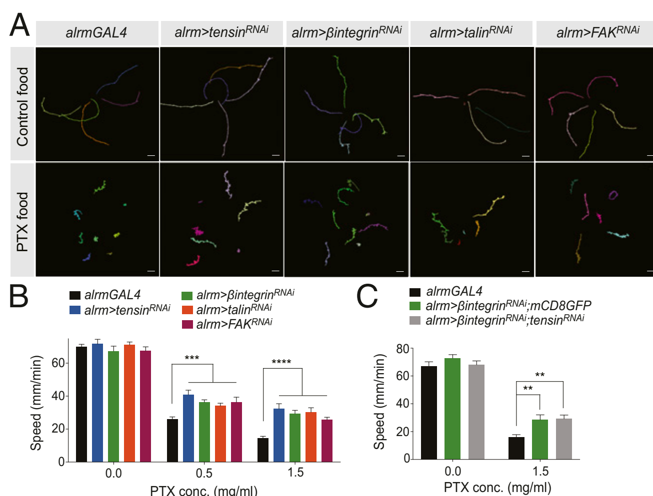

Fig. 2. Astrocyte focal adhesion molecules suppress PTX-induced locomotion defects in third instar larvae. (*A*) Raw traces of third instar larval locomotion with and without picrotoxin treatment in control and RNAi for focal adhesion components. (Scale bars: 1 cm.) (*B*) Quantification of distance traveled within 1 min. *SI Appendix, Materials and Methods*, provides the number of animals quantified for all genotypes throughout the figures. (*C*) Simultaneous knockdown of *β-integrin* and *tensin* in astrocytes had no additive effect on suppression of PTX-induced locomotion defects. $^{**}P < 0.005$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Astrocyte Matrix Metalloproteinase 1 Activity Dynamically Regulates Seizure Activity. Integrins, heterodimeric cell-surface receptors, receive signaling from the ECM. The ECM is a substrate for the matrix metalloproteinases (MMPs), extracellular zinc-dependent endopeptidases, and their enzymatic activity leads to the cleavage of ECM proteins, which can serve to activate integrin signaling (37, 38). Interestingly, increased neuronal activity or glutamate signaling has been found to elevate MMP activity and/or expression (37, 39). We explored the possibility that *Drosophila* MMPs might modulate nervous system excitability similarly to FA-signaling molecules using both loss- and gain-of-function approaches. There are only two MMPs in the *Drosophila* genome, Mmp1 and Mmp2. Mmp1 is a secreted form and is enriched in expression approximately sevenfold in astrocytes compared with neurons. We first expressed RNAi targeting *mmp1* in astrocytes in the eas^{PC80} background and assayed sensitivity to hyperexcitability-induced seizure. We found that depletion of *mmp1* by RNAi suppressed seizure-like behavior by decreasing recovery time (Fig. 3 *A* and *A*′). Reciprocally, astrocyte overexpression of Mmp1 had the opposite effect and strongly enhanced seizure-like behavior (Fig. 3 *A* and *A*′). We further found that $Mmp1^{RNAi}$ partially suppressed PTX-induced decreases in larval locomotion (Fig. 3*B*), and the same suppression was observed when PTX-treated animals were cofed the MMP inhibitor GM 6001 (Fig. 3*C*).

Since MMPs are secreted as a pro-MMP form requiring extracellular activation to become enzymatically active (40), we speculated that Mmp1 activates integrin receptors extracellularly, which then signals with focal adhesion molecules to modulate excitability. To test this model, we assayed for phenotypic interactions between Mmp1, β-integrin, and Talin in PTX-induced seizure assays. We found that addition of the MMP inhibitor did not enhance the rescuing effect of $\beta\text{-}integrin^{RNAi}$ and that Mmp1 overexpression did not modify the seizure suppression afforded by $\beta\text{-}integrin^{RNAi}$ (Fig. 3*E*), arguing that Mmp1 acts upstream of β-integrin in the same genetic pathway in astrocyte regulation of neuronal excitability (Fig. 3*D*). Talin is a cytosolic adaptor protein that can act downstream of β-integrin, and its expression is not required for β-integrin localization to the cell surface (41). We found that the rescuing effect of $talin^{RNAi}$ in astrocytes was suppressed by Mmp1 overexpression (Fig. 3*F*), indicating that increasing extracellular Mmp1 can overcome the effect of Talin depletion in astrocytes.

Focal-Adhesion–Signaling Molecules Regulate Astrocyte Coverage of the Neuropil. Focal adhesion molecules serve critical roles in cell migration and tissue development, and integrin-mediated signaling

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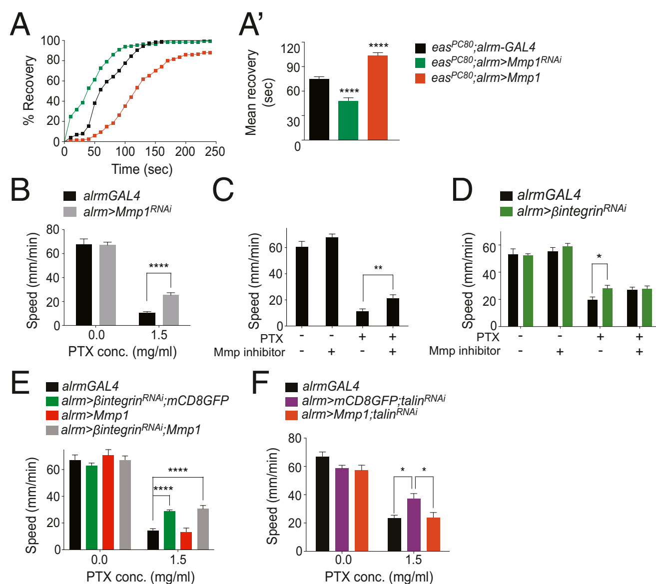

Fig. 3. (*A*) Astrocytic Mmp1 modulates seizure severity. (*A*) Mmp1 overexpression or knockdown in astrocytes modulated seizure activity in eas^{PC80} mutants. (*A′*) Mean recovery time based on *A*. $n > 100$ flies quantified for all genotypes. (*B*) Astrocyte knockdown of Mmp1 suppressed PTX-induced seizure-like behavior in larvae. (*C*) Pharmacological inhibition of MMPs during PTX feeding suppressed PTX-induced seizure. (*D*) MMP inhibitor had no additive effect on the suppression phenotype by astrocyte knockdown of *β-integrin*. (*E*) Mmp1 overexpression was not able to reverse phenotypes associated with depletion of astrocyte β-integrin in seizure assays. (*F*) Mmp1 overexpression in astrocytes reversed the effect of *talin* knockdown in seizure assays. $*P < 0.05$; $**P < 0.005$; $****P < 0.0001$.

in astrocytes is known to govern astrocyte cell spreading and morphology in vitro (42, 43). To determine whether manipulations of astrocyte FA components altered astrocyte morphology in vivo, we labeled astrocytes with membrane-tethered GFP and coimmunostained for presynaptic protein Bruchpilot (Brp) in RNAi backgrounds and controls (*SI Appendix*, Fig. S4*A*). We found that *tensin*^{RNAi} resulted in no overt defects in astrocyte morphology or synapse distribution (*SI Appendix*, Fig. S4*A*). Quantitative analysis of Brp levels using Western blots did not reveal significant alteration in the level of this synaptic marker (*SI Appendix*, Fig. S4*C*), nor were there any overt changes in the level of the astrocyte GABA transporter, Gat, in *tensin*^{RNAi} animals (*SI Appendix*, Fig. S4*B*).

FA-signaling molecules could be required for the regulation of astrocyte processes that are beyond the limits of detection by light microscopy (44), so we examined the fine morphology of astrocytes in the CNS neuropil using transmission electron microscopy (TEM). We prepared third instar larval brains from control and *β-integrin*^{RNAi} animals and focused our analysis on the ventral nerve cord where astrocyte morphology has been described previously (27, 45). Astrocyte processes were recognized by their electron-dense cytoplasm (27, 28), and the identification of astrocytes under electron microscopy was shown by 3D reconstruction (45). Our genetic ablation assay showed significant loss of astrocyte processes in the neuropil, supporting our ability to identify astrocyte processes in TEM images (*SI Appendix*, Fig. S5). Compared with controls, astrocyte *β-integrin*^{RNAi} animals exhibited a reduction in the total area covered by the processes under normal physiological conditions (Fig. 4 *A* and *B*). Consistent with our observations by light microscopy and Western blots, we found no change in the number of synapses in *β-integrin*^{RNAi} animals compared with controls (Fig. 4*D*). Thus, β-integrin is essential for the establishment of normal coverage of the neuropil by fine astrocyte processes but not for synapse formation. We next explored how FA molecules like β-integrin would regulate astrocyte processes in response to CNS hyperexcitability. We found that, while exposure of control animals to PTX did not change astrocyte coverage of the neuropil, astrocyte *β-integrin*^{RNAi} animals exhibited a marked increase in neuropil coverage by astrocyte profiles and a decrease in the distance between astrocyte membranes and synapses (Fig. 4 *A*–*C*). These changes in astrocyte morphology occurred in the absence of changes in the total number of synapses in the neuropil (Fig. 4*D*). Depletion of astrocyte β-integrin therefore leads to an increase in astrocyte coverage of the synaptic field.

Astrocyte Focal-Adhesion–Signaling Molecules Regulate Levels of the Excitatory Amino Acid Transporter EAAT1. Changes in excitatory or inhibitory neurotransmitter transporters on astrocytes could also significantly impact behavioral responses to hyperexcitability. Interestingly, we found that depletion of either β-integrin or Tensin from astrocytes by RNAi resulted in a decrease in EAAT1, the sole high-affinity glutamate transporter in *Drosophila* (46), under normal physiological conditions (Fig. 5 *A* and *B*). Therefore, FA molecules are required to establish normal basal levels of EAAT1 in the CNS. Surprisingly, we found that treatment of animals with picrotoxin to induce seizure had the opposite effect: induction of hyperexcitability led to a roughly twofold increase in EAAT1 levels in astrocyte *β-integrin*^{RNAi} and *tensin*^{RNAi} animals (Fig. 5 *A* and *C*). These data indicate that, under conditions of hyperexcitability, FAs negatively regulate EAAT1 levels, and we propose that increases in EAAT1 levels enhance glutamate uptake and help suppress the seizure activity that we see in animals where FA-signaling molecules have been inhibited. Consistent with this interpretation, we found that feeding larvae the EAAT inhibitor DHK suppressed the ability of *β-integrin*^{RNAi} or *tensin*^{RNAi} to rescue animals from seizure-induced immobility (Fig. 5*D*). Likewise, application of DHK blocked the ability of *Mmp1*^{RNAi} to suppress seizure phenotypes in larval locomotion (Fig. 5*E*).

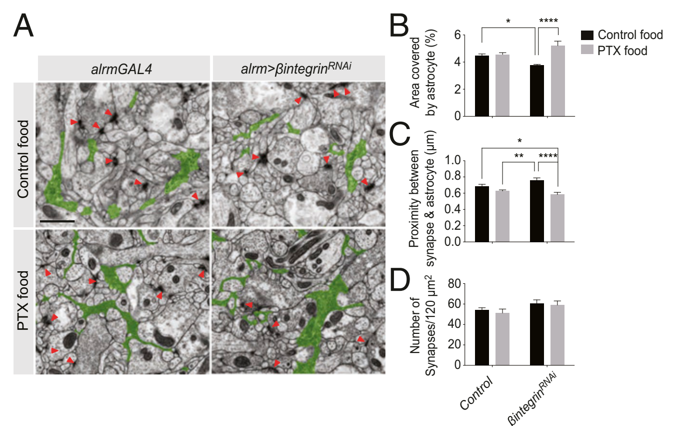

Fig. 4. Focal adhesion molecules regulate astrocyte morphology and neuropil coverage. (*A*) Ultrastructure of the larval ventral nerve cords. Astrocyte processes are pseudocolored in green. (Scale bar: 1 μm.) (*B*) Quantification of the percentage of area covered by astrocyte processes. (*C*) Quantification of the distance from a synapse to the nearest astrocyte process. (*D*) Quantification of number of synapses. *alrmGAL4* (0.0 mg/mL PTX), $n = 15$ sections from four animals; *alrm>β-integrin*^{RNAi} (0.0 mg/mL PTX), $n = 15$ sections from four animals; *alrmGAL4* (1.5 mg/mL PTX), $n = 16$ sections from four animals; *alrm>β-integrin*^{RNAi} (1.5 mg/mL PTX), $n = 16$ sections from four animals. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$; $****P < 0.0001$.

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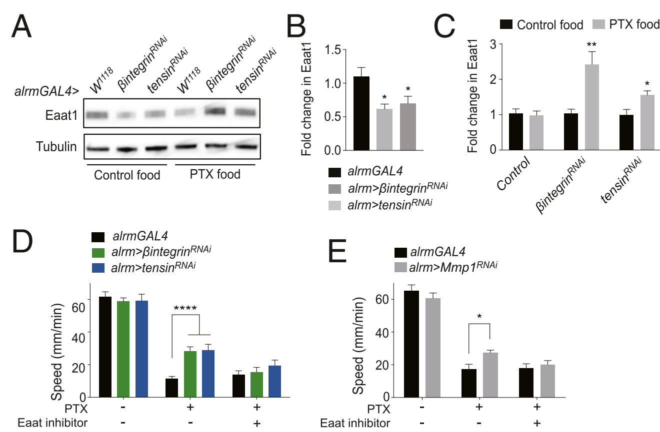

Fig. 5. Astrocyte focal adhesion molecules regulate EAATs under normal conditions and during hyperexcitability. (*A*) Western blot analysis on larval CNS lysates probed with anti-EAAT1. (*B*) Quantification of Eaat1 levels in Western blot normalized to Tubulin signal and to the control lane ($n = 6$). (*C*) Quantification of Eaat1 levels in Western blot normalized to Tubulin signal and to the control lane ($n \geq 6$). (*D*) Pharmacological inhibition of EAAT function blocked the effect of *β-integrin* or *tensin* knockdown in PTX-induced seizure. (*E*) EAAT inhibitor prevented the suppression of PTX-induced seizure by astrocyte-specific knockdown of Mmp1. *$P < 0.05$; **$P < 0.005$; ****$P < 0.0001$.

Discussion

Astrocyte processes infiltrate all synapse-rich regions of the brains of complex metazoans and are poised to globally regulate synaptic activity. In this study, we explored how astrocyte-expressed molecules regulate nervous system physiology in response to neuronal hyperactivation using genetic and pharmacological models of seizure in *Drosophila*. We found that astrocyte depletion of FA molecules strongly suppressed seizure-like behavior at both larval and adult stages. Astrocyte depletion of Mmp1, a matrix metalloproteinase known to regulate β-integrin activation, also suppressed seizure activity, while overexpression of Mmp1 had the opposite effect and enhanced seizures. Blockade of FA signaling under normal physiological conditions led to decreased coverage of the synaptic neuropil by astrocyte processes and reduced expression of EAAT1. However, upon induction of neuronal hyperactivity, depletion of FAs led to enhanced coverage of the synaptic field by astrocyte processes and a twofold increase in EAAT1. Given that the rescuing effect of FA depletion after seizure was suppressed by pharmacological blockade of EAAT1, we propose that the elevated levels of EAAT1 in FA knockdown animals, together with enhanced astrocyte coverage of the neuropil, are responsible for the decreased recovery times that we observe in seizure assays.

A Comprehensive Screen for Astrocyte-Expressed Genes That Modify Seizure Activity in Vivo. We identified astrocyte-expressed genes that regulate CNS physiology in the context of hyperexcitability by assaying for modification of seizure-like behavior using the well-characterized bang-sensitive mutants (32). The collection of RNAi lines that we used targeted the vast majority of transmembrane, secreted, and signaling molecules encoded in the *Drosophila* genome. While we focused our analysis in this study on FA molecules, a few additional classes of molecules were found repeatedly in our collection of enhancers or suppressors (*SI Appendix*, Fig. S1 and Tables S1 and S2). For example, ∼40% of the suppressors of seizure activity were transporters, while ∼25% were transmembrane receptors. Similarly, ∼30% of the enhancers fell into the transporter class, and ∼30% were transmembrane receptors (*SI Appendix*, Fig. S1). In many species, astrocytes have been found to express a wide array of transporters (26, 27, 47–52), but for the most part the in vivo activity and role of these molecules has not been explored in detail, with the exception of EAATs and GATs. Transmembrane receptors, particularly those that bind neurotransmitters, have been the focus of a number of recent studies exploring how astrocytes directly respond to neurotransmitters (12). Our work suggests that a deeper analysis of this class of molecules could shed important light on how astrocytes modulate CNS excitability and signaling.

Focal Adhesion Molecules Regulate Astrocyte Coverage of the Synaptic Neuropil, EAAT Expression, and Seizure Behavior. We provide multiple lines of evidence that molecules associated with FAs function in astrocytes to regulate seizure-like behavior in vivo. Astrocyte-specific depletion of FA-signaling molecules led to significant suppression of seizure-related behavior in the bang-sensitive seizure model in adults and picrotoxin-induced hyperexcitability in larvae. Precisely how FA-signaling molecules modulate seizure-related behavior, astrocyte process extension, and EAAT1 expression remains unclear. Whether they indeed form FAs in vivo to execute these functions remains an open question. Our analysis of astrocyte markers and morphology revealed that blockade of FA molecules under normal physiological conditions led to reduced coverage of the synaptic neuropil by astrocyte fine processes and to reduced levels of EAAT1 expression. We suspect that both increased EAAT1 and astrocyte coverage of the neuropil are important factors in enhancing recovery from seizure.

How astrocytes infiltrate synapse-rich regions of the brain and balance levels of EAATs is not well understood (53–55), and our study identifies FAs as molecules required for these processes. We suspect that an overall reduction in the adhesive properties of astrocytes could account for the phenotypes that we observe with respect to astrocyte growth. First, a reduced adhesiveness would be predicted to lead to reduced stabilization of astrocyte processes during development or growth, and thereby to an overall reduction in astocyte infiltration under basal conditions. Reciprocally, upon stimulation of hyperexcitability, if astrocyte processes begin to extend toward synapses, an overall decrease in astrocyte adhesive properties might allow for a more robust extension of astrocyte membranes into the neuropil. FA molecules also regulate levels of EAAT1 in vivo under both basal conditions and during hyperactivation of circuits. Under basal conditions blockade of FA signaling led to reduced EAAT1 levels, similar to our observations with astrocyte coverage. To our surprise, FA knockdown led to a dramatic increase in EAAT1 levels (approximately twofold) after picrotoxin exposure, which also corresponds to our observations with respect to astrocyte process growth. Astrocyte coverage and EAAT1 levels did not change in response to picrotoxin exposure alone, which might indicate that FAs normally stabilize these astrocyte phenotypes during hyperexcitability, with their knockdown allowing for more plasticity in astrocyte responses. The modulatory effect of FAs on neurotransmitter transporters is apparently specific to EAAT1, as levels of the sole *Drosophila* GABA transporter GAT appeared unchanged in FA knockdown animals. A recent mammalian study also identified an important role for astrocyte β1-integrin in the regulation of circuit excitability and astrocyte function (53, 56). Conditional elimination of β1-integrin from astrocytes using *GFAP-Cre* induced astrogliosis (56) and ultimately spontaneous seizure (53). We see no evidence that β-integrin depletion leads to the activation of glial reactive responses in *Drosophila* astrocytes. Nevertheless, these studies, combined with our observations, identify an important in vivo role for β-integrin signaling in the context of seizure activity and nervous system hyperexcitability.

It is possible that activation of EAAT1 expression and the genes required for astrocyte growth are downstream of the FA molecule signaling via FAK, which is a well-known regulator of transcriptional activity (57). We suspect that the simultaneous increase in EAAT1 and astrocytic coverage of synapses underlie the enhanced recovery from seizure-related behavior. Indeed, we found that overexpression of EAAT1 alone did not block the ability of picrotoxin to induce seizure-related behavior (*SI*

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Appendix, Fig. S6*D*). EAAT1 function is certainly important for modulating seizure activity, given that blockade of EAAT1 with the inhibitor DHK suppressed the rescuing effects of astrocytic FA knockdown on seizure-related behavior. In mammals, alteration of GLT-1 alone was sufficient to promote changes in long-term synaptic plasticity, but not basal synaptic activity (22). Interestingly, under a condition where there was a simultaneous increase in EAAT1 and astrocyte synaptic coverage, significant changes in basal synaptic activity were observed (55). Moreover, astrocyte coverage was modulated by changes in cell adhesion, which are required for astrocyte-driven in basal synaptic activity (55). We propose that the combined effect of increased EAAT1 with closer association with synapses leads to the suppression of seizure that we observed, and that this is likely due to enhanced clearance of extracellular glutamate. Whether suppression of FA signaling in the context of a seizure represents a viable therapeutic approach is an interesting question for the future.

Materials and Methods

Detailed experimental procedures, fly strains, and antibodies used in this study are provided in *SI Appendix*.

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