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Astrocytic regulation of seizure-like behavior

A Dissertation Presented

By

Sukhee Cho

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Science, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(DEC 14, 2017)

Astrocytic regulation of seizure-like behavior

A Dissertation Presented

By

Sukhee Cho

This work was undertaken in the Graduate School of Biomedical Sciences

(Program in Neuroscience)

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Abstract

Astrocytes are emerging as important regulators of neural circuit function and behavior in the healthy and diseased nervous system. In a screen for astrocyte molecules that modulate neuronal hyperexcitability we identified multiple components of focal adhesion complexes (FAs) as potent suppressors of genetically- or pharmacologically-induced seizure-like activity. Depletion of astrocytic Tensin, β-integrin, Talin, Focal adhesion kinase (FAK), or matrix metalloproteinase 1 (Mmp1), which degrades extracellular matrix to activate β-integrin receptors, resulted in enhanced recovery from, or resistance to seizure activity. Reciprocally, promoting FA signaling by overexpression of Mmp1 in astrocytes led to enhanced-seizure severity. Blockade of FA signaling in astrocytes led to reduced-astrocytic coverage of the synaptic neuropil and reduced expression of the excitatory amino acid transporter EAAT1. However, upon seizure induction, depletion of FA signaling components resulted in enhanced astrocyte coverage of the synaptic neuropil and a ~2-fold increase in EAAT1 levels compared to controls. Our data indicate that FAs promote astrocyte coverage in neuropil and EAAT1 expression under normal physiological conditions, but in the context of hyperexcitability, FAs negatively regulate the extent of astrocytic processes within neuropil and EAAT1 expression, thereby inhibiting a more rapid recovery from conditions of excessive neuronal activity.

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Sukhee Cho, Allie M Muthukumar, Tobias Stork, and Marc R Freeman. Focal adhesions regulate astrocyte morphology and glutamate transporters to suppress seizure-like behavior

List of Abbreviations

alrm	astrocytic leucine-rich repeat molecule
CNS	central nervous system
EAAT	excitatory amino acid transporters
ECM	extracellular matrix
eas	easily shocked
FA	Focal adhesion
GABA	γ-aminobutyric acid
GAT	GABA transporter
LTP	long-term potentiation
MMP	matrix metalloproteinase
PG	perineural glia
PTX	picrotoxin
RGCs	retinal ganglion cells
SPG	subperineural glia
TSP	Thrombospondin
VNC	ventral nerve cord

Preface

All work described in this thesis was performed at University of Massachusetts Medical School in the lab of Marc Freeman. The screen described in Chapter 2 was performed by Allie Muthukumar and Sukhee Cho. Allie Muthukumar contributed to the part of Figure 3.1 A, 3.2 C, 3.3 B, 3.5

Chapter 1: Introduction

The nervous system requires intricate connections between cells and the constant communication between them governs network activity and function of the brain. Neurons are one of the main residents in the nervous system and they are connected to one other through synapses. Transmitting electrical signals is a unique property of neurons and understanding how signals from different neurons work together to generate circuit output has been a great interest in our efforts to understand the cellular and molecular basis of information processing in the brain.

The two main types of neurons are excitatory and inhibitory, whose firing rates help establish the excitatory and inhibitory (E/I) balance in the brain. The maintenance of a balanced (E/I) state in neuronal networks throughout the lifetime of an organism is essential: deregulation of the E/I balance is associated with a variety of disorders including epilepsy, autism, schizophrenia, and Alzheimer's disease (Belforte et al., 2009; Heinemann, 2004; Rubenstein, 2010; Yizhar et al., 2011).

However, understanding neuronal contributions to E/I balance is only part of the puzzle. Glia, another resident brain cell type, adds a new dimension to the regulation of brain physiology, including E/I balance. Glial cells, which take up more than half of the volume of the human brain (Eroglu and Barres, 2010), used to be thought of as "Nervenkitt" (the German word for nerve glue) (Jäkel and

Dimou, 2017). Unlike the name suggests, they are now known to be indispensible for the structural and functional development as well as maintenance of a healthy state of the nervous system (Freeman et al., 2003; Zuchero and Barres, 2015). An explosion of research on glial function in the nervous system over the past decade has greatly enlightened us about the importance of glial cell functions in the orchestration of proper neuronal network activity.

There are different primary subtypes of glia in the mammalian brain, and each has their own unique properties. Oligodendrocytes and Schwann cells are myelinating glia that electrically insulate axons to control conduction velocity and provide trophic support for the axons. Thus they regulate how fast and/or efficient neuronal signal transmission occurs over long distances in the nervous system. Microglia, the resident immune cells in the CNS function as regulators of activity-dependent pruning of neuronal synapses on top of their traditional roles regarding immune responses during inflammation and diseases (Schafer and Stevens, 2015; Stevens et al., 2007). Finally, astrocytes, also known as a third component of synapses (to compose tripartite synapse), have tight coupling with neuronal synapses and they execute a wide range of functions to sculpt neuronal network activity (Chung et al., 2015; Halassa and Haydon, 2010). The precise roles that astrocytes play in regulating neuronal activity are not clear. Astrocyte membranes are filled with ion channels, receptors, and transporters for neurotransmitters, suggesting diverse mechanisms for circuit modulation, but

functions for very few of these astrocytic molecules have been defined *in vivo*. Considering this intimate structural and functional relationship between astrocytes and neuronal synapses, it is not surprising that astrocytes have a strong presence in neural circuit function and behavior. Below I provide a brief overview of the mechanisms by which astrocytes regulate neuronal activity and synaptic function, and other aspects of brain physiology.

Astrocyte regulation of neuronal metabolism, connectivity and function

The blood-brain barrier (BBB)

Exchange of ions and molecules between the brain and the blood vessels is tightly regulated by the blood-brain barrier (BBB). This physical barrier is critical for proper neuronal function allowing the brain maintain the ionic homeostasis, supply nutrients, and keep out toxins and pathogens (Daneman and Prat, 2015). Three cell types compose the BBB including endothelial cells, pericytes, and astrocytes. Astrocytes form cellular extensions, called endfeet, that make contact with and together coat almost the entire outer surface of the vasculature through the dystroglycan-dystrophin complex (Noell et al., 2011). This point of contact is thought to be the site of exchange of materials between astrocytes and the vasculature, which allows the remainder of the astrocyte tree of cellular processes to exchange materials with neurons. Studies from brain acute slices

and isolated retina provide strong evidence that astrocytes can regulate blood flow in response to neuronal activity through prostaglandins and epoxyeicosatrienoic acids, which can dilate blood vessels (Metea, 2006; Zonta et al., 2002). The obvious implication is that increased activity requires increased energy, with vasodilation increasing rates of exchange. While there is controversy over astrocyte roles in the initial formation of the BBB, it is clear that astrocytes perform critical roles in BBB functions such as regulating the blood flux and maintaining proper barrier for the homeostasis. Implications of BBB dysfunction during pathological conditions and diseases like cerebral ischemia, brain trauma, glioblastoma, stroke, multiple sclerosis, epilepsy, Alzheimer and Parkinson's diseases highlight astrocytes as a key component of this barrier to maintain a healthy and functional brain (Barreto, 2014).

Metabolic support

Astrocytes are ubiquitous in the nervous system and associate with nearly all types of neurons, at the same time they are tightly associated with the vasculature, and astrocytes form extensive gap junctions with each other, thereby allowing rapid exchange of ions and metabolic intermediates throughout the astrocyte population and, in turn, the brain. One of the first roles of astrocytes that was recognized is their capability for maintaining energy homeostasis in the brain, and this ability depends heavily on their morphology. The brain only represents 2% of the human body mass, but it utilizes about 20%

of the oxygen and glucose in our systems. Neurons are believed to receive their energy supplies through the lactate shuttle, a key proposed operational model to keep up with such a high-energy requirement in the nervous system (Magistretti, 2006). Briefly, the endfeet of astrocytes, which line cerebral microvessels, take up glucose through astrocyte-specific transporters. Glucose taken up by astrocytes is then stored in the form of glycogen via glycogenesis or shuttled back to the neurons as lactic acid after glycolysis (Tsacopoulos and Magistretti, 1996). Astrocytes aid in this diffusion of glucose due to their extensive coupling through gap junctions. Gap junctions are specialized intercellular conduits that allow the diffusion of glucose (and ions) to neighboring astrocytes. Genetic ablation studies shows connexins, gap junction proteins in astrocytes are required for activity-dependent changes in synaptic transmission and neuronal metabolism (Rouach et al., 2008). Because of the highly networked properties of astrocytes in the brain, glucose taken up by a few astrocytes can rapidly diffuse throughout the brain (Kacem et al., 1998; Signaling at the Gliovascular Interface, 2003; Theis et al., 2005), and thus allow the brain to keep up with metabolic demand in neurons.

Although glucose metabolism has been studied most extensively, it is believed that astrocyte networks function in similar ways throughout the brain to drive the central/peripheral exchange of additional bioenergetics intermediates, metabolic byproducts, and gases (O_2/CO_2). The extent of exchange remains unclear, but astrocytes membranes are equipped with a range of transporters

and channels (Minelli et al., 1996; Rothstein et al., 1994; Seifert et al., 2016; Theis et al., 2005), suggesting a broad array of substrates can be mobilized to and from neurons.

Ion and pH balance

In the nervous system, changes in pH are caused by neuronal activity and directly modulate neuronal excitability and synaptic transmission by affecting the release of neurotransmitters, conductance of voltage-gated channels and ligand-gated channels, K^+ and Ca^{2+} channels, proton gated channels as well as glutamate receptors (Obara et al., 2008). While there are multiple barrier mechanisms to keep the proper pH balance in the brain, astrocytes serve a key role, as they are equipped with several molecules by which they can regulate pH. For instance, astrocytes membranes are decorated with the Na^+/H^+ exchanger, bicarbonate transporters, monocarboxylic acid transporters, and the vacuolar-type proton ATPase, all of which are directly involved in transporting H^+ ions (Sofroniew and Vinters, 2009). The presence of these mechanisms for H+ regulation render astrocytes sensitive to pH fluctuations, and provide a means for astrocytic regulation of extracellular pH (Chesler, 2003).

Astrocyte control of synapse formation and maturation

The synapse is the basic functional unit for communication in the brain and there is significant interest in understanding how synapses form and exhibit plasticity.

Over the past decade, significant evidence has been mounting that demonstrates a central role for astrocytes in nearly every aspect of synapse formation and physiology.

Synapse formations are the initial stage of their development and there is strong evidence from various studies to show astrocytes are required for this process. Elegant studies from the Barres laboratory first revealed that astrocyte-secreted factors are potent modulators of synapse formation. Using an *in vitro* culture system Barres and colleagues attempted to identify factors in astrocyte-conditioned medium that would enhance synapse formation or efficacy, and this was extremely successful (Buard et al., 2009; Diniz et al., 2012; Hughes et al., 2010; Pfrieger and Barres, 1997; Ullian et al., 2004; Xu et al., 2009).

Thrombospondins (TSPs) are secreted by astrocytes to control synapse formation, and their expression in astrocytes is observed *in vivo* (Christopherson et al., 2005). In particular, TSP1 and TSP2 expression coincide with the timing of synaptogenesis and genetic depletion of both TSP1 and TSP2 show impairment of synapse formation in the mouse. TSPs exert their pro-synaptogenic effects through binding and activation of $\alpha 2\delta$ receptors that are expressed in neurons (Eroglu et al., 2009). Additional factors secreted from astrocytes that control synapse number are hevin and SPARC. While hevin can promote synaptogenesis, SPARC negatively regulates synapse formation by antagonizing the function of hevin (Kucukdereli et al., 2011). In addition to secreted factors , contact-dependent regulation of synapse formation by astrocytes has also been

proposed (Hama et al., 2004). This contact-mediated signaling activates neuronal integrin receptors resulting in the activation of protein kinase C signaling pathway in hippocampal neurons (Hama et al., 2004), and developing retina ganglion cells are suggested to utilize different contact-mediated signals to promote synapse formation (Barker et al., 2008). It appears that the regulation of synaptogenesis by astrocytes is conserved in invertebrates as recent genetic ablation studies in *Drosophila melanogaster* provide direct evidence of astrocytic involvement of synapse formation during the development of fly brain (Muthukumar et al., 2014).

Synapse formation occurs through the stepwise process of assembly of structural synapses (i.e. silent synapses), followed by activation of signaling properties. TSPs are sufficient to induce the formation of silent synapses, but not functionally active ones (Xu et al., 2009). In a subsequent analysis of pro-synaptogenic factors in astrocyte-conditioned medium, Allen and colleagues discovered a number of molecules capable of inducing fully mature synapses. Using a similar *in vitro* biochemical approach they identified glypicans, a family of heparin sulphate proteoglycans as necessary and sufficient for synapse maturation (Allen et al., 2012). Glypicans are tethered to the cell plasma membrane by glycosyl-phosphatidylinositol linkages and endogenous phospholipases can cleave to release the proteins (Clarke and Barres, 2013). Mouse astrocytes express glypican 4 and 6 *in vivo* and mice with glypican 4 depletion have a defects in synapse formation, and function: the amplitude of

excitatory postsynaptic currents is dramatically decreased with an impaired recruitment of AMPA receptors (Allen et al., 2012). The glypicans are well-conserved molecules throughout different species. *Drosophila melanogaster* has a homologue of glypican 4 called Dally-like which binds to the protein tyrosine phosphatase receptor leukocyte antigen-related receptor (LAR) in neurons to regulate the maturation of synapses (Johnson et al., 2006). Another study in mammalian hippocampal neurons also suggests LAR signaling is required for recruitment of AMPA receptors to synapses (Dunah et al., 2005). With these interesting insights, further studies will provide underlying mechanisms of how astrocyte glypicans act on neuronal receptors to induce synapse maturation.

Maintenance of proper synapse number requires removal of excess synaptic connections during nervous system development (Kano and Hashimoto, 2009) and the failure of synapse eliminations alters synaptic connectivity and is associated with devastating neurological disorders including autism and schizophrenia (Boksa, 2012; Zoghbi and Bear, 2012). Mounting evidence shows microglia, the resident immune cells in the brain, are major players in synapse elimination and pruning in the mouse retinogeniculate system (Schafer et al., 2012). Briefly, microglia express the complement receptor CR3, through which they recognize and phagocytose C1q-tagged weaker “loser” synapses (Schafer et al., 2012). At the same time, astrocytes also help prune synapses in the retinogeniculate system: astrocytes express MEGF10 (mouse Draper) and MERTK, and these receptors act in a partially redundant fashion to engulf

synapses destined for elimination (Chung et al., 2014). Interestingly, C1q has recently been proposed to be the ligand for MEGF10 (Iram et al., 2016), which might explain how CR3-bearing microglia and MEGF10/MERTK-bearing astrocytes recognize common synaptic targets. *Drosophila* astrocytes also efficiently phagocytose pruned synapses during neuronal remodeling (Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014), and this is where the Draper signaling cascade was first implicated in synaptic pruning (Awasaki et al., 2006). Interestingly, Mcr, the sole compmlement-like molecule in *Drosophila* appears to act genetically upstream of and potentially as a ligand for Draper in the activation of autophagy (Lin et al., 2017), and therefore Mcr/C1q tagging of engulfment targets for internalization by Draper/MEGF10 may be an additional conserved feature in phagocytic functions in the brain.

Astrocyte regulation of neuronal activity

Synaptic signaling to astrocytes

Do astrocytes receive signaling information from synapses? This is a central question in the field but has remained quite controversial. Astrocytes express numerous receptors for neurotransmitters and stimulation of astrocytes with neurotransmitters trigger increases in intracellular second messengers such as calcium and cyclic AMP (Fiacco and McCarthy, 2006). Calcium transients in astrocytes in response to neuronal activity were initially thought to be too slow to be regulating fast synaptic transmission and it seemed that only sustained and

intense neuronal activity could increase astrocyte calcium signaling (Perea, 2005; Wang et al., 2006). This slower response appears to act on a time scale more consistent with neuromodulation (seconds to minutes), and indeed recent studies have shown that astrocytic calcium signaling in *Drosophila* and mouse can be activated by octopamine/tyramine and norepinephrine, respectively (Ding et al., 2013; Duffy and MacVicar, 1995; Ma et al., 2016; Paukert et al., 2014; Salm and McCarthy, 1990). In the *Drosophila* CNS, astrocytes have been shown to express the receptor for Oct/Tyr-R, activate calcium signaling in response to Oct/Tyr application, and be required for execution of neuromodulation in a dopaminergic circuit at both the electrophysiological and behavioral levels (Ma et al., 2016).

The role of faster time course microdomain calcium imaging events are less clear, although they are mediated by calcium release from mitochondria in some way (Agarwal et al., 2017). Some evidence exists for their regulation by activity: two-photon imaging allowed for observation of astrocyte calcium transients that occur on a similar timescale to synaptic activity upon single-synapse stimulation. This type of astrocyte activation involves metabotropic glutamate subtype 5 receptors (Clarke and Barres, 2013; Panatier et al., 2011), but its physiological relevance remains unclear. Finally, astrocytes have also been reported to detect the inhibitory neurotransmitter γ-aminobutyric acid (GABA) via metabotropic GABA receptor ($GABA_BR1/2$), with signaling through

this pathway modulate GABA transporter (GAT) expression and therefore GABA uptake rates in neural circuits (Muthukumar et al., 2014).

Gliotransmitters

One of the qualities that enable astrocytes to be active participants in the neural circuit behavior is their ability to release chemical transmitters called gliotransmitters. Neurotransmitters released from presynaptic terminals can activate astrocytes to release various gliotransmitters including glutamate, D-serine, ATP, and neuropeptide Y (Henneberger et al., 2010; Jourdain et al., 2007; Mothet et al., 2005; Parpura et al., 1994; Pascual et al., 2005b; Schwarz et al., 2017), although *in vivo* evidence for release of any of these compounds is scarce. Several studies suggest astrocytes release glutamate in a Ca^{2+}-dependent manner that is mediated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins including vesicle-associated membrane proteins-2 (VAMP2), syntaxin-1, synaptosome-associated protein-23, and synaptobrevin II (Harada et al., 2016; Montana et al., 2006; Schwarz et al., 2017). While other release mechanisms, including reverse operation of plasma membrane glutamate transporters, cell swelling-induced anion transporter opening, release via P2X7 receptors, and gap junction-mediated extrusion have been reported (Harada et al., 2016). D-serine in astrocytes has been proposed to be stored in the synaptic-like vesicles and released also in a Ca^{2+}-dependent manner (Martineau et al., 2013). Perhaps the best characterized potential gliotransmitter is ATP. While the ATP release mechanisms still remain

controversial, several studies suggested a gap junction channel, connexin 43 (Torres et al., 2012) and secretory lysosomes (Verderio et al., 2012) as potential release mechanisms for ATP in astrocytes.

Neurotransmitter clearance

The major excitatory and inhibitory neurotransmitters in the mammalian brain are glutamate and GABA, respectively. Their concentrations at synapses determine the extent of postsynaptic receptor activation. Maintaining an appropriate extracellular concentration of these neurotransmitters is critical to maintain a high signal to noise ratio in synaptic and extrasynaptic modes of neurotransmission (Danbolt, 2001; Danbolt, 2013). Cellular uptake is the only known mechanism by which cells can maintain Glu or GABA concentrations in the extracellular space, and there is thought to be no extracellular breakdown of either GABA or glutamate in the CNS. Astrocytes express transporters for both Glu and GABA, and are the primary cell type in the brain that clears them from the extracellular space (Iversen and Johnston, 1971; Iversen and Kelly, 1975; Logan and Snyder, 1971).

Glutamate uptake by glutamate transporters (Excitatory amino acid transporters, EAATs) is driven by the ion gradients of K^+ and Na^+ and the uptake of GABA by its transporters (GATs) is driven by the gradients of Na^+ and Cl^-. There are five high-affinity glutamate transporters in mammals, EAAT1 through 5 and they belong to the *slc1*-family. EAAT2 also known as GLT-1 was the first

one to be purified and characterized and it immediately became clear that they are highly expressed in astrocytes (Chaudhry et al., 1995; Danbolt et al., 2002). Both transporter activity blockade and knockout studies show that EAAT2 is responsible for 95 % of the total glutamate uptake activity (Danbolt et al., 1992; Tanaka et al., 1997). EAAT2 knockout animals further implicated EAAT2 in preventing excitotoxicity, without its action, increased glutamate levels induce epilepsy and cell death (Tanaka et al., 1997). Furthermore, depletion of EAAT1 and EAAT2 in mice perturbed overall brain development including stem cell proliferation, radial migration of neurons, neuronal differentiation, and neuronal survival, presumably because of the buildup in extracellular glutamate (Matsugami et al., 2006). These studies clearly suggest critical roles of astrocyte glutamate transporters in normal network activity, which directly correlates with brain development and health.

The first identified neurotransmitter transporter was the GABA transporter in mammals: GAT1, (slc6a1), and this was followed by the identification of another three members of the GABA transporters family (GAT2, GAT3, and BGT1) (Danbolt, 2013; Liu et al., 1993; López-Corcuera et al., 1992). GAT3 is selectively expressed in astrocytes throughout the brain while GAT1 expression is both astrocytic and neuronal (Conti, 2014; Minelli et al., 1996; Ribak et al., 1996; Yadav et al., 2015). GAT1 deletion leads to reduced aggression, hypoalgesia, reduced anxiety, and depression-like behavior possibly because of increased extracellular GABA levels and enhanced inhibitory signaling (Danbolt,

2013). While remaining astrocytic expression of GAT3 might complicate the interpretation of the above studies, recent studies in *Drosophila melanogaster* highlight critical roles of astrocytic GABA transporters in the function of the nervous system *in vivo*. Gat encodes the sole slc6-family GABA transporter family member in *Drosophila* (Neckameyer and Cooper, 1998; Thimgan et al., 2006) and its expression is exclusively astrocytic throughout development (Stork et al., 2014). Flies with Gat deletion are embryonic lethal, but re-expression of Gat in astrocytes rescues these animals to adulthood. Further support for the notion that balancing levels of GAT are critical for neurophysiology come from studies of GAT expression during development. During *Drosophila* metamorphosis Gat expression in astrocytes is activated through $GABA_BR1/2$ signaling pathway. Decreases in $GABA_BR1/2$ signaling led to reduced astrocytic Gat expression resulting in suppression of seizure-like behavior. These observations imply that GABA transporters play a central role in regulation of excitatory/inhibitory balance in the fly nervous system (Muthukumar et al., 2014).

Astrocyte morphology

Astrocyte' fine processes are intimately associated with synapses. Ultrastructural studies in mammalian hippocampus showed about 60 % of synapses are in contact with astrocyte processes (Ventura and Harris, 1999). Just like neuronal synapses, astrocyte processes undergo rapid morphological remodeling. One of the striking observations supporting important roles for dynamic changes in

astrocyte processes comes from the studies of the hypothalamic supraoptic nucleus (SON)(Oliet et al., 2001). In this study, researchers take advantage of the massive neurological remodeling that occurs in this system in response to specific physiological stimulations like lactation and dehydration. Under these conditions, most of the astrocyte processes are moved away from synapses allowing enhanced synaptic communications. Astrocytes resume their close association with synapses once the stimulation has been removed (Montagnese et al., 1990; Theodosis, 2002). This structural plasticity in astrocytes upon synaptic remodeling changes the proximity between astrocytes and neighboring synapses to bring alterations in circuit behavior (Benediktsson et al., 2005; Haber, 2006; Hirrlinger et al., 2004). Furthermore the correlation between dendritic spine stability and astrocyte contacts suggests important roles in astrocyte process motility and synapse maintenance (Haber, 2006).
Regulation of astrocytes' fine processes in association with synapse proximity also has a great impact on buffering of the glutamate released at the synapse. Astrocyte glutamate transporters expressed in their fine processes are the major mechanism for clearance of glutamate during synaptic transmission (Danbolt, 2001). Therefore, changes in the proximity to the synapses from astrocyte processes likely influence the concentration of glutamate, which can directly modify synaptic transmission. In fact, reduced astrocyte contact with dendritic spines was observed during spine head remodeling and pharmacological blockade of astrocyte glutamate transporters prevented spine remodeling in

mouse hippocampal slices (Verbich et al., 2012). Despite the importance of astrocyte association with synapses for CNS function, we know almost nothing about how astrocytes regulate morphological changes in response to neural activity or changes in circuit needs.

Astrocytes and epilepsy

Brain network activity maintains the balance between excitation and inhibition to precisely encode and deliver information. Epilepsy is a prevalent neurological disorder that is characterized by the unpredictable and spontaneous seizures (Thurman et al., 2011). Seizures are caused by an imbalance between excitatory and inhibitory synaptic activity resulting in neuronal network hyperexcitability (Jefferys, 1990; Pinto, 2005; Robel et al., 2015). Therapeutic effort has been made to control epileptiform activity by directly targeting neuronal activity using general CNS depressants (Binder and Steinhäuser, 2017). However one third of patients become medically refractory and often their cognition is affected by current therapeutic approaches that are neuron centric (Binder and Steinhäuser, 2017; Wahab et al., 2010). Therefore, there is a need for the development of more specific therapeutics, which requires better understanding of the cellular and molecular mechanisms underlying epileptic seizures.

Alterations in astrocytic structure and function in epilepsy have been well characterized (Bedner et al., 2015; Bordey and Sontheimer, 1998; de Lanerolle

et al., 2010; Robel et al., 2015; Ye et al., 1999). These alterations include distinct changes in astrocytic morphology, expression, subcellular localization and function of K^+ and water channels, glutamate transporters and glutamine synthetase (GS) in the epileptic brain (Seifert and Steinhäuser, 2013). Accordingly, astrocytes need to be included in the equation to understand neuronal hyperexcitability in seizure.

Maintenance of proper extracellular [K^+] is crucial to shape neuronal activity and maintain network balance and it heavily depends on astrocytic K^+ and water channels (Kofuji and Newman, 2004). Studies in epileptic human hippocampus suggest that altered expression in astrocytic Kir channels resulted in the impaired K+ buffering and enhanced seizure susceptibility (Hinterkeuser et al., 2000; Kivi et al., 2000). These Kir channels show spatial overlap with the water channel, aquaporin 4 (AQP4) in astrocytes (Nielsen et al., 1997) and AQP4 knockout mice are impaired in K^+ buffering and prolong seizure duration (Binder et al., 2006). These findings indicate that impaired water channels together with decreased expression of Kir channels in astrocytes are responsible for loss of K^+ buffering and increase in seizure susceptibility.

Another key mechanism to balance network activity is proper clearance of neurotransmitters at the synaptic cleft. In particular removing excess glutamate by glutamate transporters in astrocytes is a fundamental mechanism to terminate excitatory synaptic signal and keep the balance between excitation and inhibition (Seifert and Steinhäuser, 2013). Impaired activity of the astrocytic transporters,

EAAT1 and EAAT2 and a consequent increase in extracellular glutamate are common features of epilepsy (Glass and Dragunow, 1995; Seifert et al., 2006). Studies using inhibitors of glutamate transporters suggested that astrocytic control of ambient glutamate is critical for the genesis and maintenance of seizure activity (Nyitrai et al., 2010; Seifert and Steinhäuser, 2013). In addition to glutamate transporters, glutamine synthetase (GS) in astrocytes plays an important role in glutamate clearance. This astrocyte specific enzyme converts glutamate into glutamine that is glutamate receptor-inactive. Its alteration that is accompanied by increased extracellular glutamate levels is observed in epilepsy (Eid et al., 2008; Hammer et al., 2008; Seifert and Steinhäuser, 2013). Glutamate to glutamine conversion by GS is also essential for GABA synthesis and its synaptic release to regulate inhibitory synaptic activity (Liang et al., 2006). Decreased inhibitory tone by impaired GS expression resulted in hyperactivity and seizure (Ortinski et al., 2010).

Mounting evidence demonstrates altered astrocyte function in human and rodent models of epilepsy. However, a lack of available tools in mammalian systems for precisely manipulating astrocytic function makes it challenging to answer critical questions including whether these alterations are causative or not and mechanisms underlying these alterations.

Drosophila as a model system to study astrocytes

Astrocytes contribute to virtually every aspect of nervous system development and function. Despite this notion, our understanding of how and why astrocytes do what they do is nowhere near complete. To accelerate our understanding of astrocytes the field needs to embrace model organisms like *Drosophila*. Over the last two decades it has become clear that astrocyte biology changes dramatically when these cells are removed from their *in vivo* context (Balasubramanian et al., 2016; Foo et al., 2011; Götz et al., 2015). Studying these cells in intact circuits *in vivo* will therefore be essential if we wish to unravel the nature of astrocyte-neuron and astrocyte-vasculature interactions.

Advantages of *Drosophila* as a model organism

Foundational reasons to use *Drosophila* to study the biology of astrocytes in the context of circuit behavior include full access to their annotated genome sequence and substantial gene conservation with humans (Adams et al., 2000). In addition, it has been estimated that about 75% of human disease related genes have conserved orthologs in flies (Lloyd and Taylor, 2010; Reiter et al., 2001). A comparison of conserved functional domains between a fly and a human in a given gene suggests that the homology is in fact even higher, approaching 80 to 90 % (Pandey and Nichols, 2011). A simplified genome compared to humans also gives less redundancy, reducing complications in data interpretation during genetic analysis (Adams et al., 2000).

Over 100 years of history in using *Drosophila melanogaster* in modern biological sciences have accumulated resources for easy genetic manipulations. By far, the most appreciated milestone for genetics is the introduction of the GAL4/UAS system by Brand and Perrimon (1993). It utilizes the yeast transcriptional activator GAL4 that is flanked by a defined promoter to activate a transgene of interest under the control of the upstream activation sequence (UAS), the target of GAL4. This cell type–specific molecular genetics, along with the development of reliable GAL4 driver lines for subsets of glia (Stork et al., 2011) allows for a new level of examination in astrocytic contribution to brain function *in vivo*. A further expansion has been made in terms of reverse genetic analysis since the development of transgenic fly lines carrying a UAS-regulated transgene expressing RNAi for almost every single gene in *Drosophila* genome (Dietzl et al., 2007; Ojelade et al., 2014). These resources not only allow for rapid testing of any genes involved in a given biological process but also give a chance to perform a large-scale screen, which can be a powerful approach to find novel genes mediating a pathway of interest.

The rapid life cycle of flies cannot be overlooked. A single reproductive pair can generate hundreds of eggs and it takes 10 to 12 days for these offspring become reproductive adults. Compared to rodent models that only produce a handful of offspring every 3 to 4 months, this is a great advantage to facilitate a study (Pandey and Nichols, 2011). Defined developmental stages of *Drosophila* include the embryo, the larva, the pupa, and the adult and each stage provides

unique experimental advantages. The embryo has been a great platform to study cell fate determination, cellular development, and organogenesis. The larva, the wandering third instar larva in particular has been popular to study developmental and physiological processes. Furthermore easy tissue extraction and simple behavioral patterns will invite even more studies of circuit level questions. Researchers have already recognized the great advantage of using the pupa to investigate glial roles in neural circuit remodeling (Muthukumar et al., 2014; Tasdemir-Yilmaz and Freeman, 2014). Finally the discreet circuits in adult flies mediate complex behaviors including circadian rhythms, learning and memory, courtship, feeding, aggression, grooming, and flight navigation and they are suggested to be a great potential platform for drug screening (Pandey and Nichols, 2011).

Drosophila CNS glia

The *Drosophila* nervous system is relatively simple, but houses excitatory and inhibitory neurons that are remarkably similar to their mammalian counterparts in terms of their development, mechanisms of synaptic release, and major neurotransmitter systems used (Freeman, 2015). The glia to neuron ratio in mouse is ~55% glia to ~45% neurons (averaged across the entire brain), while in *Drosophila* glia make up only ~10-15% of all the cells in the nervous system (Awasaki et al., 2008; Ito et al., 1995; Pfrieger and Barres, 1995). The *Drosophila* CNS can be divided into two parts: the neuronal cell cortex where all

the cell bodies sit, and the neuropil where axons and dendrites form synapses (Freeman, 2015). Glial cells in *Drosophila* are characterized according to their position, morphology and association with neurons. The outermost layer of the CNS is surrounded by perineural glia (PG) that function as a chemical and physical barrier for the CNS (Carlson et al., 2000). Beneath the PG is the layer of subperineural glia (SPG), which form pleated septate junctions with one another to establish and maintain the blood-brain barrier (BBB) (Freeman, 2015; Stork et al., 2011). Within the domain of the BBB there are three major glial cell types that form intimate associations with neurons and mediate neural circuit assembly, function, plasticity, and behavior. Cortex glia infiltrate the cortex region to surround all neuronal cell bodies and the proximal regions of neuronal processes (Coutinho-Budd et al., 2017; Ito et al., 1995). Ensheathing glia reside right along the boundary between the cortex and neuropil to help separate these discrete anatomical compartments (Dumstrei et al., 2003). One of the most recognized functions of ensheathing glia is the injury induced response they exhibit after axotomy, which is mediated the Draper/Src-family kinase signaling cascade (Awasaki et al., 2006; Doherty et al., 2009; MacDonald et al., 2006; Ziegenfuss et al., 2012). Together with cortex glia, ensheathing glia are also critical for CNS development, in particular for proper positioning and survival of neuronal cell bodies and formation of major axon tracts (Dumstrei et al., 2003; Spindler et al., 2009).

Astrocytes are found within the synaptic neuropil and have direct association with neuronal processes and synapses. Their tufted morphology and tiling behavior is highly reminiscent of mammalian protoplasmic astrocytes (Awasaki et al., 2008; Doherty et al., 2009; Stork et al., 2014; Tasdemir-Yilmaz and Freeman, 2014), and they serve very similar functional roles. During metamorphosis, larval neural circuits undergo major remodeling. After deconstruction and clearance of the circuits, adult CNS starts to be formed by massive synaptogenesis which is coordinated by astrocytes (Freeman, 2015; Muthukumar et al., 2014). These results strongly suggest that just like their mammalian counterparts, *Drosophila* astrocytes play a crucial role in synapse formation during CNS development. During this dramatic remodeling in the larval CNS, astrocytes become highly phagocytic by increasing the expression of engulfment receptor, Draper and engulf pruned axons, dendrites, and synapses (Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014). Interestingly, pruning in the mammalian retinogeniculate system is activated during remodeling by astrocyte secretion of TGF-β (Bialas and Stevens, 2013), and astrocyte secretion of TGF-β (Myoglianin, Myo) is necessary and sufficient to initiate neuronal pruning in *Drosophila* mushroom body (Awasaki et al., 2011).

Drosophila astrocytes also play important roles in neurotransmitter clearance at synaptic cleft to modulate network activity and behavior. They express transporters to take up glutamate (EAAT1), GABA (Gat), and enzymes (glutamine synthetase and GABA transaminase) to metabolize them once they

are taken up (Freeman et al., 2003; Rival et al., 2004; Stacey et al., 2010; Stork et al., 2014). Genetic depletion of EAAT1 in glia leads to impaired synaptic transmission and locomotion in larvae (Stacey et al., 2010) and excitotoxicity-induced neurodegeneration in adult flies (Rival et al., 2004). Knockdown of Gat expression in astrocytes causes severe locomotion defects in larvae and adults and complete removal of Gat leads to animal lethality (Stork et al., 2014). These data suggest neurotransmitter transporters in *Drosophila*, like mammals, play critical roles in E/I balance, but mechanisms regulating their expression and function remain poorly defined.

The field has achieved remarkable progress in identifying astrocytes as dynamic signaling elements that have impact on neuronal circuit formation and maintenance. However, the detailed cellular and molecular mechanisms of such regulations during different conditions like physiological or pathological conditions are still elusive. In this thesis, through *in vivo* behavioral screen, we identified astrocytic genes governing astrocyte functions in regulating neuronal network activity.

Chapter 2: *In vivo* RNAi screen for astrocytic factors to regulate neuronal activity using a genetic model of seizure

Introduction

Astrocytes are now a well-recognized cell type in the nervous system for their critical roles in neuronal network homeostasis. Astrocytes give metabolic support to neurons, buffer ions and pH, and removal of neurotransmitters.(Kimelberg and Nedergaard, 2010). Their tufted morphology has been determined to be important for many of their functions. Especially, the distal fine processes of astrocytes compose 80% of their membrane and are filled with ion channels, neurotransmitter receptors, and transporters. They infiltrate into the neuropil, where synapses are and communicate with neurons and modulate synaptic activity (Chung et al., 2015; Halassa and Haydon, 2010; Stork et al., 2014). Astrocytes alter their structural organization and functions in response to neuronal activity to accommodate and participate in a changing network environment (Theodosis et al., 2008). However, the precise mechanisms through which astrocytes change their morphological and functional features and the molecules required in astrocyte-neuron crosstalk have only started to be discovered. *In vivo* observation in particular, remains to be a major challenge in decoding the roles of astrocytes in brain function (Haydon and Nedergaard, 2014).

Drosophila is a great model system to overcome the limitations and challenges of studying astrocyte function *in vivo* and accelerate the growth of the field. The *Drosophila* nervous system is relatively simple compared to mammalian counterparts yet they share a number of glial characteristics

including molecular basis, functions, and morphology (Doherty et al., 2009; Freeman et al., 2003; Murai and van Meyel, 2007; Muthukumar et al., 2014; Stork et al., 2014). A phalanx of genetic tools available in the fly will allow us to specifically label and manipulate astrocytes *in vivo* and decipher their roles within the neuronal network in an intact brain.

To first identify astrocyte genes required for modulating neuronal activity using *Drosophila*, we performed an *in vivo* candidate screen in adult flies with RNA interference (RNAi). Considering that astrocyte functions heavily rely on their transmembrane proteins including channels, receptors, and transporters (Araque et al., 1999), we selected about 2,000 *Drosophila* transgenic UAS-RNAi lines from Vienna Drosophila Resource Center (VDRC) targeting mostly genes coding for secreted, transmembrane or membrane associated proteins (Dietzl et al., 2007). Then, to achieve astrocyte-specific RNAi expression, we used an astrocyte-specific Gal4 line, *alrm-Gal4* (Doherty et al., 2009), to drive UAS-RNAi from the chosen library.

As a tool to manipulate neuronal activity *in vivo,* we took advantage of a sensitized genetic background in which neuronal hyperexcitability-mediated seizure can be induced by simple mechanical shock (Pavlidis et al., 1994). A bang-sensitive mutation, *easily-shocked* (eas^{PC80}) is a well-established model to study neuronal hyperexcitability and seizure in the *Drosophila* nervous system (Parker et al., 2011; Pavlidis et al., 1994). The loss-of-function of ethanolamine

kinase disrupts the metabolism of phosphatidylethanolamine which is one of the prevalent membrane lipids (Lim et al., 2011; Pavlidis et al., 1994).
These mutant flies display seizure-like behaviors characterized by initial seizure, temporary paralysis, and recovery seizure when electrical or mechanical shock, “bang” is introduced (Benzer, 1971; Ganetzky and Wu, 1982) and this *Drosophila* model of seizure has a significant similarities with human seizures (Parker et al., 2011). Electrophysiological recordings on the mutant flies show 5 times lower in their seizure threshold compared to wild-type flies suggesting hyperexcitability in their neuronal network (Kuebler and Tanouye, 2000). In addition, this fly strain carrying a mutation in ethanolamine kinase suffers from hyperexcitability and paralysis resulting in loss of postural control, being unable to right themselves when exposed to a brief mechanical shock (e.g. 10 sec of vigorous vortexing). Following the mechanical bang, mutant flies require recovery time around 110 seconds while control flies need no recovery time to right themselves up and resume their normal activities like grooming and climbing on the culture vials (Parker et al., 2011; Pavlidis et al., 1994).

Ethanolamine kinase is suggested to play an important role in nervous system development since *eas*^{PC80} impairs proper development of mushroom body α/β lobes (Pascual et al., 2005a). However, later studies elaborated that these developmental changes were not indicative of seizure sensitivity but increased ratio of excitatory and inhibitory activity was suggested as a seizure inducing mechanism (Kroll and Tanouye, 2013a). Thus, assaying for this distinct

behavioral phenotype in *eas*^{PC80} can provide a robust platform to define genes that modify seizure sensitivity when they are depleted in astrocytes.

We identified 100 RNAi lines that suppress hyperexcitability induced seizure-like behavior when they are expressed in astrocytes and 87 lines enhanced seizures in the *eas*^{PC80} genetic background.

Results

An *in vivo* RNAi screen identifies potential astrocyte-expressed regulators of seizure-like behavior

To identify novel astrocyte genes capable of modulating neural circuit activity, we performed an *in vivo* RNAi screen in adult *Drosophila* to identify astrocyte genes that enhance or suppress seizure when they are knocked down. We took advantage of the *easily shocked* (*eas*^{PC80}) mutant, a well-studied seizure model that belongs to the bang-sensitive (BS) paralytic class. It provides a sensitized genetic background in which neuronal hyperexcitability and ultimately seizure activity can be induced by simple application of mechanical force (Pavlidis et al., 1994) providing a convenient platform for the screen. Out of three BS mutants that have been the most utilized as experimental representatives (The *Slamdance*, the *easily shocked*, and tha *bang senseless*[1]), *easily shocked* mutant shows moderate seizure sensitivity which allows us to define both suppressors and enhancers for seizure sensitivity during the screen. Our target collection of RNAi lines consisted of ~2,000 constructs that targeted genes

encoding the majority of secreted, transmembrane, or membrane-associated molecules in the fly genome (Dietzl et al., 2007). Astrocyte-specific knockdown of each gene was accomplished using the alrmGal4 driver (Doherty et al., 2009)(Fig2 b). Application of mechanical shock (i.e. vortexing of animals in culture vials for 10 seconds) has no effect on control animals, which means they immediately resume normal motor activity after the shock. In contrast, the same stimuli resulted in *eas*^{PC80} mutants experiencing broad neuronal hyperexcitability and paralysis, and they exhibit a mean recovery time of approximately 110 seconds (Pavlidis et al., 1994)(Fig 2A). We crossed individual males from *UAS-RNAi* lines to virgin *eas*^{PC80}, alrmGal4 females, and collected up to 15 male progenies that are 1 to 5 days old post eclosion in a clean vial (i.e. *eas*^{PC80}*/y; alrm-Gal4/+* and also contained a single copy of the *UAS-RNAi* construct being assayed). The next day, collected flies were transferred to a clean empty vial and recover for at least 2 hrs from any mechanical shock that may have introduced during transfer. Animals were then vortexed for 10 seconds and the percent of animals that righted themselves were scored at 1, 1.5, 2, 3, and 4 minutes (Fig. 2A). Since *eas*^{PC80} animals required about 110 sec to reach 100 % recovery from bang-induced seizure-like behavior, 4 min of total assay time allowed us collect suppressors as well as enhancers for this behavior.

We scored suppressors as *UAS-RNAi* lines that led to strong recovery within 1.5 minutes, and enhancers as *UAS-RNAi* lines that had less than 90 % recovery until after 4 min. Then, only the ones that had at least 10 flies as a total

number of animals tested made it to the final list of hits. In total, we identified 100 RNAi lines (4.6 % out of all the lines tested) that suppressed seizure activity and 87 lines (4.0 %) that enhanced seizures in the eas^{PC80} genetic background when expressed in astrocytes (Fig. 2B).

The lists of potential suppressors and enhancers (Table 1 and 2) were subjected for protein functional categorization using PANTHER classification system (Mi et al., 2013). 91 genes out of 100 scored as suppressors and 78 out of 87 genes scored as enhancers had defined molecular functions based on the database. From both suppressors and enhancers, major portions of genes were suggested to function as transporters (34.5 % of suppressors and 24.6 % of enhancers)(Fig 2C, D). Further validation is necessary to confirm the effect of RNAi perhaps using second RNAi that targets different sequences since a handful of RNAi lines are predicted to have off-targets.

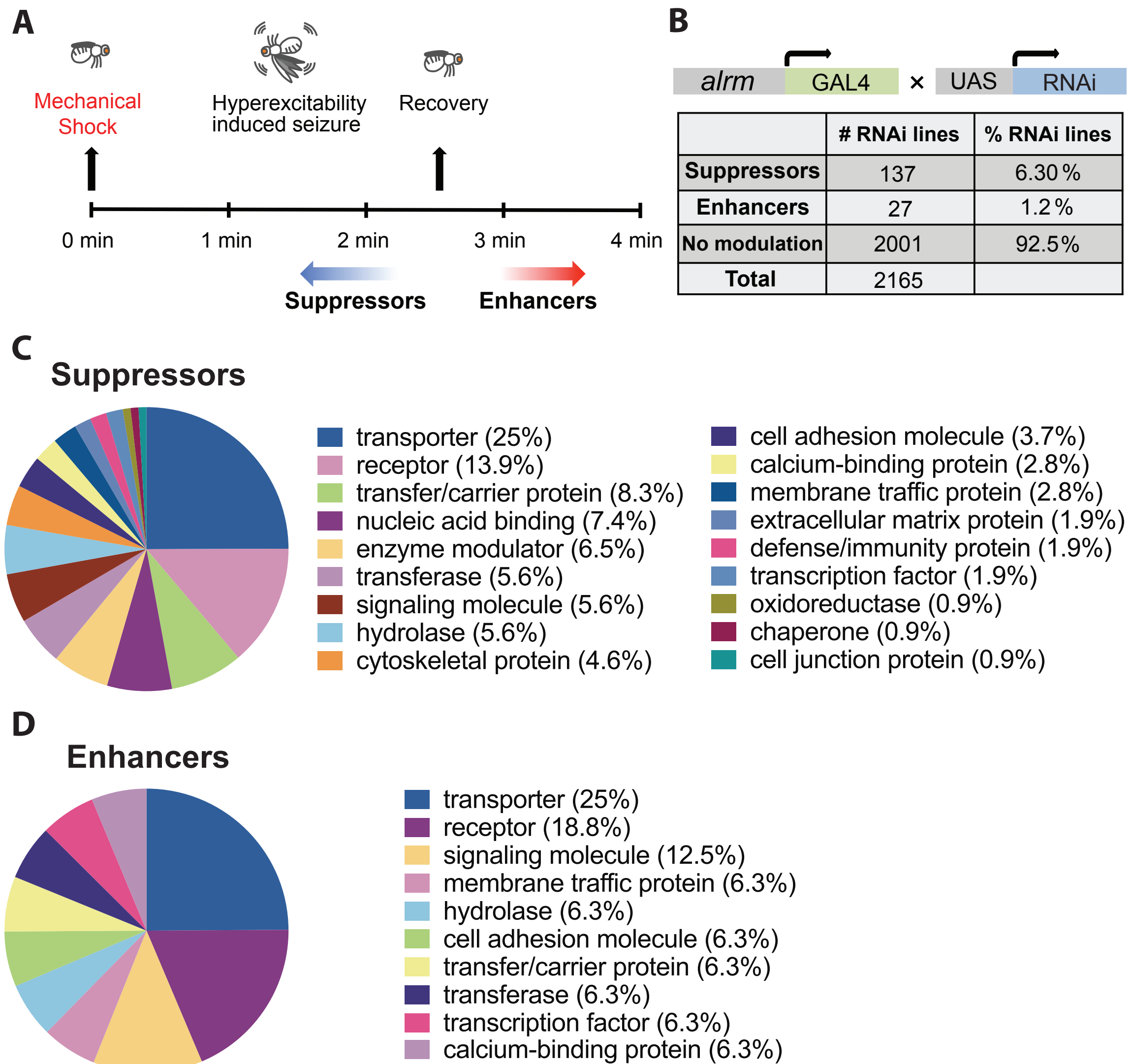


Figure 2: Schematics of the screen and summary of the results

A, Schematics of the behavioral assay using *Drosophila* bang-sensitive mutant, *eas*^{PC80} for RNAi based screen. B, Astrocyte specific knockdown utilizing GAL4/UAS system and summary of screen results. C, Pie charts represent candidate genes categorized based on their protein function. 100 suppressors were identified as genes that cause flies recover 100 % from the

hyperexcitability-induced seizure by 90 seconds when RNAi for target gene is expressed in astrocytes. Protein functional categorization was done by the PANTHER classification system (Mi et al., 2013). 91 genes that have defined function were subjected for categorizations. D. Enhancers are defined when flies with a RNAi showed less than 90 % recovery by 4 minutes which is the total length of assay. 78 genes out of 87 genes were subjected for categorizations.

Discussion

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 (Pavlidis et al., 1994). While the precise mechanism to induce network hyperexcitability by the genetic seizure model (eas^{PC80}) used in our screen is not completely understood, studies with cell-type specific rescues suggests a role for *eas+* in excitatory rather than inhibitory transmission (Kroll and Tanouye, 2013b). Therefore, the imbalance of the network activity supposedly comes from enhanced excitation rather than decreased inhibition and the hits from our screen would act either by decreasing excitation or increasing inhibition to ameliorate network imbalance. Our collection of RNAi lines targeted the vast majority of transmembrane, secreted, and signaling molecules encoded in the *Drosophila* genome. While we focused our further analysis on FA molecules (see Chapter 3), a few additional classes of molecules were found repeatedly in our collection of enhancers or suppressors

(Fig 2, Table 1, 2). For instance, ~40% of the suppressors of seizure activity were transporters or transfer/carrier proteins, while ~9% were transmembrane receptors. Similarly, ~25% of the enhancers fell into the transporter class, and ~12% were transmembrane receptors (Fig 2C, D). In many species astrocytes have been found to express a wide array of transporters (Amara and Kuhar, 1993; Aroeira et al., 2013; Doherty et al., 2009; Hanu et al., 2000; Malynn et al., 2013; Martin and Krantz, 2014; Raiteri et al., 2008; Stork et al., 2014; Suzuki et al., 2011), but the *in vivo* activity and role of these molecules has not been explored in detail, perhaps with the exception of Excitatory amino acid transporters (EAATs) and γ-aminobutyric acid transporters (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. Our work suggests a deeper analysis of this class of molecules could shed important new light on how astrocytes modulate central nervous system (CNS) excitability and signaling.

Materials and Methods

Fly strains. The following Drosophila strains were used: *w*^{1118} Canton S, *alrm-GAL4*(Doherty et al., 2009), *eas*^{PC80}(Pavlidis et al., 1994).

UAS-RNAi lines were from Vienna *Drosophila* Resource Center (Vienna, Austria).

Bang-sensitive behavioral assay. The behavioral assay was modified from Song et al. (2008) and described in Muthukumar et al. (2014). Adult male flies after 3-7 days of eclosion were collected in fresh food vials the night before experiments. Vials containing 10 – 15 flies were subjected to mechanical stimulus using a VWR Vortex Mixer (VWR International, West Chester, PA) at maximum speed for 10 sec to induce paralysis and seizure in bang-sensitive mutant, *eas*^{PC80}. The numbers of flies standing and resuming normal behavior was noted at 10 s intervals for 4 min. Mean recovery time was calculated as the average time taken by an individual fly to recover from paralysis.

CHAPTER 3: Tensin and other focal adhesion associated molecules as a regulator of seizure-like behavior

Introduction

Astrocytes are highly branched non-neuronal cells that tile with one another and densely infiltrate nearly all synapse rich regions of the brain. The fine membranes 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 (Kimelberg and Nedergaard, 2010). Through dynamic changes in calcium signaling, astrocytes act as important regulators of neural activity (Khakh and McCarthy, 2015; Ma et al., 2016) and neuro-vascular coupling (Daneman and Prat, 2015). Despite our growing appreciation for the importance of astrocytes in neural circuit function and maintenance (Chung et al., 2015; Halassa and Haydon, 2010), 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. 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 extra-synaptic space (Danbolt, 2013). Efficient astrocyte clearance of NTs is important for proper termination of synaptic signaling to avoid, for instance, chronic receptor desensitization at synpases (Pita-Almenar et al., 2012; Rothstein et al., 1996;

Stacey et al., 2010), regulation of the spillover of neurotransmitters between synapses (Danbolt, 2013; Kersanté et al., 2013; Pál, 2015), and for setting proper basal excitatory/inhibitory (E/I) tone (Muthukumar et al., 2014; Tanaka et al., 1997; Zeng et al., 2010). Conditional elimination of EAATs from astrocytes in mouse results in fatal epilepsy (Petr et al., 2015), and mutations in human EAATs cause episodic ataxia and seizure (de Vries et al., 2009).

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 (Gegelashvili et al., 1996) , neuronal contact (Swanson et al., 1997), or neuronal activity (Genoud et al., 2006; Pannasch et al., 2014; Perego et al., 2000). Pathological changes in EAATs have been associated with disease including epilepsy and seizure (Tanaka et al., 1997), neurodevelopmental disorders (Higashimori et al., 2016), ataxia (de Vries et al., 2009; Parinejad et al., 2016) and ALS (Rothstein et al., 1995), where dysregulation of EAATs is thought to drive changes in E/I balance. Treatments that upregulate GLT1 (the astrocyte EAAT2 in human) expression *in vitro* show neuroprotective effects (Rothstein et al., 2005), arguing that rebalancing EAAT levels may be a valuable therapeutic approach to reestablish 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 (Carmona et al.,

2009; Omrani et al., 2009; Rothstein et al., 2005), presumably because glutamate levels were not sufficiently high to drive synaptic strengthening. From our comprehensive *in vivo* RNAi screen in *Drosophila* to identify astrocyte-expressed genes that could phenotypically modify a genetically induced seizure model, focal adhesion molecule called tensin has emerged as a strong suppressor.

Focal adhesions are multi-protein complexes in which integrins have a central role in linking the extracellular matrix (ECM) with the actin cytoskeleton. The cytoplasmic side of focal adhesions is composed of multiple adaptor proteins, which connect the cytosolic tail of β-Integrin to the actin cytoskeleton (Lo, 2006). Studies in mice fibroblasts show that the dynamic nature of focal adhesions enables them to mediate cell motility and membrane protrusion by rearranging the actin cytoskeleton (Le Clainche and Carlier, 2008; Lin et al., 1994). In response to extracellular stimuli, a trigger of actin signaling is mediated by focal adhesions to drive changes in cell shape and membrane protrusion. Dynamic formation of focal adhesions would be expected to account for actin-dependent cellular processes (Jovceva et al., 2007). In cultured astrocytes, activation of Eph receptor tyrosine kinases increases the number of focal adhesions along with cytoskeletal reaarangement (Puschmann and Turnley, 2010). Another study suggests treatment of the thyroid hormone, thyroxine (T4) which has an essential role in the growth and development of the mammalian brain increases the number and length of focal adhesions by promoting integrin

clustering in astrocytes (Siegrist-Kaiser et al., 1990). While focal adhesions have been analyzed in cultured astrocytes it is not known if focal adhesions play important roles for the establishment or maintenance of the complex morphology of astrocytes and their functions in relation to neuronal activity *in vivo*.

With a further analysis, we found that astrocyte-specific depletion of other components of focal adhesion complexes (FAs) and a matrix metalloproteinase potently suppressed seizure activity, and this suppression further expanded to a pharmacologically induced model of seizure (i.e. picrotoxin exposure). We provide evidence that under normal physiological conditions FAs are required for proper establishment of astrocyte coverage of the synaptic neuropil and expression of EAAT1, but they do not regulate the sole *Drosophila* GAT molecule. Interestingly, in the context of hyperexcitability, FAs negatively regulated EAAT1 levels and astrocyte coverage, and their elimination led to enhanced EAAT1 expression, increased astrocyte coverage of the synaptic neuropil, and more rapid recovery from seizure.

Results

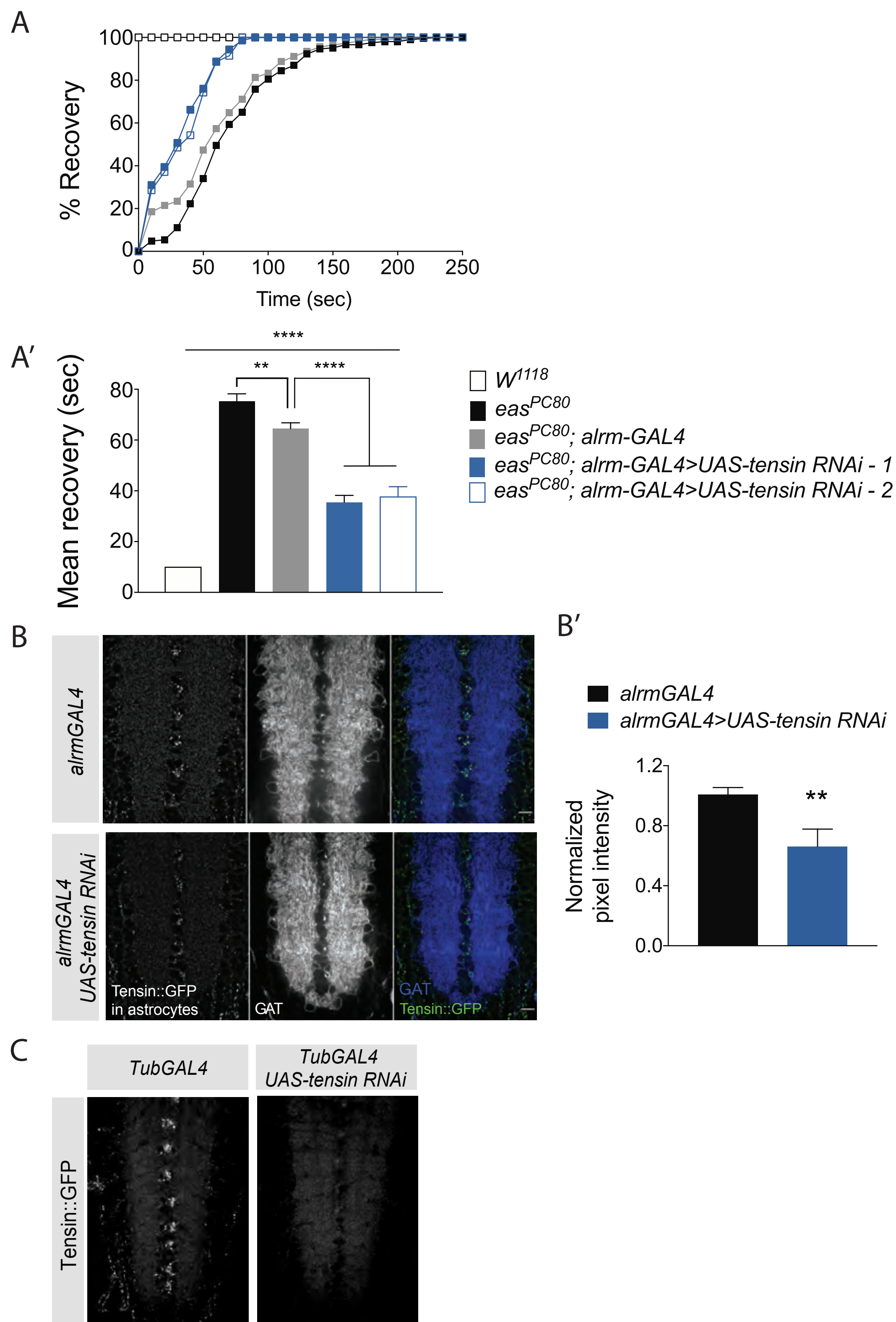
An *in vivo* RNAi screen identifies tensin as an astrocyte-expressed regulator of seizure activity

In our primary screen we found that astrocyte-specific knockdown of tensin, an essential component of focal adhesion complexes (FAs) and regulator of cell-extracellular matrix interactions, led to mean recovery times of less than 1.5 min.

We repeated the *eas*^{PC80} seizure assay with multiple *tensin*^{RNAi} lines, in this case recording recovery rates at 10 sec intervals. We noticed a dramatic shift of recovery curve with astrocyte-specific expression of *tensin*^{RNAi} compared to controls (Fig 3.1A) and a decrease in mean recovery time with astrocyte *tensin*^{RNAi} (Fig 3.1A').

To determine the pattern of Tensin localization in astrocytes we visualized Tensin using an endogenously GFP-tagged *tensin* line, and labeled astrocyte membranes with anti-Gat antibodies in 3rd instar larva ventral nerve cords (Fig 3.1B). We found that Tensin-GFP was localized in punctate structures in astrocyte cell bodies and astrocyte processes as they projected into the neuropil area among neuronal circuits. To determine whether our *tensin*^{RNAi} lines were indeed targeting *tensin*, we quantified Tensin-GFP levels in astrocytes in controls and animals expressing *UAS-tensin*^{RNAi} under the control of alrmGAL4. To selectively examine astrocyte Tensin-GFP we focused on the quantification of Tensin-GFP that fell within the domains of anti-Gat immunoreactivity (i.e. the Tensin within astrocytes). We found that Tensin-GFP levels were decreased by 38 percent in *tensin*^{RNAi} animals compared to its control (Fig 3.1B'). Furthermore, when we expressed *UAS-tensin*^{RNAi} under the control of tubulin-GAL4, Tensin-GFP punctae were not detectable (Fig 3.1C). These data demonstrated that Tensin is expressed in astrocytes, localized to punctate structures within astrocytes, and that our RNAi lines successfully targeted *tensin* expression. We

conclude that Tensin is a novel astrocyte molecule that can modulate seizure-like behavior *in vivo*.


A
% Recovery
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W1118
easPC80
easPC80; alrm-GAL4
easPC80; alrm-GAL4>UAS-tensin RNAi - 1
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B
alrmGAL4
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Tensin::GFP in astrocytes
GAT
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TubGAL4 UAS-tensin RNAi
Tensin::GFP

Figure 3.1: Tensin, a focal adhesion molecule potently suppresses seizure-like behavior when knocked down in astrocytes

A, Percent of recovery is measured every 10 sec following 10 sec of mechanical shock. Wide-type flies are not affected by the shock so their recovery is 100 % in 10 sec while eas^{PC80} displays seizure-like behavior requiring time to recover. Knockdown of astrocyte focal adhesion molecule, *tensin* suppresses seizure-like behavior in bang-sensitive mutant. Two RNAi lines that are targeting different sequences were tested. A’, Mean recovery time is measured based on data shown in A. Error bars represent SEM. **P<0.005, ****P<0.0001; one-way ANOVA and Dunnett’s multiple comparisons. *N* > 100 flies quantified for all genotypes. B, Tensin signal is visualized with GFP that is genetically tagged and astrocyte membrane is labeled using α-Gat antibody in control and astrocyte tensin knockdown. B’, Knockdown efficiency by RNAi is quantified by measuring the intensity of tensin::GFP signal in astrocyte membrane. Error bars represent SEM. **P<0.01; unpaired t test. *alrmGAL4/+, N* = 8; *alrmGAL4>UAS-tensin RNAi, N* = 6 animals quantified. C, Expressing tensin RNAi with TubGAL4 driver removed Tensin::GFP punctae throughout the ventral nerve cord of 3rd instar larvae. *TubGAL4/+, N* = 8; *TubGAL4>UAS-tensin RNAi, N* = 7 aniamls. Scale bars represent 10 μm in B and C.

Multiple components of focal adhesion complexes function in astrocytes to modulate seizure activity

Tensin is a component of FAs, which are large multi-protein complexes that include heterodimeric integrin receptors that interact with ECM, and adaptor proteins that connect integrin receptors to the actin cytoskeleton (Fig 3.2A). We sought to determine whether additional known FA molecules functioned in astrocytes to modulate hyperexcitability-induced seizure behavior.

Integrins are the central players of focal adhesions and composed of heterodimeric α- and β- subunits. The cytosolic tail of β-integrin serves as an anchor to multiple adaptor proteins in the focal adhesions (Le Clainche and Carlier, 2008). In *Drosophila,* there is only one βintegrin called βPS as opposed to αintegrin with five different subunits and βintegrin is sufficient to initiate a signaling pathway in the developing embryo (Martín-Bermudo and Brown, 1999). To address roles of integrins as a focal adhesion in astrocytes and their functions in seizure-like behavior, we choose to look at βintegrin that is unlikely to complicate the interpretation of data with redundancy. Immunofluorescent labeling with anti-βintegrin antibodies revealed that βintegrin colocalized with Tensin-GFP^{+} punctae within ventral nerve cord (Fig 3.2B), which supports the notion that Tensin protein is largely associated with βintegrin containing FAs.

We next screened well defined FA components in the adult *eas*^{PC80} assay and found that RNAi knockdown of adaptor protein, Talin or focal adhesion kinase (FAK), as well as βintegrin in astrocytes strongly suppressed seizure behavior at levels similar to *tensin*^{RNAi} (Fig 3.2C, C'). These results further support the notion that FAs in astrocytes regulate nervous system hyperexcitability and seizure behavior.

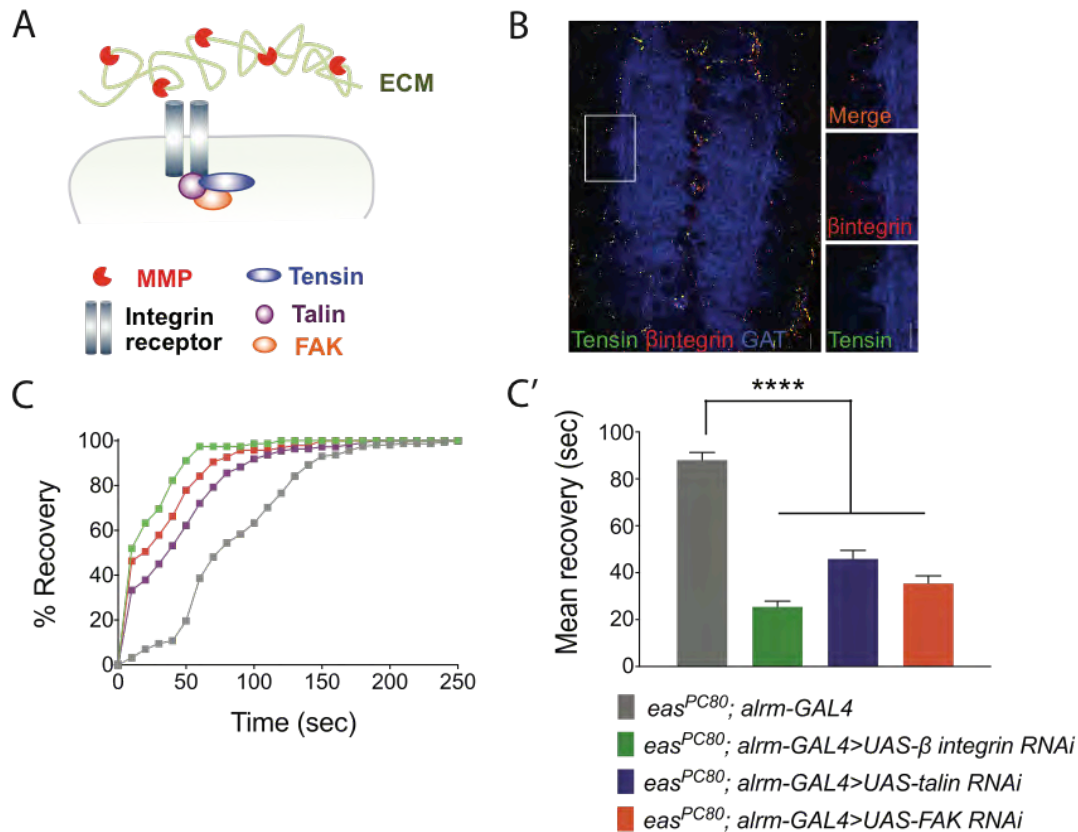


Figure 3.2: Multiple components of focal adhesion complexes function in astrocytes to modulate seizure-like behavior

A, Schematics of focal adhesions (only components that are discussed in this study are shown). B, Confocal image shows colocalization of tensin and βintegrin in the ventral nerve cord of 3rd instar larvae. Tensin signal is genetically tagged with GFP and βintegrin signal is detected by α-βPS antibody. *N* = 19 animals. C, Mutiple components of focal adhesions including βintegri, talin, and FAK showed similar suppression tensin in the adult behavioral assay when they are depleted in astrocytes using RNAi. C', Quantification of mean recovery time

from data C. Error bars represent SEM. ****P<0.0001; one-way ANOVA and Dunnett's multiple comparisons. *N* > 100 flies quantified for all genotypes.

Seizures induced by pharmacological blockade of GABA-A receptors are modulated by astrocyte focal adhesions

To complement our studies using the *eas*^{PC80} genetic model of seizure we turned to pharmacological blockade of GABA-A receptor signaling. 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 (Stilwell et al., 2006). This pharmacological model of seizure has a precise mechanism of action because of the specificity of the inhibitor and an easy *in vivo* delivery in *Drosophila* larvae gives us a great advantage to control the action of drug in terms of concentration and exposure time (Stilwell et al., 2006). We collected 3rd instar larvae and allowed them to consume PTX-laced or control food for a period of 9 hrs, and we compared sham animals to animals treated with two different doses of PTX (0.5 or 1.5 mg/ml). We observed no changes in baseline motility in animals that were sham treated, and no alterations in baseline motor function after astrocyte-specific knockdown of *tensin*, *βintegrin*, *talin*, or *FAK* (Fig 3.3A, B). Therefore depletion of FA signaling components in astrocytes did not appear to alter baseline motor behavior in crawling assays. We observed a dose-dependent decrease in motility in control animals, with velocity (mm/min) being decreased by 65% after exposure to 0.5 mg/ml PTX, and further decreased by 83% after exposure to 1.5 mg/ml PTX (Fig 3.3A, B). In contrast, PTX-induced decreases in larval motility were partially but significantly suppressed at both doses in animals with

astrocyte-specific RNAi depletion of *tensin*, *βintegrin*, *talin*, or *FAK* (Fig 3.3A, B). Since PTX was delivered with food, the possibility of impairment of animal feeding behavior by RNAi was explored. Using food coloring, when larvae were subjected to 9hr of food consumption, the relative food intake measured by spectrophotometry was comparable across genotypes (Fig 3.4A) suggesting the amount of PTX delivered into the system is not accountable for the RNAi effect on the behavior. When we only expressed *UAS-RNAis* to control the insertion of *UAS-RNAi* construct, the suppression effect on PTX-induced decrease in larval locomotion was absent (Fig 3.4B). To overcome the concerns for expressing two UAS-RNAi at the same time, we utilized the tensin null animal (by^{33C}), which showed the same suppression in the PTX induced seizure as $tensin^{RNAi}$ along with a defect in normal locomotion (Fig 3.5). Finally, RNAi effects were validated by utilizing non-overlapping RNAi lines for *tensin*, *βintegrin*, *talin*, or *FAK* (Fig 3.4C).

The strength of suppression was not further enhanced by simultaneous expression of RNAi constructs for *βintegrin* and *tensin* (Fig 3.3C), which is consistent with these molecules functioning in the same genetic pathway. These data indicate that astrocyte FA molecules modulate changes in neural excitation/inhibition balance at multiple developmental stages, and in response to both genetic and pharmacological induction of hyperexcitability.

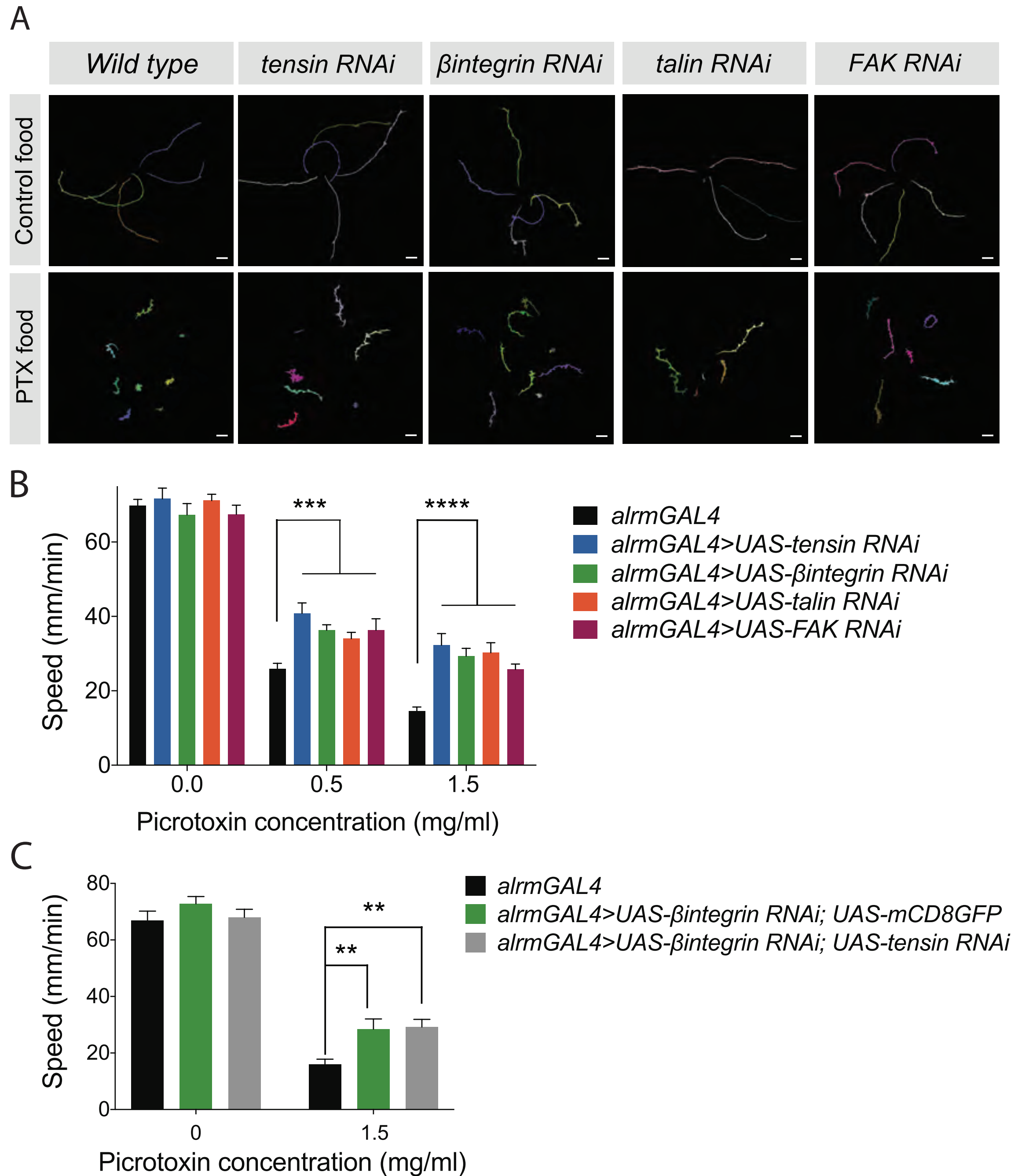


Figure 3.3: Seizure induced by pharmacological blockade of GABA-A receptors are modulated by astrocyte focal adhesions

A, Raw traces of 3rd instar larval locomotion with and without picrotoxin treatment in control and RNAis for focal adhesion components. Scale bars represent 1 cm. B, A distance traveled within one minute is measured to quantify locomotion speed for each RNAi. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 18; *alrmGAL4/+* (0.5 mg/ml PTX), *N* = 96; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 66; *alrmGAL4>UAS-tensin RNAi* (0.0 mg/ml PTX), *N* = 15; *alrmGAL4>UAS-tensin RNAi* (0.5 mg/ml PTX), *N* = 33; *alrmGAL4>UAS-tensin RNAi* (1.5 mg/ml PTX), *N* = 24; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX), *N* = 4; *alrmGAL4>UAS-βintegrin RNAi* (0.5 mg/ml PTX), *N* = 58; *alrmGAL4>UAS-βintegrin RNAi* (1.5 mg/ml PTX), *N* = 45; *alrmGAL4>UAS-talin RNAi* (0.0 mg/ml PTX), *N* = 9; *alrmGAL4>UAS-talin RNAi* (0.5 mg/ml PTX), *N* = 55; *alrmGAL4>UAS-talin RNAi* (1.5 mg/ml PTX), *N* = 22; *alrmGAL4>UAS-FAK RNAi* (0.0 mg/ml PTX), *N* = 17; *alrmGAL4>UAS-FAK RNAi* (0.5 mg/ml PTX), *N* = 21; *alrmGAL4>UAS-FAK RNAi* (1.5 mg/ml PTX), *N* = 54 animals quantified. C, Knockdown of βintegrin and tensin in astrocytes simultaneously had no additive effect on suppression phenotype. Error bars represent SEM. **P<0.005; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 6; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 17; *alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP* (0.0 mg/ml PTX), *N* = 12; *alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP* (1.5 mg/ml PTX), *N* = 19; *alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi* (0.0

mg/ml PTX), *N* = 5; *alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi* (1.5 mg/ml PTX), *N* = 13 animals quantified.

Full statistical details are provided in Table 3.

Astrocyte matrix metalloproteinase 1 (Mmp1) activity dynamically regulates seizure activity

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that can degrade components of extracellular matrix (ECM), which then activate integrin signaling (Michaluk et al., 2009; Park and Goda, 2016)(Fig 3.2A). Interestingly, increased neural activity or glutamate signaling has been found to elevate MMP activity and/or expression (Huntley, 2012; Nagy et al., 2006; Wilczynski et al., 2008). We explored the possibility that *Drosophila* MMPs might modulate nervous system excitability through FA signaling pathways using both loss- and gain-of-function approaches. We first expressed RNAi lines targeting *mmp1* in astrocytes in the eas^{PC80} background and assayed sensitivity to bang-induced seizure. We found depletion of *mmp1* by RNAi suppressed hyperexcitability-induced seizure behavior by decreasing recovery time (Fig 3.6A, A'). Reciprocally, astrocyte overexpression of MMP1 had the opposite effect and strongly enhanced seizure-like behavior (Fig 3.6A, A'). We further found that $Mmp1^{RNAi}$ partially suppressed PTX-induced decreases in larval crawling behavior, (Fig 3.6B), and the same suppression was observed when PTX-treated animals were co-fed the MMP inhibitor, GM 6001 (Fig 3.6C). We demonstrated that expressing UAS-Mmp1 RNAi under the control of the pan-neuronal driver elav-GAL4 resulted in no significant suppression of PTX-induced seizure behavior (Fig 3.7), arguing for an astrocyte-specific role for Mmp1.

Since MMPs are secreted as a pro-MMP form requiring extracellular activation to become enzymatically active form (Vandenbroucke and Libert, 2014), the simplest model would be that Mmp1 activates integrin receptors extracellularly, which then signals through focal adhesion complex to modulate excitability. To test this model we assayed for phenotypic interactions between Mmp1, βintegrin, and Talin in PTX-induced seizure assays. We found addition of the MMP inhibitor did not enhance the rescuing effect of *integrin*^{RNAi}, arguing Mmp1 and βintegrin are in the same genetic pathway in astrocyte regulation of excitability (Fig 3.8A). Talin is a cytosolic adaptor protein and its expression is not required for the presence of integrins on the cell surface (Brown et al., 2002), we found the rescuing effect of *talin*^{RNAi} in astrocytes was suppressed by Mmp1 overexpression (Fig 3.8C). Finally, we found that Mmp1 overexpression could not suppress the seizure suppression afforded by *integrin*^{RNAi} (Fig 3.8B). The simplest interpretation of these data is that Mmp1 exerts its effects through βintegrin-mediated FA signaling.

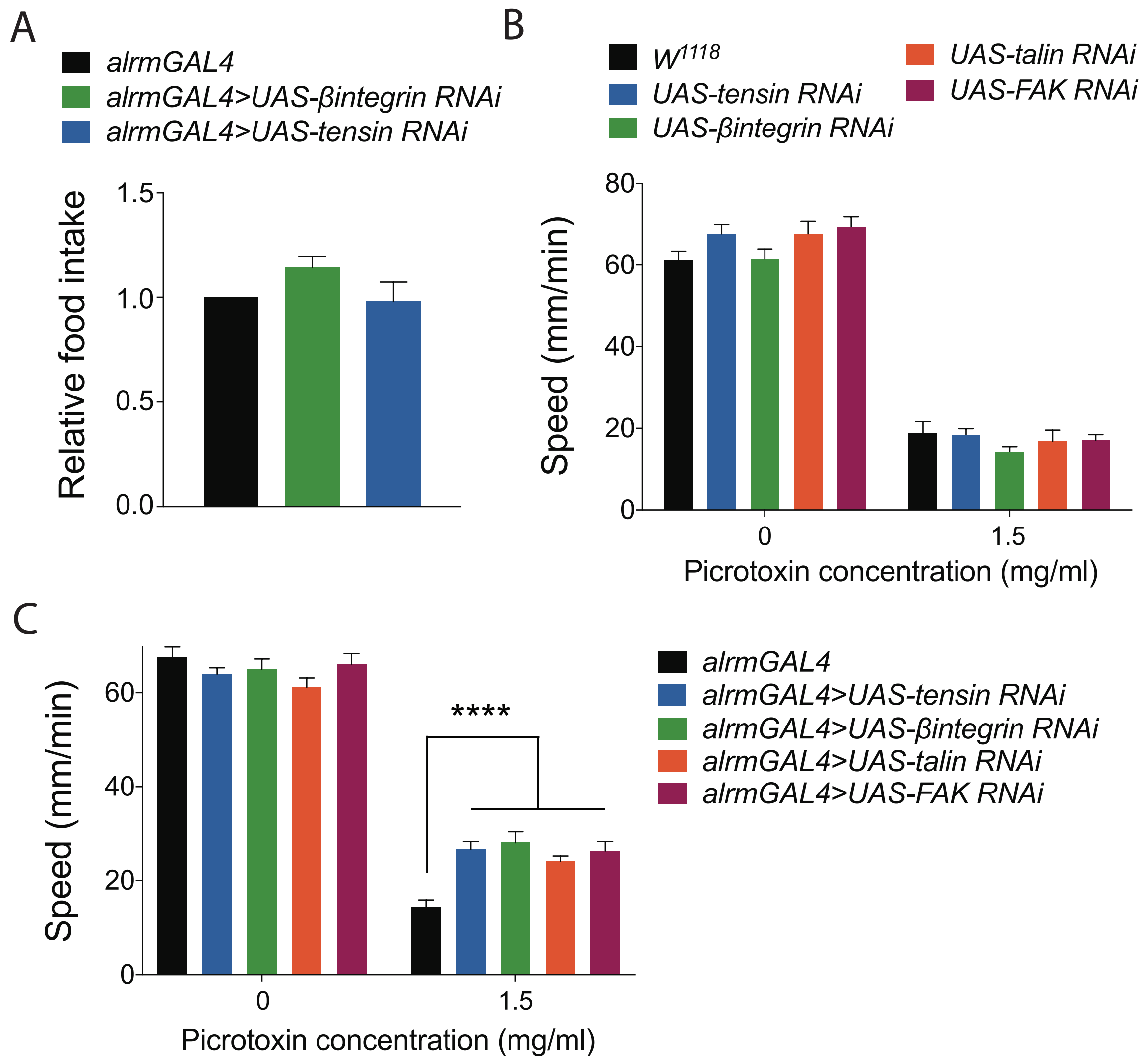


Figure 3.4: Confirmation of RNAi effects

A, Relative food intake was comparable across genotypes. Error bars represent SEM. One-way ANOVA. *N* = 3. B, Expressing RNAi alone in astrocytes had no effect on PTX based behavioral assay. Error bars represent SEM. Two-way ANOVA and Tukey's multiple comparisons. W^{1118} (0.0 mg/ml PTX), *N* = 4; W^{1118} (1.5 mg/ml PTX), *N* = 11; *UAS-tensin RNAi* /+ (0.0 mg/ml PTX), *N* = 13; *UAS-*

tensin RNAi/+ (1.5 mg/ml PTX), *N* = 46; *UAS-βintegrin RNAi /+* (0.0 mg/ml PTX), *N* = 11; *N* = 58; *UAS-βintegrin RNAi/+* (1.5 mg/ml PTX), *N* = 46; *UAS-talin RNAi/+* (0.0 mg/ml PTX), *N* = 10; *UAS-talin RNAi/+* (1.5 mg/ml PTX), *N* = 16; *UAS-FAK RNAi/+* (0.0 mg/ml PTX), *N* = 10; *UAS-FAK RNAi/+* (1.5 mg/ml PTX), *N* = 37 animals quantified. C, RNAi lines that are independent from the ones used in rest of the study displayed the same suppression effect on PTX assay. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 15; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 46; *alrmGAL4>UAS-tensin RNAi* (0.0 mg/ml PTX), *N* = 42; *alrmGAL4>UAS-tensin RNAi* (1.5 mg/ml PTX), *N* = 54; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX), *N* = 24; *alrmGAL4>UAS-βintegrin RNAi* (1.5 mg/ml PTX), *N* = 27; *alrmGAL4>UAS-talin RNAi* (0.0 mg/ml PTX), *N* = 32; *alrmGAL4>UAS-talin RNAi* (1.5 mg/ml PTX), *N* = 46; *alrmGAL4>UAS-FAK RNAi* (0.0 mg/ml PTX), *N* = 25; *alrmGAL4>UAS-FAK RNAi* (1.5 mg/ml PTX), *N* = 35 animals quantified. Full statistical details are provided in Table 3.

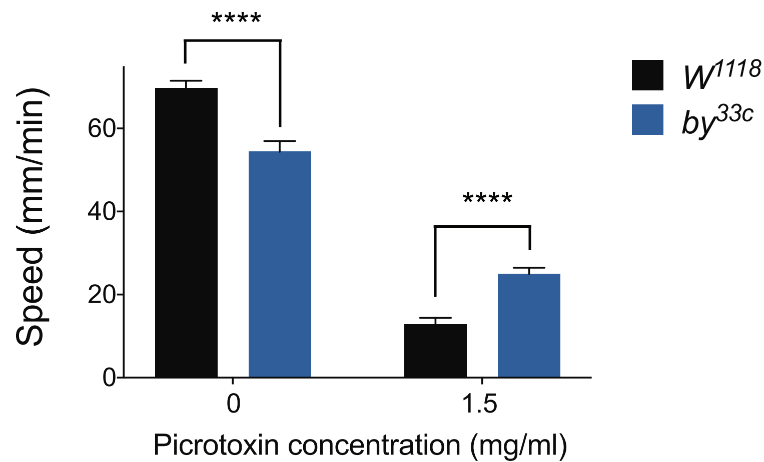


Figure 3.5: *tensin* null, by^{33c} phenocopies RNAi results

tensin null animals (*by*^{33c}) showed significant suppression in locomotion defect by PTX administration. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. *W*^{1118} (0.0 mg/ml PTX), *N* = 17; *W*^{1118} (1.5 mg/ml PTX), *N* = 20; *by*^{33c} (0.0 mg/ml PTX), *N* = 21; *by*^{33c} (1.5 mg/ml PTX), *N* = 32 animals quantified. Full statistical details are provided in Table 3.

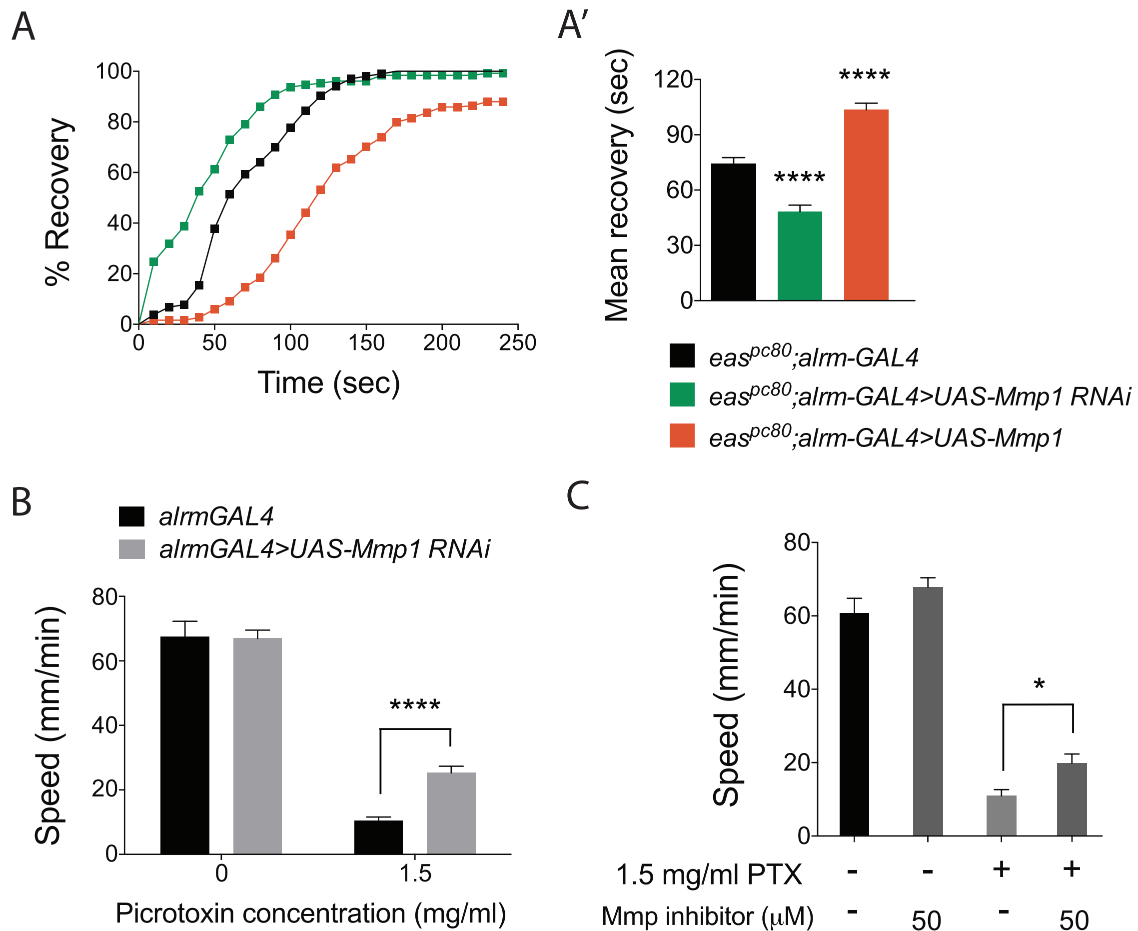


Figure 3.6: Astrocyte matrix metalloproteinase 1 (MMP1) dynamically regulates seizure activity in genetic and pharmacological models

A, Opposite effects of astrocyte MMP over-expression and knockdown on hyperexcitability induced behavior. A', Mean recovery time is measured based on data shown in A. Error bars represent SEM. ****P<0.0001; one-way ANOVA and Dunnett's multiple comparisons. *N* > 100 flies quantified for all genotypes. B, Genetically knocking down astrocyte Mmp1 suppressed PTX-induced locomotion defect. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 4;

alrmGAL4/+ (1.5 mg/ml PTX), *N* = 50; *alrmGAL4>UAS-Mmp1 RNAi* (0.0 mg/ml PTX), *N* = 15; *alrmGAL4>UAS-Mmp1 RNAi* (1.5 mg/ml PTX), *N* = 43 animals quantified. C, Pharmacological inhibition of MMPs during PTX feeding showed locomotion defect suppression. Error bars represent SEM. *$P<0.05$; one-way ANOVA and Dunnett's multiple comparisons. W^{1118} (0.0 mg/ml PTX), *N* = 5; W^{1118} (0.0 mg/ml PTX, 50 μM inhibitor), *N* = 4; W^{1118} (1.5 mg/ml PTX), *N* = 19; W^{1118} (1.5 mg/ml PTX, 50 μM inhibitor), *N* = 19 animals quantified. Full statistical details are provided in Table 3.

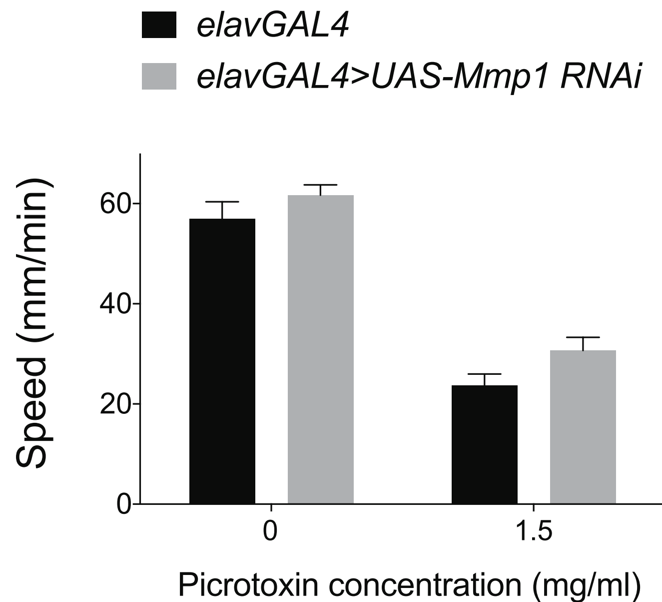


Figure 3.7: Neuronal knockdown of MMP1 has no effect on PTX-induced locomotion defect

Genetically knocking down neuronal Mmp1 does not alter PTX-induced locomotion defect. Error bars represent SEM. Two-way ANOVA and Tukey's multiple comparisons. *elavGAL4/+* (0.0 mg/ml PTX), *N* = 6; *elavGAL4/+* (1.5 mg/ml PTX), *N* = 15; *elavGAL4>UAS-Mmp1 RNAi* (0.0 mg/ml PTX), *N* = 6; *elavGAL4>UAS-Mmp1 RNAi* (1.5 mg/ml PTX), *N* = 12 animals quantified. Full statistical details are provided in Table 3.

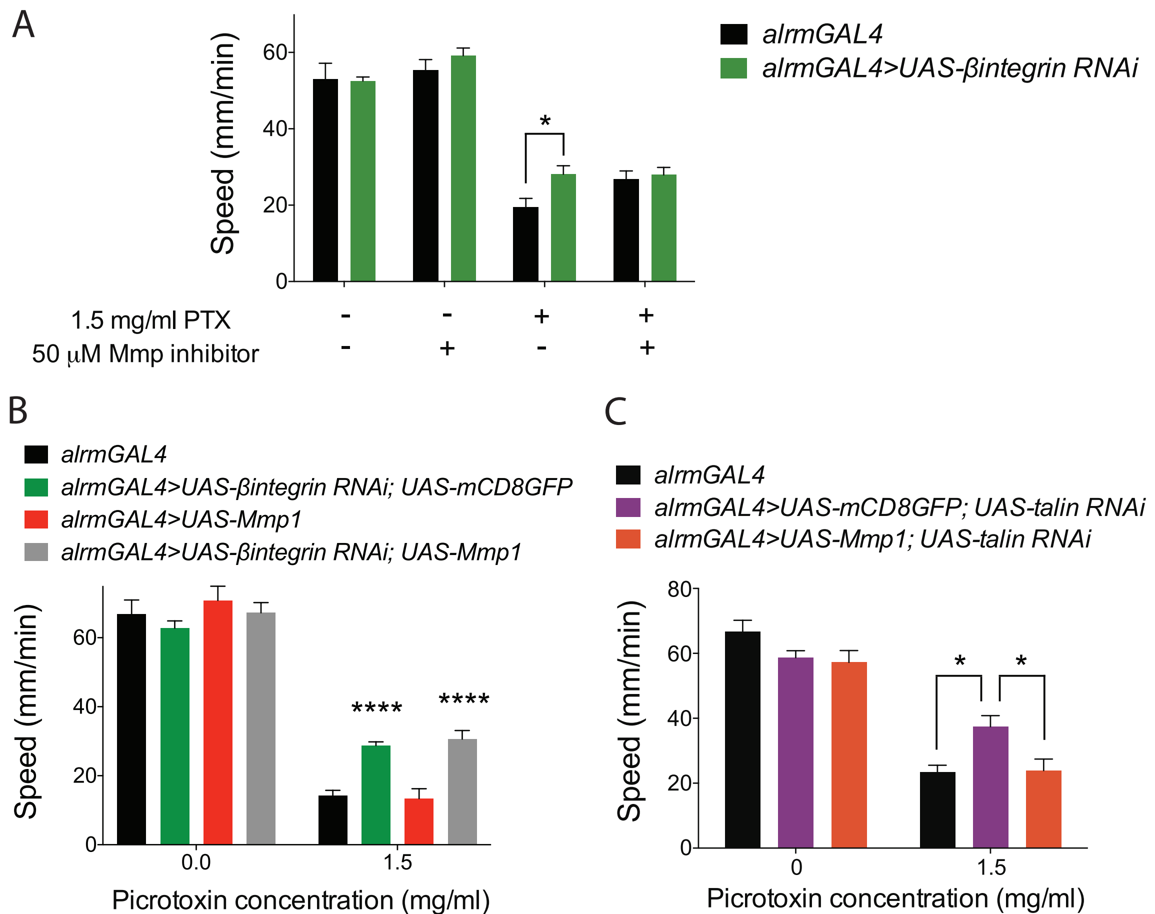


Figure 3.8: Matrix metalloproteinase1 (Mmp1) is a potential component of focal adhesion signaling pathway in astrocytes

A, MMP inhibitor had no additive effect on βintegrin knockdown phenotype. Error bars represent SEM. *P<0.05; two-way ANOVA and Bonferroni's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 7; *alrmGAL4/+* (0.0 mg/ml PTX, 50 μM inhibitor), *N* = 9; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 14; *alrmGAL4/+* (1.5 mg/ml PTX, 50 μM inhibitor), *N* = 11; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX), *N* = 6; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX, 50 μM inhibitor), *N* = 8; *alrmGAL4>UAS-βintegrin RNAi* (1.5 mg/ml PTX), *N* = 15; *alrmGAL4>UAS-*

βintegrin RNAi (1.5 mg/ml PTX, 50 μM inhibitor), *N* = 13 animals quantified. B, The suppression level by βintegrin depletion in astrocytes in PTX assay had no change with overexpression of Mmp1. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Dunnett's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 12; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 30; *alrmGAL4>UAS-Mmp1* (0.0 mg/ml PTX), *N* = 10; *alrmGAL4>UAS-Mmp1* (1.5 mg/ml PTX), *N* = 12; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX), *N* = 18; *alrmGAL4>UAS-βintegrin RNAi* (1.5 mg/ml PTX), *N* = 60; *alrmGAL4>UAS-βintegrin RNAi; UAS-Mmp1* (0.0 mg/ml PTX), *N* = 12; *alrmGAL4>UAS-βintegrin RNAi; UAS-Mmp1* (1.5 mg/ml PTX), *N* = 33 animals quantified. C, Mmp1 over-expression in astrocytes reversed the effect of *talin* knockdown in PTX induced behavior. Error bars represent SEM. *P<0.05; two-way ANOVA and Bonferroni's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 11; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 7; *alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi* (0.0 mg/ml PTX), *N* = 11; *alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi* (1.5 mg/ml PTX), *N* = 12 animals quantified. Full statistical details are provided in Table 3.

Focal adhesion signaling regulates astrocyte membrane coverage of the synaptic neuropil

Focal adhesions serve critical roles in cell migration and tissue development and integrin mediated focal adhesion signaling in astrocytes is known to govern astrocyte cell spreading and morphology *in vitro* (Kong et al., 2013; Leyton et al., 2001). To determine whether our manipulations of astrocyte FA molecules altered astrocyte morphology, we examined astrocyte processes in the CNS by transmission electron microscopy. We prepared 3rd instar larval brains from control and *βintegrin*^{RNAi} animals, and focused our analysis on ventral nerve cord where astrocyte morphology has been well-described (Stork et al., 2014). Astrocyte processes were identified by their electron-dense cytoplasm. Compared to controls, astrocyte *βintegrin*^{RNAi} animals exhibited a reduction in the total area covered by the processes under normal physiological conditions (Fig 3.9A, B). Consistent with our observations by light microscopy and Western blots (data performed by Allie Muthukumar not presented here), we found no change in the number of synapses in *βintegrin*^{RNAi} animals compared to controls (Fig 3.9D). Thus, βintegrin is essential for the establishment of normal coverage of the synaptic neuropil by fine astrocyte processes.

We next sought to explore how FA molecules like βintegrin would regulate astrocyte membrane responses to hyperexcitability. While exposure of control animals to PTX did not change astrocyte coverage of the synaptic neuropil, astrocyte *βintegrin*^{RNAi} animals exhibited a marked increase in neuropil coverage

by astrocyte profiles and a decrease in the distance between astrocyte membranes and synapses (Fig 3.9A, B, C), and this was in the absence of changes in the total number of synapses in the neuropil (Fig 3.9A, D).

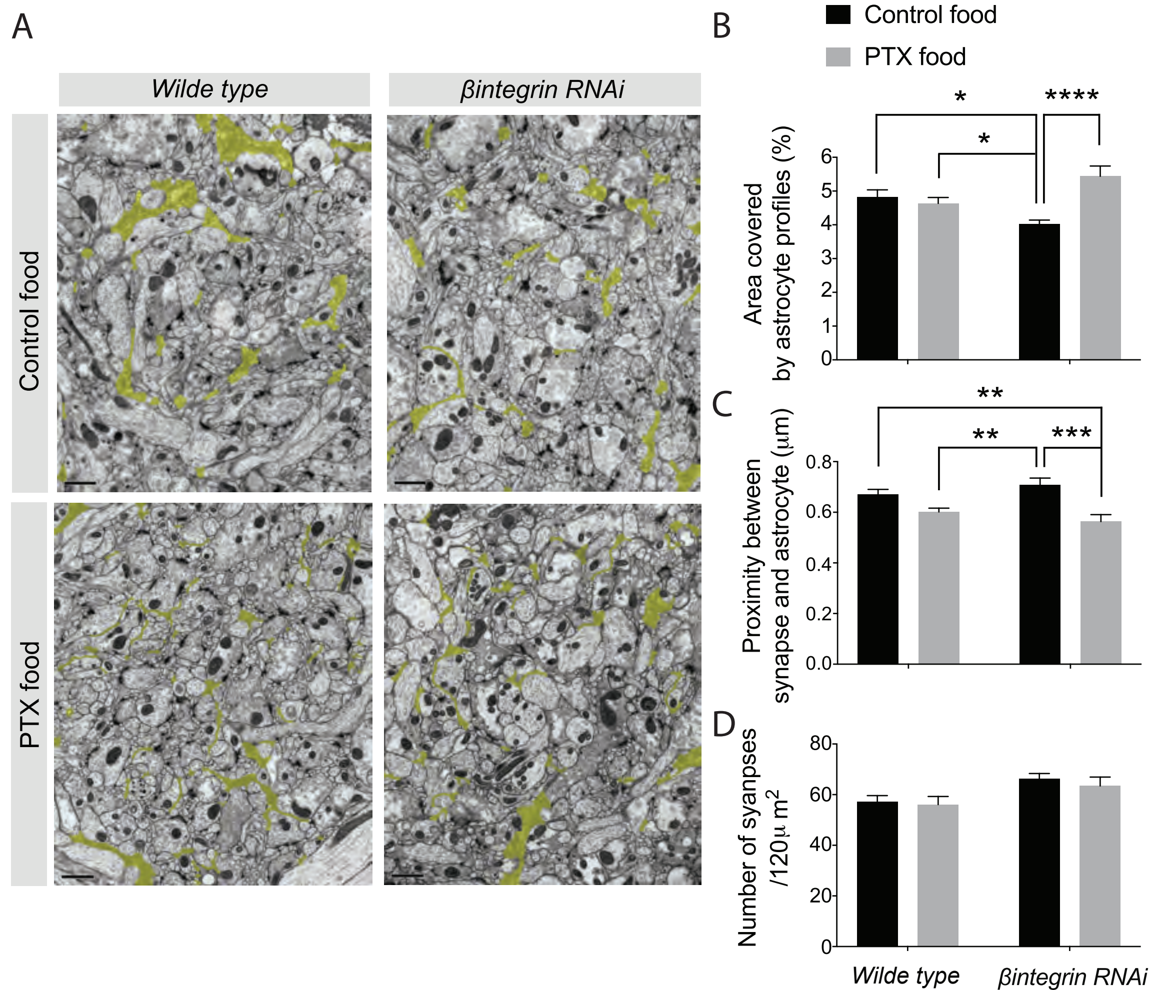


Figure 3.9: Focal adhesions regulate astrocyte morphology and synapse coverage

A, Ultrastructure of neuropil area from Drosophila 3rd instar ventral nerve cord by transmission electron microscopy. Putative astrocyte processes are pseudo colored in yellow. Three different animals were analyzed for each group. B, Quantification of percent area covered by astrocyte processes. C, Quantification of the distance from a synapse to the nearest astrocyte process. D,

Quantification of number of synapses. Error bars represent SEM. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. 18 VNC sections from three different animals were quantified for each group.

Astrocyte focal adhesion signaling regulates levels of the excitatory amino acid transporter EAAT1

In addition to changes in astrocyte coverage of the synaptic neuropil, changes in excitatory or inhibitory neurotransmitter transporters on astrocytes could also significantly impact physiological responses to hyperexcitability. Consistent with previous studies in the adult brain, EAAT1 showed prevalent expression in astrocytes in ventral nerve cord of 3rd instar larvae (Fig 3.10A). Astrocyte-specific knockdown of EAAT1 significantly impaired larval locomotion, which implies astrocytic EAAT1 plays an important role in regulating glutamate transmission that is required for proper movement of the animal (Fig 3.10C). The above observations prompted us to investigate the possibility of changes in astrocytic EAAT1 as a potential mechanism for the suppression behavior in PTX-induced locomotion defect in focal adhesion molecule knockdowns.

We found that depletion of either βintegrin or Tensin from astrocytes by RNAi resulted in a decrease in EAAT1, the sole high-affinity glutamate transporter in *Drosophila* (Besson et al., 2000), under normal physiological conditions (Fig 3.11A, B). In contrast, Western blot analysis revealed that astrocytic GABA transporter (Gat) levels were not altered when we compared astrocyte *tensin*^{RNAi} animals to controls (data performed by Allie Muthukumar not presented here). These are consistent with our findings above that FA molecules are required in astrocytes to promote normal coverage of the synaptic neuropil by fine processes of astrocytes. Surprisingly, we found that treatment of animals

with picrotoxin to induce seizure had the opposite effect: it led to a roughly 2-fold increase in EAAT1 levels in both astrocyte *βintegrin*^{RNAi} and *tensin*^{RNAi} animals (Fig 3.11A, C). This observation indicates that FA regulation of EAAT1 is different under normal physiological conditions compared to hyperexcitability, and implies that increases in EAAT1 levels may be responsible for the suppression of seizure activity we see in these animals. Consistent with this interpretation, we found that feeding larvae the EAAT1 inhibitor DHK suppressed the ability of *βintegrin*^{RNAi} or *tensin*^{RNAi} to rescue animals from seizure-induced immobility while the administration of DHK alone did not affect the locomotion behavior (Fig 3.10B and Fig 3.12A). Likewise, application of dihydrokainic acid (DHK) blocked the ability of *mmp1*^{RNAi} to suppress seizure phenotypes in larval crawling (Fig 3.12B). Overexpression of EAAT1 in astrocytes was not able to phenocopy the effect of the focal adhesion knockdowns in the PTX assay (Fig 3.13), which suggests there might be differences between acute versus chronic upregulation of EAAT1. Overall, these data argue that under normal conditions FA molecules are required to establish appropriate levels of EAAT1 on astrocyte membranes, but under conditions of hyperexcitability, FAs negatively regulate EAAT1 expression, and their depletion allows for a more robust increase in astrocyte EAAT1 and rescue of seizure-like behavior.

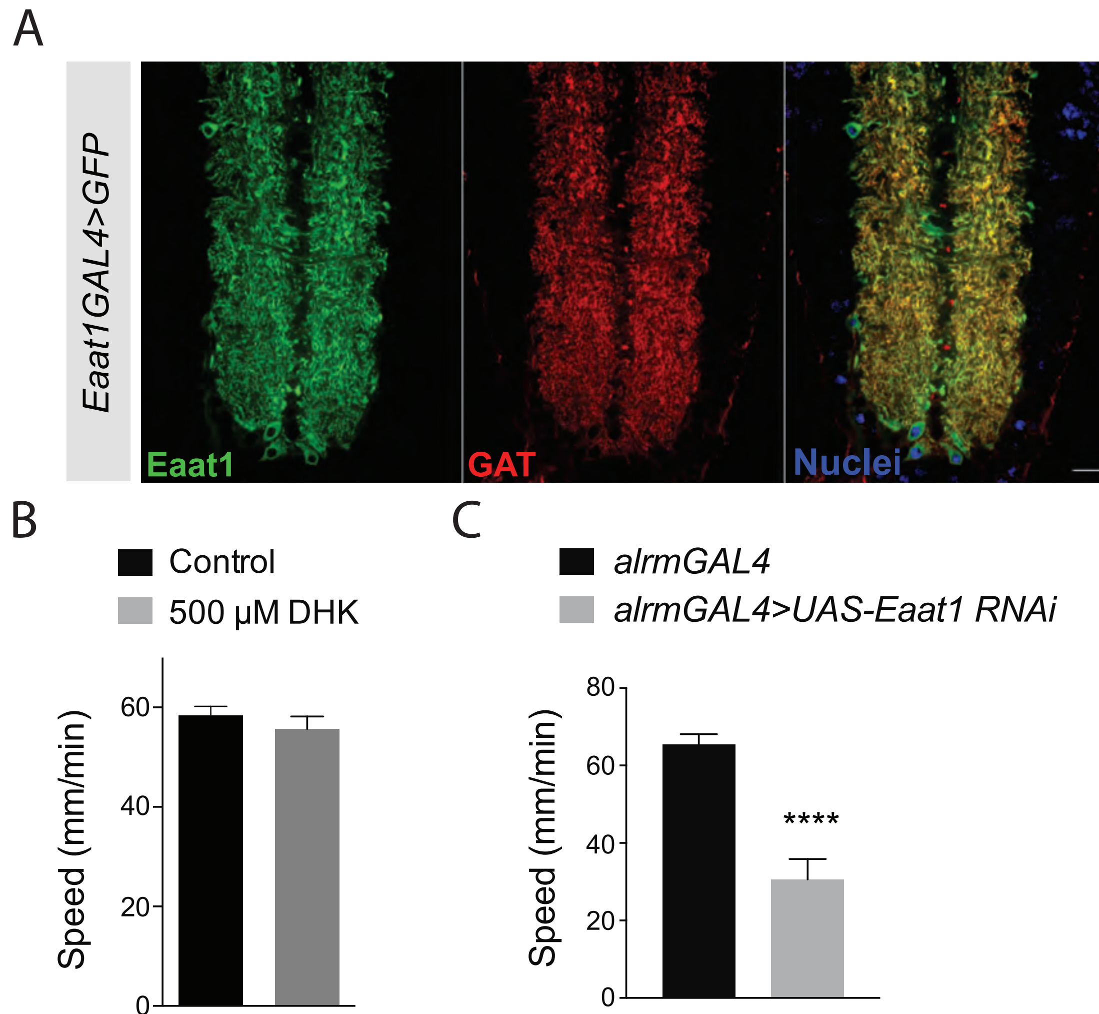


Figure 3.10: Glutamate transporter, EAAT1 function in astrocytes

A, EAAT1-GAL4 driver with UAS-mCD8GFP to show the localization of EAAT1 in astrocytes. EAAT1 expressing cells are labeled with membrane tethered GFP and astrocyte membrane is labeled by α-Gat antibody. B. Locomotion speed quantification from larvae fed either with vehicle or 500 μM DHK. Error bars represent SEM. Unpaired t test. W^{1118} (0.0 μM inhibitor), *N* = 11; W^{1118} (500 μM inhibitor), *N* = 29 animals quantified. C, Larval locomotion was impaired when

EAAT1 was depleted in astrocytes. Error bars represent SEM. ****P<0.0001; unpaired t test. *alrmGAL4/*+, *N* = 16; *alrmGAL4>UAS-Eaat1 RNAi*, *N* = 9 animals quantified.

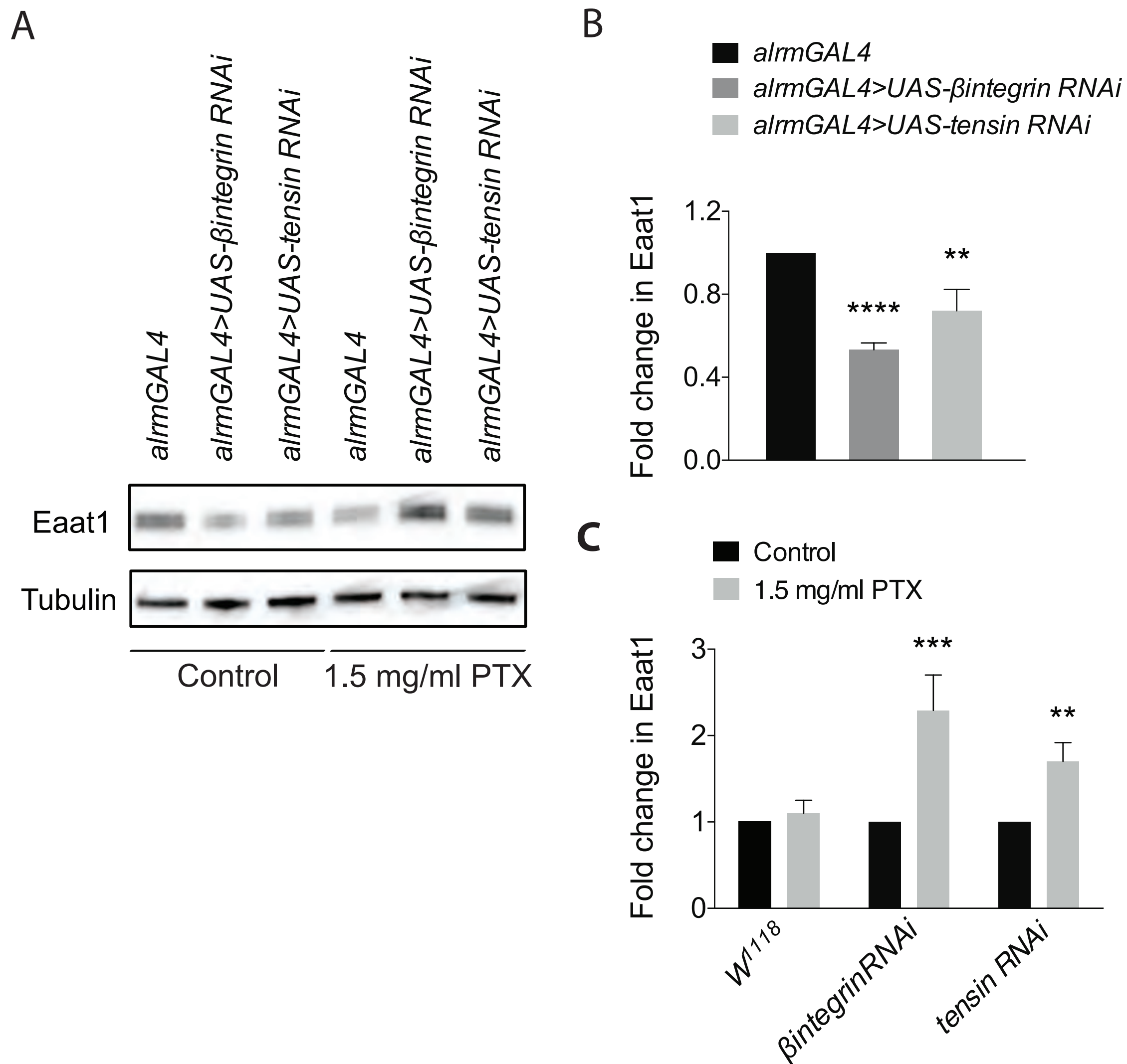


Figure 3.11: Astrocyte focal adhesion signaling regulates levels of the excitatory amino acid transporter EAAT1

A, Western blot analysis on 3rd instar 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). Error bars represent SEM. **P<0.005, ****<P0.0001; one-way ANOVA and Dunnett's multiple comparisons. C, Quantification of Eaat1

levels in western blot normalized to Tubulin signal and to the control lane ($N \geq 6$).

Error bars represent SEM. **P<0.005, ***<P0.001; Unpaired t test.

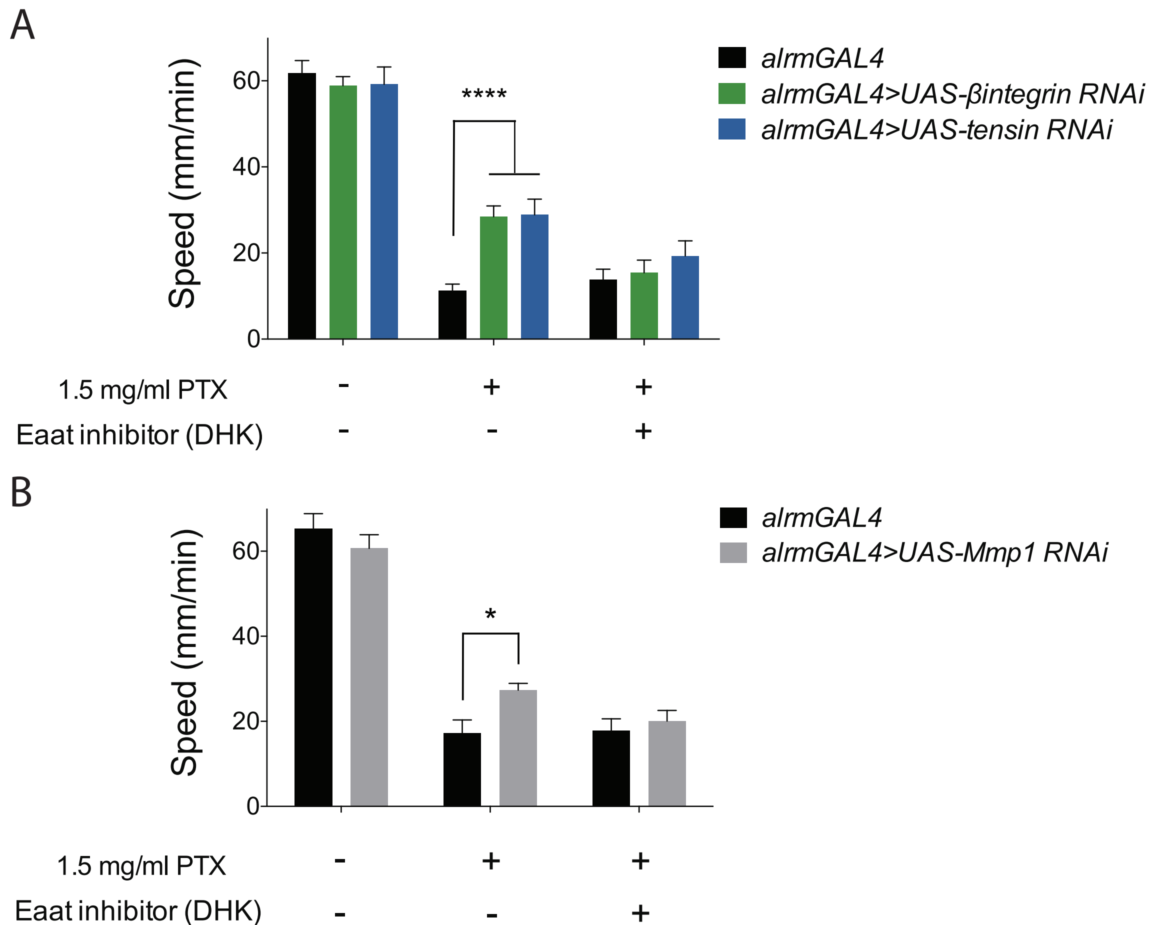


Figure 3.12: Glutamate transporter inhibitor reverses the behavioral effect of astrocyte depletion of focal adhesion genes or Mmp1

A, Pharmacological inhibition of EAAT function blocked βintegrin or tensin knockdown effect on PTX induced locomotion behavior. Dihydrokainic acid (DHK) was applied along with PTX and feeding period was 9 hr. Error bars represent SEM. ****P<0.0001; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 13; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 25; *alrmGAL4/+* (1.5 mg/ml PTX, 500 μM inhibitor), *N* = 16; *alrmGAL4>UAS-βintegrin RNAi* (0.0 mg/ml PTX), *N* = 12; *alrmGAL4>UAS-*

βintegrin RNAi (1.5 mg/ml PTX), *N* = 25; *alrmGAL4>UAS-βintegrin RNAi* (1.5 mg/ml PTX, 500 μM inhibitor), *N* = 18; *alrmGAL4>UAS-tensin RNAi* (0.0 mg/ml PTX), *N* = 8; *alrmGAL4>UAS-tensin RNAi* (1.5 mg/ml PTX), *N* = 17; *alrmGAL4>UAS-tensin RNAi* (1.5 mg/ml PTX, 500 μM inhibitor), *N* = 10 animals quantified. B, EAAT inhibitor also prevented suppression of PTX induced locomotion defect by astrocyte specific knockdown of Mmp1. Error bars represent SEM. *$P<0.05$; two-way ANOVA and Bonferroni's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 8; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 18; *alrmGAL4/+* (1.5 mg/ml PTX, 500 μM inhibitor), *N* = 20; *alrmGAL4>UAS-Mmp1 RNAi* (0.0 mg/ml PTX), *N* = 10; *alrmGAL4>UAS-Mmp1 RNAi* (1.5 mg/ml PTX), *N* = 21; *alrmGAL4>UAS-Mmp1 RNAi* (1.5 mg/ml PTX, 500 μM inhibitor), *N* = 28 animals quantified. Full statistical details are provided in Table 3.

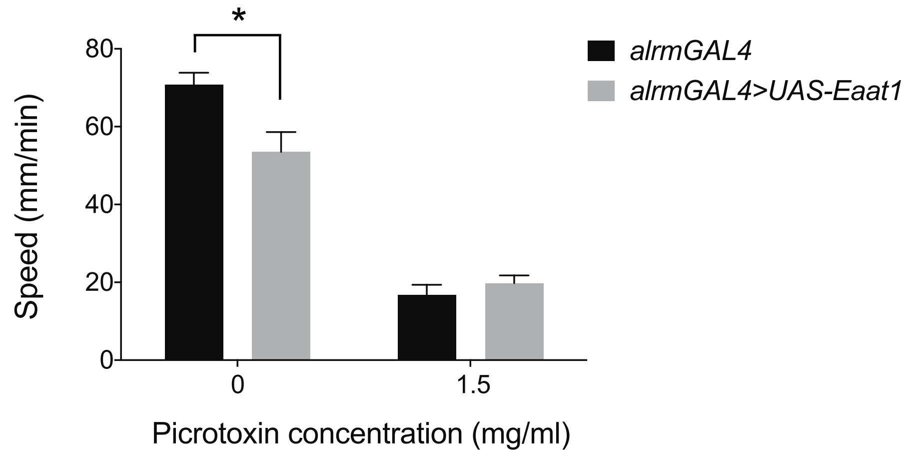
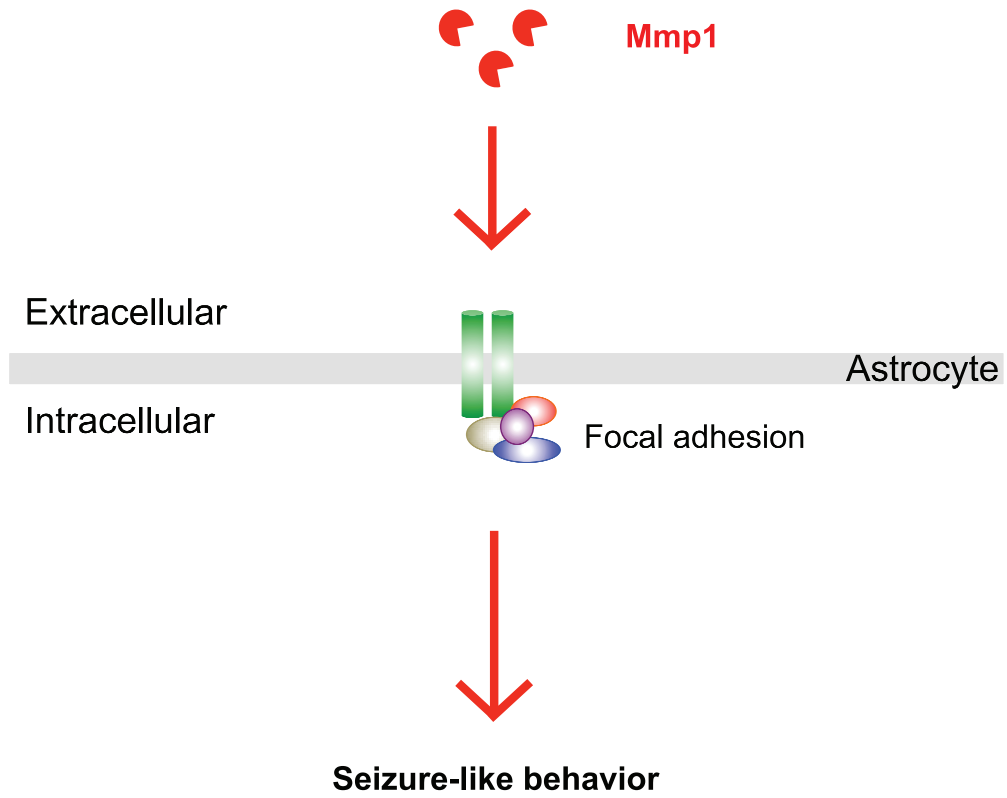


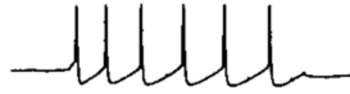
Figure 3.13: Genetic overexpression of Eaat1 in astrocytes affects normal locomotion and fails to suppress locomotion defect by PTX administration

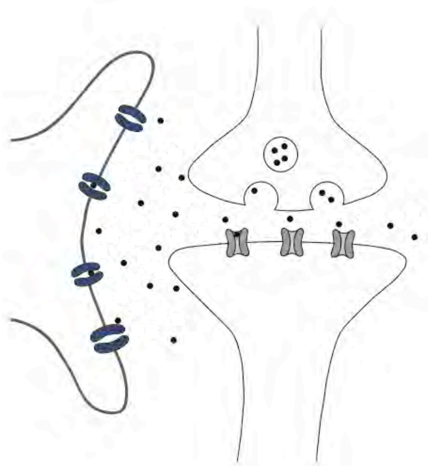
Overexpression of EAAT1 in astrocytes altered normal locomotion and did not suppress PTX-induced locomotion defect. Error bars represent SEM. **P<0.005; two-way ANOVA and Tukey's multiple comparisons. *alrmGAL4/+* (0.0 mg/ml PTX), *N* = 10; *alrmGAL4/+* (1.5 mg/ml PTX), *N* = 19; *alrmGAL4>UAS-Eaat1* (0.0 mg/ml PTX), *N* = 6; *alrmGAL4>UAS-Eaat1* (1.5 mg/ml PTX), *N* = 5 animals quantified. Full statistical details are provided in Table 3.


A
Mmp1
Extracellular
Astrocyte
Intracellular
Focal adhesion
Seizure-like behavior

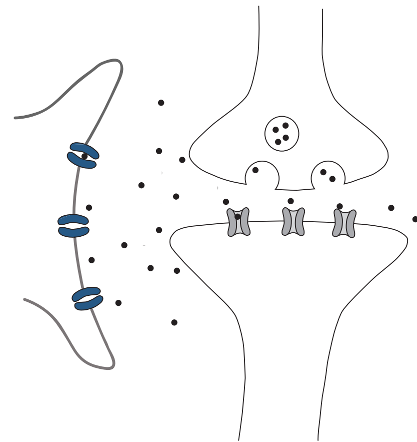
B

Glutamatergic synapse

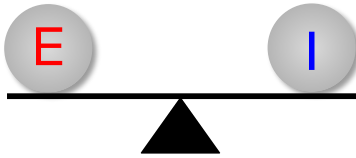


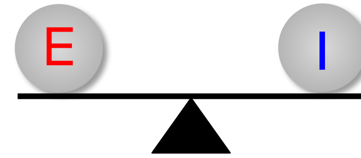


Wild-type astrocyte



FA signaling attenuated astrocyte





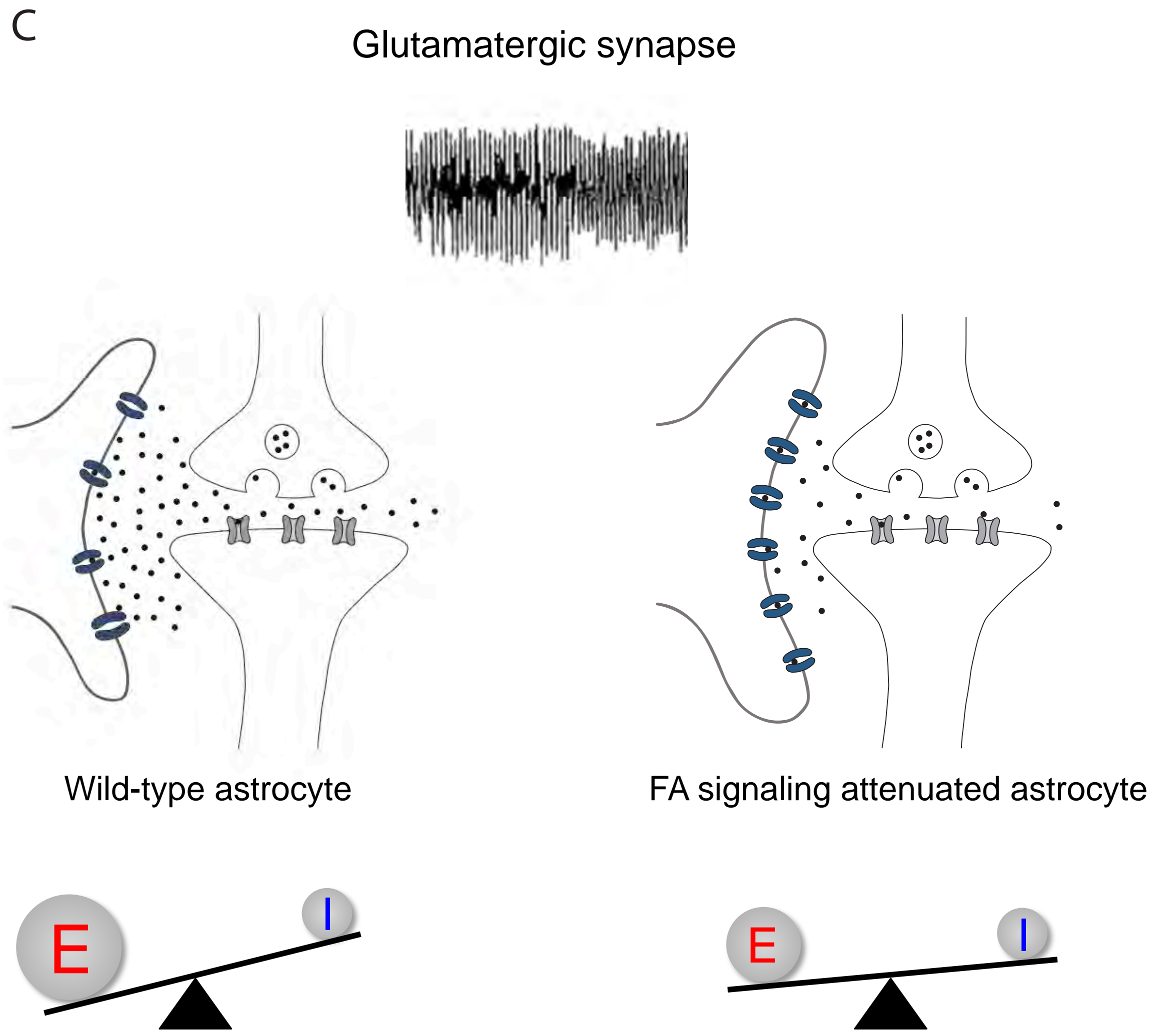


Figure 3.14: Working model

A model suggesting how focal adhesions in astrocytes might modulate glutamatergic synapses to regulate the balance between excitation and inhibition by altering their morphologies and EAAT1 expression during network hyperexcitability. A, Our data suggest that Mmp1 works upstream of the focal adhesion signaling in astrocytes to regulate seizure-like behavior that is induced genetically or pharmacologically. B, blockade of focal adhesion signaling in

astrocytes at basal levels of CNS excitability resulted in reduced astrocytic coverage of the neuropil and reduced expression of the excitatory amino acid transporter EAAT1. C, induction of hyperexcitability after depletion of focal adhesion signaling components resulted in enhanced astrocyte coverage of the neuropil and significant increase in EAAT1 levels, which might directly affect excitatory glutamatergic synapses to show the suppression of seizure-like behavior.

Discussion

We provide multiple lines of evidence that FA molecules regulate seizure behavior *in vivo*. Astrocyte-specific depletion of FA signaling molecules led to significant suppression of seizure behavior in the bang-sensitive model of hyperexcitability in adults and picrotoxin-induced hyperexcitability in larvae. A deeper analysis of astrocyte markers and morphology revealed that blockade of FA signaling under normal physiological conditions led to reduced coverage of the neuropil by astrocytes' fine processes and reduced levels of EAAT1 expression. This modulatory effect of FAs appears to be specific to EAAT1, as levels of the sole *Drosophila* GABA transporter GAT appeared unchanged in FA knockdown animals. These findings are consistent with a mammalian study showing astrocyte knockout of β1integrin leads to impaired glutamate uptake by astrocytes with decreased GLT-1 levels resulting in reactive astrogliosis and spontaneous seizures (Robel et al., 2015). However, decreased levels of EAAT1

by βintegrin or tensin knockdowns displayed normal locomotion, while EAAT1 depletion in astrocytes by RNAi caused significant defect in crawling behavior (Fig 3.3B, Fig 3.10C). There might be a delayed effect on changes in EAAT1 levels by focal adhesion knockdowns, which enables animals to bypass developmental defects. In fact, GLT-1 downregulation started to appear when β1integrin knockout mice were 6 months old (Robel et al., 2015). Nevertheless, our data suggest under normal physiological conditions FAs promote expression of EAAT1 in astrocytes and normal astrocyte coverage of the neuropil.

Under conditions of hyperexcitability FAs appear to negatively regulate EAAT1 expression and astrocyte infiltration of the neuropil. FA knockdown increased EAAT1 levels ~2-fold and led to greater coverage of the neuropil by astrocyte processes. EAAT1 does not appear to be regulated directly by picrotoxin-induced hyperexcitability, as picrotoxin exposure alone did not alter EAAT1 levels. We suspect that the simultaneous increase of astrocytic coverage of synapses and EAAT1 together underlie the enhanced recovery from seizure behavior. For instance, overexpression of EAAT1 alone did not block the ability of picrotoxin to induce seizure behavior (Fig 3.13). Therefore increased EAAT1 in astrocytes alone is not sufficient to account for the phenotypic rescue we observe in FA knockdown animals. 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-like behavior. In mammals, alteration of GLT-1 alone was sufficient promote

changes in long-term synaptic plasticity, but not basal synaptic activity (Filosa et al., 2009; Omrani et al., 2009). Interestingly, under conditions where there was a simultaneous increase in EAAT and astrocyte synaptic coverage, significant changes in basal synaptic activity were observed (Pannasch et al., 2014). Moreover, astrocyte coverage was modulated by changes in cell adhesion (Pannasch et al., 2014). We suspect the combined effect of increased EAAT1 with closer association with synapses leads to the suppression of seizure we observe, and that this is likely due to enhanced clearance of extracellular glutamate.

Precisely how FAs modulate astrocyte process extension and EAAT1 expression is an important next question. It is possible that activation of EAAT1 expression and genes required for astrocyte growth are downstream of FA activation. FAK is certainly a well-known regulator of transcriptional activity (Parsons, 2003). It is also possible that the decreased adhesive properties of cells in which FA signaling molecules have been depleted can respond more dynamically with process extension in response to pathological changes in neuronal activity. Connexin30 is a key modulator of cell adhesion in astrocytes and is required for astrocyte process extension (Pannasch et al., 2014), but whether this is regulated by FAs or neuronal activity remains an open question.

Materials and Methods

Fly strains. The following Drosophila strains were used: *w*^{1118} Canton S, *alrm-GAL4* (Doherty et al., 2009), *Eaat1-GAL4* (Doherty et al., 2009), *UAS-mCD8::GFP* (Lee et al., 1999), *eas*^{PC80} (Pavlidis et al., 1994), *by*^{33c} (Torgler et al., 2004), *tensin-GFP* (Torgler et al., 2004).

The following UAS-RNAi lines were from Vienna *Drosophila* Resource Center (Vienna, Austria): *UAS-byRNAi*$^{\#22823}$, *UAS-mysRNAi*$^{\#103704}$, *UAS-rheaRNAi*$^{\#40399}$, *UAS-FAKRNAi*$^{\#108608}$. The following lines were from Bloomington *Drosophila* Stock Center (Bloomington, IN): *UAS-Mmp1*$^{\#58700}$, *UAS-Mmp1RNAi*$^{\#31489}$, *UAS-Mmp2RNAi*$^{\#31371}$, *UAS-byRNAi*$^{\#38288}$, *UAS-mysRNAi*$^{\#33642}$, *UAS-rheaRNAi*$^{\#33913}$, *UAS-FAKRNAi*$^{\#29323}$.

Immunohistochemistry and confocal microscopy. *Drosophila* larval CNSs were dissected in PBS and fixed for 25 min at room temperature. 4 % paraformaldehyde was used for a routine fixation and Bouin's fixative was used to prepare samples for anti-Eaat1 immunohistochemistry. The following primary antibodies were used: rabbit anti-Eaat1 ((Peco et al., 2016)1:4000), rabbit anti-Gat ((Stork et al., 2014)1:2000), mouse anti-Bruchpilot (Developmental Studies Hybridoma Bank: nc82,1:20), mouse anti-Repo (Developmental Studies Hybridoma Bank: 8D12, 1:10), mouse anti-myospheroid (Developmental Studies Hybridoma Bank: CF.6G11, 1:50).

Immunoblotting. Drosophila CNSs were dissected in PBS and homogenized in SDS loading buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue). Samples were centrifuged at 16,000g for 10 min at 4 °C to collect supernatants. After boiling at 95 °C for 5 min, each samples were run on SDS-PAGE gels and transferred to nitrocellulose membranes. Appropriate primary antibody was diluted in blocking buffer (5% non-fat dry milk, 0.01 % Tween 20 in PBS) to incubate membranes overnight at 4 °C. Following with three 10-min washes, membranes were incubated with appropriate HRP-conjugated secondary antibody at room temperature for 1 hr. Chemiluminescence detection (ECL Plus Amersham) system was used to image signal with Fujifilm Luminescent Image Analyzer LAS-4000. Blots were analyzed using ImageJ Software. The following antibodies were used: 1:8,000 rabbit anti-Eaat1 (Peco et al., 2016); 1:10,000 rabbit anti-Gat (Stork et al., 2014); 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).

Bang-sensitive behavioral assay. The behavioral assay was modified from Song et al. (2008) and described in Muthukumar et al. (2014). Adult male flies after 3-7 days of eclosion were collected in fresh food vials the night before experiments. Vials containing 10 – 15 flies were subjected to mechanical stimulus using a VWR Vortex Mixer (VWR International, West Chester, PA) at a

maximum speed for 10 sec to induce paralysis and seizure in bang-sensitive mutant, *eas*^{PC80}. The numbers of flies standing and resuming normal behavior was noted at 10 s intervals for 4 min. Mean recovery time was calculated as the average time taken by an individual fly to recover from paralysis.

Picrotoxin feeding. Picrotoxin was dissolved in acetone and added to microwaved standard cornmeal agar to mix and make final concentrations of 0.5 and 1.5 mg/ml. Broad spectrum MMP inhibitor, GM6001 and EAAT2 (GLT-1) inhibitor, dihydrokainic acid were each prepared in DMSO and water respectively to mix in with food. The food with appropriate drugs was prepared the night before experiments. Early third instar larvae were collected to place in prepared drug containing food and 9 hr of feeding was allowed.

Larval locomotion behavior. Locomotion behavior was analyzed using FTIR-based Imaging Method (FIM) (Risse et al., 2013). Third instar larvae were collected and washed gently with water before placed on arena made of 0.8 % agar. Larval behavior was filmed with 10 frames per second for one minute. Crawling trajectory was analyzed with FIMTrack Software and the speed of locomotion was quantified using NeuronJ, ImageJ plugin.

Transmission electron microscopy. Experiments were conducted at the University of Massachusetts Medical School Electron Microscopy core facility.

For each condition, at least three animals were prepared for TEM procedure and analysis. Larval CNSs were dissected in PBS and immediately fixed in 2.5% gluteraldehyde in 0.1 M Sodium Cacodylate buffer pH 7.2. Briefly, fixed samples were moved into fresh 2.5% gluteraldehyde in 0.1 M Sodium Cacodylate buffer and left overnight at $4^{o}C$. The samples were then rinsed twice in the same fixation buffer and post-fixed with 1% osmium tetroxide for 1h at room temperature. Samples were then washed twice with DH_2O for 5 minutes and then dehydrated through a graded ethanol series of 20% increments, before two changes in 100% ethanol. Samples were then infiltrated first with two changes of 100% Propylene Oxide and then with a 50%/50% propylene oxide / SPI-Pon 812 resin mixture. The following day three changes of fresh 100% SPI-Pon 812 resin were done before the samples were polymerized at $68^{o}C$ in plastic capsules. The samples were then reoriented, and thin sections were taken at approximately 100 microns from the posterior tip of the ventral nerve cord. The thin sections (approx. 70 nm) were placed on copper support grids, and contrasted with Lead citrate and Uranyl acetate. Sections were examined using the FEI Tecani 12 BT with 100Kv accelerating voltage, and images were captured using a Gatan TEM CCD camera.

Statistical analysis. Graphpad Prism software was used to perform statistical analysis. Two-tailed Student's t-test or two-way ANOVA with either Dunnett's or

Tukey's post hoc test was performed and $P < 0.05$ was considered significant.

Error bars in bar graphs indicate SEM.

CHAPTER 4: 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 focal adhesion (FA) molecules strongly suppressed seizure-like behavior at both larval and adult stages. Astrocyte depletion of Mmp-1, a matrix metalloproteinase known to regulate β-integrin activation, also suppressed seizure activity, while overexpression of Mmp-1 had the opposite effect and enhanced seizures. Blockade of FA signaling under normal physiological conditions led to decreased coverage of the neuropil by astrocyte processes and reduced expression of EAAT1. However, upon induction of neuronal hyperactivity, depletion of FAs led to enhanced coverage of the neuropil by astrocyte processes and a 2-fold increase in EAAT1. Given that the rescuing effect of FA depletion after seizure was suppressed by pharmacological blockade of EAAT1, we propose the elevated levels of EAAT1 in FA knockdown animals, together with enhanced astrocyte coverage of the neuropil, is responsible for the behavioral rescue in seizure assays.

Astrocyte focal adhesion signaling during periods of hyperexcitability

Based on our RNAi screen in *Drosophila* adults with a mutation causing hyperexcitability induced seizure-like behavior, Tensin, an adaptor protein in a focal adhesion complex emerged as a candidate to suppress seizure when knocked down in astrocytes (Fig 3.1A). To confirm the specificity of the behavioral effect by tensin RNAi, a second RNAi line was tested and resulted in similar suppression (Fig 3.1A). When RNAi-mediated tensin knockdown efficiency in astrocytes was analyzed under confocal microscopy, we observed about a 40 % decrease in tensin level compared to control (Fig3.1B,B'). We suspect the actual efficiency of RNAi knockdown might be greater because we focused on the quantification of Tensin-GFP that fell within the domains of anti-Gat immunoreactivity, which only labels the membrane. In fact, when we express tensin RNAi under the control of tubulin-GAL4, a distinct pattern of tensin::GFP punctae was not detectable (Fig 3.1C).

Other components of focal adhesions including βintegrin, talin, and FAK phenocopied the behavioral effect by *tensin*^{RNAi} suggesting astrocyte focal adhesion signaling plays an important role in regulating hyperexcitability induced behavior (Fig 3.2). Characterization of seizure behavior induced by picrotoxin (PTX) in 3rd instar larvae corroborates the effect of astrocyte specific knockdown of FA molecules in the context of general network hyperexcitability (Fig 3.3A, B). Finally, the strength of suppression was not further enhanced by simultaneous expression of RNAi for β-integrin and tensin suggesting these molecules might function in the same genetic pathway. Though we would lose cell-type specificity

in that manipulation, in the future we could test if astrocyte depletion of βintegrin in tensin null mutant backgrounds had an additional suppression compared to *βintegrin*^{RNAi} alone.

Studies in rodents with conditional knock-out of β1integrin in ependymal zone stem cells have shown increased expression of Glial fibrillary acidic protein (GFAP) and astrocytic differentiation without altering oligodendrocyte or neuronal differentiation (North et al., 2015), which argues a somewhat specific role for integrin signaling in astrocytes rather than other glia. Indeed, another study found more direct linkage between integrin and astrocyte function in regulation of neuronal network (Robel et al., 2015). Using a GFAP-cre line, researchers knocked out β1integrin in astrocytes to induce astrogliosis and then investigated the effect of activated astrocytes on spontaneous seizure. While the genetic deletion of β1integrin was not entirely astrocyte-specific, the authors showed a strong correlation between the conditional knockout of β1integrin and development of seizure by neuronal hyperexcitability (Robel et al., 2015). Although we see no evidence that βintegrin depletion leads to reactive gliosis in *Drosophila* astrocytes, our data along with mammalian studies provide a strong argument that integrin associated focal adhesion in astrocytes plays an important role in a context of seizure and hyperexcitability.

How might focal adhesions signal in astrocytes?

Focal adhesions are macromolecular complexes at the cell-matrix interface to link actin-containing microfilaments and extracellular environment (Hotulainen, 2006). They change assembly and turnover rates rapidly in response to environmental cues, including gradients of chemokines, growth factors, or extracellular matrix (ECM) molecules to adapt cell shape and function (Le Clainche and Carlier, 2008; Lele et al., 2008; Lin et al., 1994; Lo, 2006; Ridley et al., 2003).

Adhesion is mediated by transmembrane receptors called integrins, a family of heterodimeric proteins consisting of α- and β-integrins. Upon integrin receptor activation, actin binding proteins, also known as adaptor proteins such as vinculin, talin, and tensin, are recruited to form adhesions and tightly link to control actin assembly and disassembly (Le Clainche and Carlier, 2008; Lin et al., 1994). Dynamic structural reorganization or remodeling of actin filaments governs a variety of cellular processes, such as cell motility, membrane dynamics, cell cycle control, cellular structure and cell signaling in various cell types including astrocytes (Farwell et al., 1995; Nicchia et al., 2008; Oberheim et al., 2008; Siegrist-Kaiser et al., 1990; Zhu, 2005).

Actin is a major cytoskeletal component that is essential to maintain and sculpt cell shape, and dynamic changes of actin cytoskeleton drive a variety of processes such as cell motility, cell division, and intracellular protein trafficking. Because of their dynamic nature, actin molecules in dendritic spines are intensively studied in the context of spine morphology and synaptic plasticity. In

addition to their functions in morphological changes, actins also play a critical role in providing a scaffold for neurotransmitter receptors and facilitating the trafficking of synaptic machinery (Cingolani and Goda, 2008). Actins are enriched in astrocytes, especially in distal fine processes, where they can mediate rapid shape changes (Lau et al., 2011; Lavialle et al., 2011; Stork et al., 2014). Do changes in activity alter actin dynamics and in turn morphology of astrocytes? *In vitro* studies have shown that treatment with the excitatory neurotransmitter glutamate can induce filopodia formation in astrocytes and increase actin cytoskeleton-based motility (Cornell-Bell et al., 1990; Lavialle et al., 2011). Long-term potentiation in the hippocampus significantly changed the ramification of astrocyte processes by increasing surface area (Wenzel et al., 1991) and an *in vivo* study shows motor skill learning induces an increase in astrocytic volume in the cerebellar cortex (Kleim et al., 2007). Given that focal adhesions provide a direct link between the extracellular space and actin, I propose that changes in the actin cytoskeleton downstream of focal adhesion signaling drives morphological changes of astrocytes, which in turn modify their physiological interactions with synpases (e.g. increased Glu buffering). The dynamic nature of actin cytoskeleton is also associated with changes in the surface exposure of membrane proteins. In a variety of settings, reorganization of actin cytoskeleton increases membrane protein expression and trafficking to the surface (Chen et al., 2013; Gu et al., 2010; Lau et al., 2011). For instance, actin remodeling in cultured astrocytes by the Rho kinase inhibitor increases

astrocyte glutamate transporter (EAAT1 and EAAT2) levels and activity (Lau et al., 2011). Since changes of neurotransmitter transporter levels in astrocyte membranes directly shape synaptic activities (Murphy-Royal et al., 2015; Muthukumar et al., 2014), this implies changes in astrocyte morphology could have a strong association with astrocytic regulation of synapses. Finally, since integrin-mediated focal adhesions have diverse signaling functions based on their molecular components and cellular context, especially during development of the nervous system and synaptogenesis, activation of glia, or stabilization of the endothelium and blood-brain barrier (Milner and Campbell, 2002), our findings opens up a number of avenues for exploration to define the precise mechanisms by which FAs alter animal responses to hyperexcitability.

MMP as a potential activator of FA signaling in astrocytes

Integrins, heterodimeric cell surface receptors from focal adhesion complexes, receive signaling from the extracellular matrix (ECM). ECMs are the substrates for the matrix metalloproteinases (MMPs), extracellular zinc-dependent endopeptidases and their enzymatic activity leads to the cleavage of ECM proteins which then activate integrin signaling (Lukashev and Werb, 1998; Werb, 1997).

Interestingly, there is a strong correlation between neuronal activity and MMP expression and/or activity. Under normal physiological conditions, the expression of MMP9 in particular has been reported to be essential to form

neuronal activity-dependent plasticity in rodent cortices as well as hippocampus (Kaliszewska et al., 2012; Nagy et al., 2006; Spolidoro et al., 2011). In addition, increased expression and enzymatic activity of MMPs are known to be a critical element in the development of seizure and cocaine relapse and relapse-associated synaptic plasticity (Smith et al., 2014; Wilczynski et al., 2008). Finally it has been suggested that chronic elevation of network activity can increase activation of MMPs (Huntley, 2012). These observations argue that increased activity leads to increased MMP signaling, which could in turn regulate integrin receptor-mediated focal adhesion signaling in astrocytes in a hyperexcitable network. Consistent with this interpretation, by depleting Mmp1 expression in astrocytes, we were able to observe the same suppression effect as focal adhesion knockdowns on both genetically and pharmacologically induced seizure assays (Fig 3.6). When we conversely overexpressed astrocyte Mmp1, enhancement of seizure phenotype was observed in the genetic seizure model (Fig 3.6A, A'). These data provide a strong *in vivo* link between the level of MMP expression and animal responses to hyperexcitability. Based on the preliminary data from the Freeman lab comparing translating mRNA transcripts between neurons and astrocytes using a technique called Translating Ribosome Affinity Purification (TRAP), Mmp1 mRNA showed 7-fold enrichment in astrocytes compared to neurons in 3rd instar larval brains. Thus, astrocytes appear to be the major Mmp1 expressing cell type in the CNS and attempting to deplete Mmp1 from a minor source of the protein might have resulted in insufficient

knockdown. This hypothesis is also supported by the fact that the degree of suppression we observed with pharmacological inhibitor of MMP in the PTX assay (Fig 3.6C) was comparable with the result from the expression of Mmp1 RNAi in astrocytes (Fig 3.6B). From our assays for phenotypic interactions between Mmp1 and focal adhesion molecules in PTX-induced seizure-like behavior, we found that addition of the MMP inhibitor had no enhanced rescuing effect on animals expressing βintegrin RNAi suggesting Mmp1 and βintegrin work in the same genetic pathway in astrocytes to regulate this behavior (Fig 3.8A). Further pathway analysis utilizing genetic overexpression of Mmp1 in astrocytes with integrin receptor knockdown showed that Mmp1 may exert its effects through βintegrin-mediated focal adhesion signaling to regulate hyperexcitability induced behavior (Fig 3.8B). The simplest interpretation is that Mmp1 is secreted by astrocytes, where it acts outside the cells—it is secreted as a pro-MMP form and requires extracellular activation to become enzymatically active (Vandenbroucke and Libert, 2014)—to break down ECM and activate FA signaling. Consistent with this notion Mmp1 overexpression was able to reverse the effect of talin RNAi in the same behavioral assay, but not that of the receptor knockdown. One possible explanation is that expression of talin is not absolutely required for integrin expression and signaling (Brown et al., 2002; Conti et al., 2009; Liu et al., 2011). Thus, even with significant depletion of expression of talin in astrocytes, increased Mmp1 may overcome this loss due to redundancy with other FA molecules, or partial Talin knockdown, while loss of the βintegrin

receptor cannot be overcome because it is absolutely essential for Mmp1 to exert its effects.

A number of questions remain regarding the interactions between MMPs and the focal adhesion signaling in a context of hyperexcitability. First, is there elevated expression or activity of Mmp1 in response to PTX treatment? Oral consumption of PTX induces a global stimulation of neuronal activity resulting in seizure in *Drosophila* 3rd instar larvae (Stilwell et al., 2006). Electrophysiological recordings on body wall muscles of larval preparations with preserved CNS showed seizure activity with PTX application. The PTX effect was abolished when motor neurons are severed from the nerve cord, suggesting PTX affects the excitability in the CNS to elicit seizure-like behavior (Stilwell et al., 2006). Thus, our method to induce network hyperexcitability is likely robust. In rodents, astrocytes constitutively express MMPs *in vitro* as well as *in vivo* and their expression becomes upregulated when synaptic activity is heightened with administration of kainate, an agonist for non-NMDA glutamate ionotropic receptors (Szklarczyk et al., 2002). Therefore we hypothesize there is elevated expression and/or activity of Mmp1 with PTX administration in *Drosophila* 3rd instar larvae which leads to increased βintegrin signaling as MMPs degrade extracellular matrix that are ligands for integrin receptors. Based on previous reports, we would speculate that the time course of MMP activation and termination would be relatively fast (1 – 2 hours after intense stimulation) (Huntley, 2012; Nagy et al., 2006). An experiment where one examined the time

course of Mmp1 activation after PTX feeding will be required to determine the precise effects.

The primary source of MMPs (which we propose is astrocytes) and their mechanism of activation is another important area requiring clarification. MMPs can be activated by each other (Huntley, 2012) or free radicals like nitric oxide (NO) (Huntley, 2012). Increased synaptic stimuli such as long-term potentiation activates postsynaptic NMDA receptor upon presynaptic glutamate release which in turn triggers rapid release of nitric oxide (NO) into the perisynaptic environment (Huntley, 2012; Schuman and Madison, 1991). In the future it will be important to find the source that kindles the initial activation of MMPs to determine more efficient ways to regulate focal adhesion signaling *in vivo*.

Finally, what is the mechanism of integrin receptor activation by Mmp1? Is it through a specific ECM protein that is enzymatically processed by Mmp1? Or is it a direct action of Mmp1 on integrins? Degradation of ECM by MMPs activates integrin receptors (Stefanidakis and Koivunen, 2006; Werb, 1997). Studies have also shown direct enzymatic action of MMPs on integrin receptors, integrin β1 itself is shown to be a substrate for MMP2 (Kryczka et al., 2012). Thus, both direct and indirect effects of MMP activity on integrin receptors could contribute to the activation of focal adhesion signaling in astrocytes.

Astrocyte morphological changes in association with neuronal activity

Astrocytes have highly ramified morphologies to infiltrate synaptic regions. This structural characteristic enables one astrocyte contact with more than 100,000 synapses in rodents and ~1,000,000 synapses in the human brain (Oberheim et al., 2006; Singh et al., 2015). Distal processes of astrocytes are thought to be the main compartments that are responsible for astrocyte-neuron interaction. These very thin filopodia-like processes (often 50 -100 nm wide) that are actin rich were first visualized by Derouiche and colleagues in 2001 with immunolabeling of actin binding protein, ezrin (Derouiche and Frotscher, 2001). Like any other cellular processes that are actin rich, these fine processes can be rapidly extended or withdrawn from synapses in response to neuronal activity (Lavialle et al., 2011). Understanding how astrocytes develop and maintain their dynamic morphology has been a great interest because these fine processes not only position themselves close to synapses but also their membranes are densely occupied by ion channels, neurotransmitter receptors, and transporters (Vernadakis, 1996; Walz, 1989). Thus these morphological and functional characteristics of astrocyte membranes are essential components to investigate astrocytic contribution on neuronal activity and circuit behavior.

Initial observations of the dynamics of astrocyte processes in response to neuronal signaling were in cultured hippocampal astrocytes. With brief 30 sec exposure to the neurotransmitter glutamate in a culture dish where primary neurons and astrocytes sit, researchers observed formation and extension of filopodia along the edge of astrocyte membrane (Cornell-Bell et al., 1990).

These plastic changes of astrocyte processes also exist in the intact brain. In the hypothalamic supraoptic nucleus, while neurons undergo substantial remodeling during lactation, astrocytes also alter their morphologies to regulate glutamate concentration at synapses. This striking structural remodeling is highly plastic (Oliet et al., 2001). A later study, where mouse somatosensory cortex was used to investigate structural and functional plasticity of astrocytes upon neuronal activity, revealed whisker stimulation elicited increased astrocyte coverage as well as enhanced expression of GLT1 and GLAST (astrocyte specific glutamate transporters) in the cortex (Genoud et al., 2006). An additional *in vivo* study in the mouse cerebellum corroborated the notion that astrocytes alter the extent of their processes in response to neuronal stimuli: motor skill learning through consecutive training induced the hypertrophy of astrocyte processes and again the change was reversed when training was terminated (Kleim et al., 2007). Despite increasing evidence supporting the existence of plastic changes in astrocyte processes in association with altered neuronal activity, we know almost nothing about the molecular cascades regulating the morphology of fine astrocyte membranes.

We investigated changes of astrocyte processes and their association with synapses at the ultrastructural level using electron microscopy. Our ultrastructural analysis revealed that astrocyte processes undergo significant alterations in their morphological parameters by astrocyte specific depletion of βintegrin or in response to increased neuronal activity. This is the first

experiment, to my knowledge, to show morphological plasticity in mature *Drosophila* astrocytes in response to altered neuronal activity. By comparing control animals to animals expressing βintegrin RNAi in astrocytes, we found that percent of coverage by astrocyte processes in neuropil of *Drosophila* 3rd instar ventral nerve cord was decreased by ~20% suggesting βintegrin containing focal adhesion signaling regulates the extent of astrocytic processes in the neuropil (Fig 3.9 A, B). It is important to note that when we visualized astrocytes with expression of membrane-tethered GFP under confocal light microscopy, gross morphology and positioning of astrocytes was unchanged (Data not shown. Data generated by Allie K Muthukumar). In all likelihood, the fine processes of astrocytes that infiltrate deep into neuropil cannot be entirely captured by light microscopy due to resolution limitations. Interestingly, when βintegrin knockdown animals were subjected to increased neuronal activity by PTX treatment, we were able to see a significant increase in the extent of astrocyte processes compared to mock treatment while control animals showed no alterations in astrocyte coverage (Fig 3.9 A, B). These data strongly suggest that inhibition of focal adhesion signaling allows for more dramatic changes of astrocyte morphology, in other words focal adhesion signaling negatively regulates astrocyte coverage during elevated neuronal activity. Furthermore, this morphological change in βintegrin RNAi expressing animals allows astrocytes to position their membrane closer to synapses thereby enhancing astrocyte coverage of synaptic elements (Fig 3.9C). To elaborate, by changing the

distribution of astrocytic processes toward synapses, neurotransmitter transporters in astrocyte membranes are placed more closely to the neurotransmitter release sites and can more directly shape synaptic transmission.

Also worth noting, we did not observe complete wrapping of synapses by astrocyte processes as reported in certain mammalian brain regions (Genoud et al., 2006) and this is consistent with observation from another EM study in *Drosophila* 3rd instar larvae (MacNamee et al., 2016; Stork et al., 2014). We can only speculate but we do not think that this comes from differences in organisms because even in mammalian brain, the cortical region has less than 10 % of synapses ensheathed by astrocytes (Genoud et al., 2006).

An important consideration regarding our EM study is that within our sampled volumes, it is possible that the measurement comes from the astrocyte processes that belong to as few as one astrocyte. Even though a previous study suggested it is unlikely that *Drosophila* astrocytes have heterogeneity in terms of genetic programming (Stork et al., 2014), it still remains a possibility that each astrocyte responds differently with their morphology to neuronal activity depending on their neighboring neuronal cell types and molecular environment. With this in mind, it is possible that we may have missed capturing heterogeneity in terms of morphological changes that could exist between different segments or different positioning within the VNC. In the future, one could use the more high-throughput approach of super-resolution microscopy, which can give us twice the resolution (~100 nm) of regular confocal microscopy, potentially resolving

astrocyte fine processes. Combined with genetic tools to label astrocyte membrane in a clonal manner, this could allow us analyze the morphology of single astrocytes from different segments and positions (e.g. thoracic vs abdominal or dorsal vs ventral).

How can restricted expression of cell adhesion molecules permit structural plasticity in astrocytes in response to altered neuronal activity? One possibility is that βintegrin depleted astrocytes can more readily expand their processes upon PTX treatment, perhaps due to reduced adhesiveness or they simply have more room to expand since they are hypotrophic compared to wild-type astrocytes in control conditions. Astrocytes certainly appear capable of significant expansion in the *Drosophila* CNS in response to certain conditions. For instance, astrocytes organize their spatial domains not to intrude in each other's space and this so called "tiling" behavior is well conserved in *Drosophila* astrocytes (Stork et al., 2014). However, if a neighboring astrocyte is removed by genetic ablation, the remaining astrocytes extend their domain into the open neuropilar space. Having reduced cell adhesion molecules on astrocyte membranes could also enhance morphological alterations upon hyperexcitability. It is well defined that cell morphology is strongly dependent on adhesive properties and integrin containing focal adhesion complexes are the major force for the cell adhesion (Parsons et al., 2010). Previous studies on how connexin 30 in astrocytes can elongate their processes and enhance ramification through its cell adhesion regulatory behavior complement this hypothesis (Pannasch et al., 2014). Our findings certainly leave

many open questions. Could this morphological change be preferential towards certain types of synapses (glutamatergic, GABAergic, or cholinergic to name a few)? If so, are there other astrocytic molecules involved in the process other than focal adhesions? This could potentially be answered by exploring some of the remaining hits from our screen. Are there direct changes we could observe in astrocyte motilities by genetic alterations and/or neuronal activity using higher resolution microscopy methods? Astrocyte processes are capable of rapid extension and retraction via actin cytoskeleton remodeling which can be more dynamic than dendritic spines (Haber, 2006). Since focal adhesions are the main regulator of actin cytoskeleton, one can easily imagine how altering focal adhesions in astrocytes can affect motilities of their processes.

Finally, although we observed no significant changes in overall synapse number in our study by manipulation of activity or βintegrin signaling, this does not necessarily mean there are no alterations in composition of different types of synapses. For example, there maybe increased numbers of inhibitory synapses and decreased excitatory synapses so that they have hypoactive network with βintegrin knockdown in astrocytes that leads to elevated threshold for the seizure-like behaviors. Future work defining the precise synaptic changes that occur in response to astrocyte-specific alteration of FA signaling will be essential for us to answer these questions. Additionally, changes in intrinsic properties of neurons can be another factor to consider in terms of synaptic activity. Homeostatic plasticity is a cell-autonomous mechanism in neurons to balance

between excitation and inhibition by scaling up or down the postsynaptic AMPA receptor levels (Sun and Turrigiano, 2011; Turrigiano et al., 1998). Treatment of pictoroxin for 24 hr to induce chronic changes in neuronal activity leads to homeostatic adjustments of synaptic AMPA receptors resulting in reduced excitatory action (Turrigiano et al., 1998). While 9 hr of picrotoxin treatment might not be long enough to induce homeostatic changes, it might be an interesting avenue for future studies since homeostatic plasticity in *Drosophila* CNS has not been explored.

Glutamatergic signaling and EAAT1 regulation

Glutamate is the primary excitatory neurotransmitter in the vertebrate nervous system. Being such an essential component of the nervous system, understanding development and maintenance of glutamatergic synapses and mechanisms underneath their signaling cascades has been one of the main focuses in the field of neuroscience. On top of the intensive focus on understanding the physiology of glutamatergic synapses from a neuron-centric perspective, significant efforts have been put into studying glial regulation of glutamate-mediated signaling, and here astrocytes play a critical role. Once glutamate is released from presynaptic neurons, it is critical that extracellular glutamate is properly cleared from the extracellular space to terminate activation of postsynaptic receptors and prevent an excitotoxic environment. Because of the lack of degradative enzymes for glutamate at the synapses, transporter-

mediated uptake is the only known mechanism of the neurotransmitter clearance. Among five different subtypes that are identified in mammals, EAAT1 (GLAST) and EAAT2 (GLT-1) have been characterized as functionally prevalent glutamate transporter subtypes with selective expression in astrocytes (Chaudhry et al., 1995; Danbolt, 2001). Along with concentrated expression pattern near glutamatergic synapses, direct implication of the glial glutamate transporter GLT-1 and extracellular glutamate levels has been shown with genetic deletion of the transporters (Rothstein et al., 1996).

Drosophila also utilize glutamate as a major neurotransmitter and glutamatergic synapses are abundant both in CNS and PNS (Daniels et al., 2008). *Drosophila* neuromuscular junctions (NMJs) have served as an excellent system to study development and function of glutamatergic signaling because of their easy access using techniques like electrophysiology, dye labeling, and Ca^{2+} imaging. Furthermore the fly molecular genetic toolkit empowers the system by allowing rapid generation of genetic mutants with high temporal and spatial control (Collins and DiAntonio, 2007).

In the *Drosophila* CNS, abundant expression of vesicular glutamate transporters (VGLUT) in neurons and enrichment of glutamate transporter (EAATs) in neuropil glia strongly suggest an important contribution of glia to regulation of glutamatergic signaling in invertebrates (Daniels et al., 2008; Stacey et al., 2010). Recent studies unraveled critical functions of *Drosophila* glial glutamate transporter (EAAT1) in glutamate buffering and neuronal circuit

function as well as behavior (Rival et al., 2004; Stacey et al., 2010). *Drosophila* EAAT1 is highly conserved in terms of amino acid identity compared to human EAAT1 and EAAT2 and it is less likely to complicate studies of EAAT1 loss of function since it is a sole high-affinity glutamate transporter in *Drosophila* (Besson et al., 2000). In addition, it is selectively expressed in a subpopulation of CNS glial cells (Freeman et al., 2003; Soustelle et al., 2002). While other glial subtypes express EAAT1 in the fly CNS, its expression in astrocytes is prominent (Doherty et al., 2009; Peco et al., 2016) (Fig 3.10A). Previous studies have also shown that EAAT1 expression is in close proximity to VGlut synaptic punctae, which strongly imply potential modulation in glutamatergic neurotransmission by glial EAAT1 (Daniels et al., 2008; Stacey et al., 2010). Loss-of-function mutation data provide direct evidence that EAAT1 acts on glutamatergic neurotransmission regulating the rhythmic patterning of motor neuron activity to control locomotor behavior (Stacey et al., 2010). Also, inactivation of EAAT1 by RNA interference led to various neurological defects including oxidative stress, neurodegeneration, and shortened life span implying glutamate buffering by EAAT1 is required for maintenance of healthy brain (Rival et al., 2004).

We know very little about how EAATs are regulated in glia. Fringe-mediated neuron-glia communication has been implicated as a regulative mechanism of *Drosophila* EAAT1 expression within a glial subtype called longitudinal glia during development of the nervous system (Stacey et al., 2010).

However, how their expression level is controlled, particularly by astrocytes, in response to neuronal activity has not been explored much.

While EAAT1 protein expression in the whole brain from wild-type animals does not appear to be changed upon 9 hr of PTX application, there was about two-fold increase in EAAT1 level by knocking down either βintegrin or tensin in astrocytes when we compare with or without PTX treatment (Fig 3.11A, C). These findings strongly suggest during network hyperexcitability, EAAT1 expression is increased, presumably to allow for better glutamate clearance at synapses in RNAi groups resulting in suppression of seizure-like behavior.

While we did not observe any alterations in EAAT1 level in wild-type with increased neuronal activity, previous work in rodents showed increasing neuronal activity by whisker stimulation results in enhanced expression of astrocyte glutamate transporters (GLT-1 and GLAST) suggesting neuronal activity is linked to EAAT regulation (Genoud et al., 2006). This discrepancy may be explained by the differences in time scale of the experimental settings. We only waited for 9 hr after PTX administration before we performed quantitative western blot analysis, while Genoud et al. observed an increase in the transporters after 24 hr of whisker stimulation. In fact, 1 hr after longer-term potentiation (LTP) stimulation or contextual fear conditioning does not lead to any changes in protein expression of GLT-1 and GLAST in rat hippocampus (Levenson et al., 2002). We also observed a significant down regulation of EAAT1 in astrocyte knockdown of focal adhesion molecules compared to wild-type. These findings

are consistent with a mammalian study (Robel et al., 2015) showing astrocyte knockout of β1integrin leads to impaired glutamate uptake by astrocytes with decreased GLT-1 levels. However, decreased level of EAAT1 by βintegrin or tensin knockdowns displayed normal locomotion when EAAT1 depletion in astrocytes by RNAi caused significant defect in crawling behavior (Fig 3.10C). The reason for this difference is unclear, but may result from a delayed effect on changes in EAAT1 levels by focal adhesion knockdowns (i.e. RNAi), which might enable animals bypass any developmental defects. In fact, GLT-1 downregulation started to appear when β1integrin knockout mice were 6 months old (Robel et al., 2015).

To further explore the functional contribution of EAAT1 to the behavioral phenotype in focal adhesion knockdowns, we utilized pharmacological inhibitor for glutamate transporter, dihydrokainate (DHK) in our PTX-induced behavioral assay. We found that the suppression phenotype in animals expressing either βintegrin or tensin RNAi in astrocytes was significantly rescued with co-application of DHK with PTX (Fig 3.12A). Likewise, DHK application also reversed the effect of Mmp1 RNAi in the behavioral assay (Fig 3.12B). These data strongly argue that the suppression of seizure-like behavior in animals with astrocyte focal adhesion knockdown is due, at least in part, to increased EAAT1 expression that could relieve hyperexcitability by regulating extracellular glutamate concentrations. Administration of DHK itself did not affect the locomotion behavior of 3rd instar larvae (Fig 3.10B). This is not surprising

because studies in mouse neocortex have shown that glutamate uptake inhibition by pharmacological inhibitors, including the same inhibitor used in this study, does not affect the amplitude or decay kinetics of AMPA-mediated EPSCs in layer II/III pyramidal neurons (Campbell and Hablitz, 2004). However, in the presence of bicuculline, another $GABA_A$ receptor blocker lowered the threshold for evoking seizure activity and prolonged the duration of seizure activity (Campbell and Hablitz, 2004). This implies that glutamate clearance at synapses becomes more critical for the network excitability when a substantial number of neurons are activated.

Further work will be necessary to dissect which glial subtype contributes most significantly to the alteration of EAAT1 level in the larval CNS, if it is not solely astrocytes. Nevertheless, our data highlight that glial EAAT1 expression and function is regulated by focal adhesion signaling in astrocytes during network hyperexcitability.

Currently we can only speculate why overexpression of EAAT1 in astrocytes shows no effect on PTX-induced locomotion defect (Fig 3.13). It could reflect differences between acute versus chronic upregulation of EAAT1. In fact, genetic overexpression of EAAT1 in astrocytes alone has impaired larval locomotion significantly, which could mean proper regulation of extracellular glutamate is critical for controlling motor function *in vivo*; ectopic expression of this protein over the course of development could lead to compensatory changes in the network; or levels of membrane exposure are regulated post-translationally

(Schlag et al., 1998). To bypass developmental changes by EAAT1 overexpression, we could utilize temperature-sensitive GAL80 molecule to control the timing of protein overexpression or pharmacological approach with antibiotics called β-lactam. β-lactam has been shown to increase GLT1 expression in rodents to modulate neuronal plasticity, neuroprotection and behavior (Bellesi et al., 2009; Omrani et al., 2009; Rothstein et al., 2005). These alternative approaches would allow us to eliminate potential developmental compensation by chronic overexpression of EAAT1. Lastly, our favored hypothesis to explain EAAT1 overexpression data is that increasing EAAT1 without changes of astrocytic processes is not sufficient for the necessary levels glutamate clearance during intense neuronal activity to rescue behavioral defects. In fact, recent studies have shown that increasing GLT1 density on astrocytes without their protrusions toward synaptic clefts made no changes on AMPA receptor currents (Pannasch et al., 2014). To test whether both increased EAAT1 level and astrocyte coverage of the neuropil are required to suppress seizure-like behavior in the animals with focal adhesion knockdowns, we can block protein synthesis using cycloheximide during PTX feeding to see if it still suppresses the behavior. In parallel, withdraw cycloheximide afterward and examine if the suppression of the behavior comes back. If indeed both modifications in astrocytes, increased neuropil coverage and EAAT1 level are required to ameliorate the imbalance between excitation and inhibition, it is even

more plausible to think excitatory glutamatergic synapses are the most affected by astrocytes to suppress seizure-like behavior.

Going forward, significant work will be required to determine detailed mechanisms underlying EAAT1 regulation by astrocyte focal adhesion signaling. To determine whether the changes in EAAT1 protein levels are mediated by transcriptional or post-transcriptional modification, we could perform quantitative PCR to compare EAAT1 mRNA levels between genotypes (wilde-type vs astrocyte focal adhesion RNAi) and treatments (control food vs PTX containing food). Transcriptional regulators downstream of βintegrin could be involved in regulation of EAAT1 expression. The link between astrocyte glutamate transporter expression and the nuclear transcription factor nuclear factor-κB (NF-κB) has been reported previously(Lee et al., 2008; Sitcheran et al., 2005) and later it was defined that NF-κB binds directly to GLT1 promoter regions to regulate the transcript, which requires neuronal signaling (Ghosh et al., 2011). Other downstream effectors of focal adhesion signaling and their potential roles are discussed in Appendix1.

In addition, there are many levels of crosstalk between mentioned intracellular signaling pathways (Ben Haim et al., 2015) which means we need to develop more careful and elegant approaches on studying regulatory mechanisms of EAAT1 for future studies.

Beyond changes in protein expression, another mechanism to modulate transporter function is alteration of membrane surface exposure dynamics (e.g.

insertion, surface diffusion and membrane trafficking). Neuronal glutamate transporters have revealed that trafficking of the transporters from cytosol to the membrane is important mechanism by which synapses maintain appropriate synaptic strength during LTP and fear conditioning (Levenson et al., 2002). A recent study using a single nanoparticle (quantum dot,QD) tracking technique found that surface diffusion of astrocyte glutamate transporter, GLT1 was a key mechanism to maintain synaptic glutamate homeostasis *in vitro* (Murphy-Royal et al., 2015). Thus, to further improve our knowledge of the transporter contribution on regulating glutamate concentration at synapses, understanding their membrane dynamics is a necessary avenue.

Concluding remarks

The balance between excitatory/inhibitory inputs in the central nervous system has to be in a certain range to maintain normal and healthy brain function. This balance is compromised in many neurological disorders, in which neurons experience chronically elevated activity (Heinemann, 2004; Rubenstein, 2010; Yizhar et al., 2011).

Astrocytes have tight coupling with neuronal synapses and their membranes are equipped with molecules to regulate network activity. My thesis work has contributed to understanding of how astrocytes regulate neuronal activity to eventually adjust excitatory/inhibitory balance in the brain network. My work demonstrated that focal adhesion signaling in astrocytes is a critical pathway for

their association with synapses and expression of glutamate transporters in response to changing network environment. Especially the alterations of glutamate transporters can result in direct modulation of excitatory synaptic signals, which ultimately influence the balance between excitation/inhibition. Additionally, our comprehensive screen suggests a deeper analysis of astrocyte molecules to understand how astrocytes modulate CNS excitability and signaling.

Appendix

Potential downstream targets of astrocyte focal adhesion signaling

Activation of the c-jun N-terminal kinase (JNK) cascade upon integrin activation has been well documented (Jeffrey et al., 2007). Thus we expressed two different RNAis for JNK in astrocytes to test if PTX-induced seizure behavior is modified. While only one of the RNAi lines showed a significant suppression of the behavior, both RNAi lines resulted in a severe defect in basal locomotion when they were expressed in astrocytes (Fig A.1A). Given that JNK/c-Jun signaling pathway regulates such a wide range of cellular processes; it is not surprising that JNK signaling is required for normal locomotion (Figure A.1A). There is a close association between activation of focal adhesion signaling and ROCK (Rho-associated, coiled-coil containing protein kinase) signaling (Goetsch et al., 2014; Wozniak et al., 2004). In addition, ROCK regulation of astrocyte morphology and EAAT expression has been documented *in vitro* cell culture system (Lau et al., 2011). However, when we altered ROCK expression or activity with genetic and pharmacological approaches, no changes in seizure-like behavior by PTX treatment was observed (Fig A1B, C, D).

Lastly, our preliminary data suggested a possible contribution of ERK to the suppression of PTX-induced seizure (Fig A1E). While positive regulation of ERK activity by focal adhesion signaling has been widely observed (Lambert et al., 2012), the opposite mode of action has also been suggested in more non-

canonical signaling between ERK and focal adhesion signaling in *Drosophila* neuromuscular junction (Tsai et al., 2008). In our hands, both ERK overexpression and ERK RNAi resulted in suppression of the seizure behavior (though we note overexpression had much greater degree of suppression than RNAi) (Fig A.1E). Future work will be required to clarify the nature of FA/ERK signaling interactions in E/I balance and hyperexcitability in the *Drosophila* nervous system, but the strong phenotypes observed with both manipulations argue for an important role for ERK in this context.

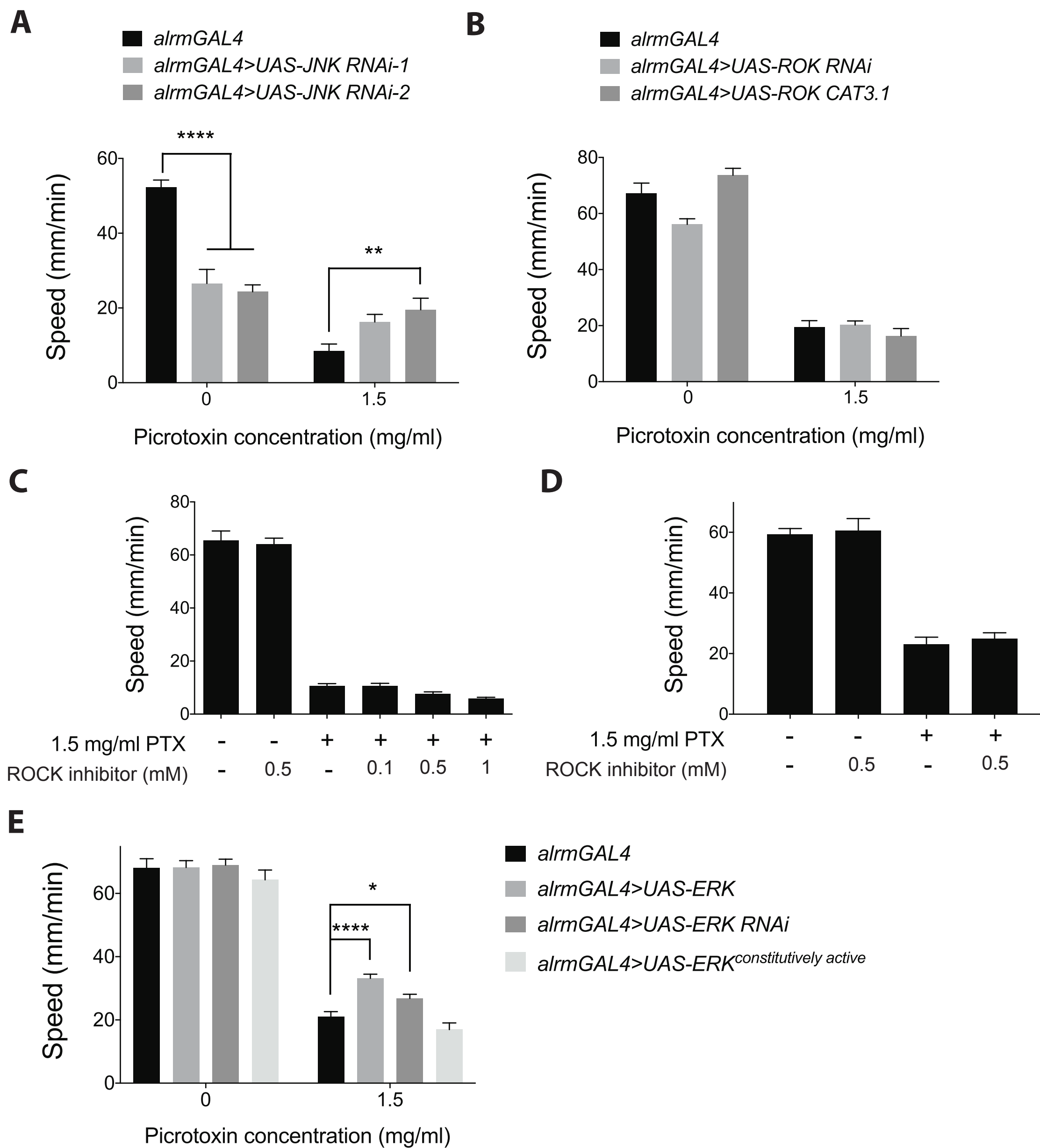


Figure A.1: Tests of potential downstream molecules of astrocyte focal adhesions

A, Depletion of JNK in astrocytes by RNAi impaired basal locomotion of larvae compared to control genotype but showed no changes in PTX induced seizure. Error bars represent SEM. **P<0.005, ****P<0.0001; two-way ANOVA and Dunnett's multiple comparisons test. B, Genetic alteration of ROK either by RNAi or overexpression of constitutively active ROK in astrocytes made no difference in both basal locomotion and PTX induced seizure compared to wilde-type. Error bars represent SEM. Two-way ANOVA and Dunnett's multiple comparisons test. C, Pharmacological inhibition of ROCK with Fasudil during PTX feeding showed no changes in locomotion defect suppression. Two-way ANOVA and Dunnett's multiple comparisons test. D, 6 hr of pretreatment of Fasudil does not affect PTX induced behavior. Error bars represent SEM. Two-way ANOVA and Dunnett's multiple comparisons test. E, Either overexpression or knockdown of ERK in astrocytes significantly suppresses seizure behavior by PTX while overexpression of constitutively active form of ERK had no effect. Error bars represent SEM. *P<0.005, ****P<0.0001; two-way ANOVA and Dunnett's multiple comparisons test.

Potential applications for FA signaling in disease

Molecular mechanisms by which one could alter E/I balance and hyperexcitability of high interest. Here we identified astrocyte focal adhesions through their regulation of EAAT as a new potential therapeutic interventions. Neurological disorders like Fragile X Syndrome (FXS) and seizure that present with phenotypes including E/I imbalance and excitotoxicity might be excellent targets. Recent studies on GLT1 and Fragile X Syndrome (Higashimori et al., 2016) have provided a strong evidence that dysregulation of astrocyte specific glutamate transporter, GLT1 is underlying pathophysiology in mouse model of FXS. To explore this potential link to the pathology of FXS, we utilized *Drosophila* FXS model with loss-of-function mutations in the *Drosophila* fragile X-related 1(dfmr1) that shows larval crawling defect (Siller and Broadie, 2011; Xu et al., 2004)(Fig A.2C, D). Interestingly, we observed the same phenomenon as in the mouse model of FXS where glial EAAT expression decreases dramatically in the disease state (Fig A.2A, B)(Higashimori et al., 2016). We then wondered if alterations of focal adhesions in astrocytes would rescue any of this pathophysiology. Surprisingly, when we deplete astrocyte βintegrin in the dfmr1 mutant background ($fmr1^{50M}$), we were able to see a significant rescue of the locomotion defect compared to the driver control (alrmGAL4; $fmr1^{50M}$)(Fig A.2G, H). However, in our attempt to test if the underlying mechanism for the behavioral rescue is alteration of EAAT1 levels, we observed no changes of EAAT1 in our driver control nor in βintegrin RNAi in the mutant background in

comparison to wild-type (Fig A.2E, F). While this might suggest no correlation between the behavioral rescue by βintegrin knockdown and EAAT1 levels, one needs to proceed in the interpretation with caution: I found the *alrm-GAL4* driver itself had an effect on EAAT1 level in the mutant background, which complicates the matter.

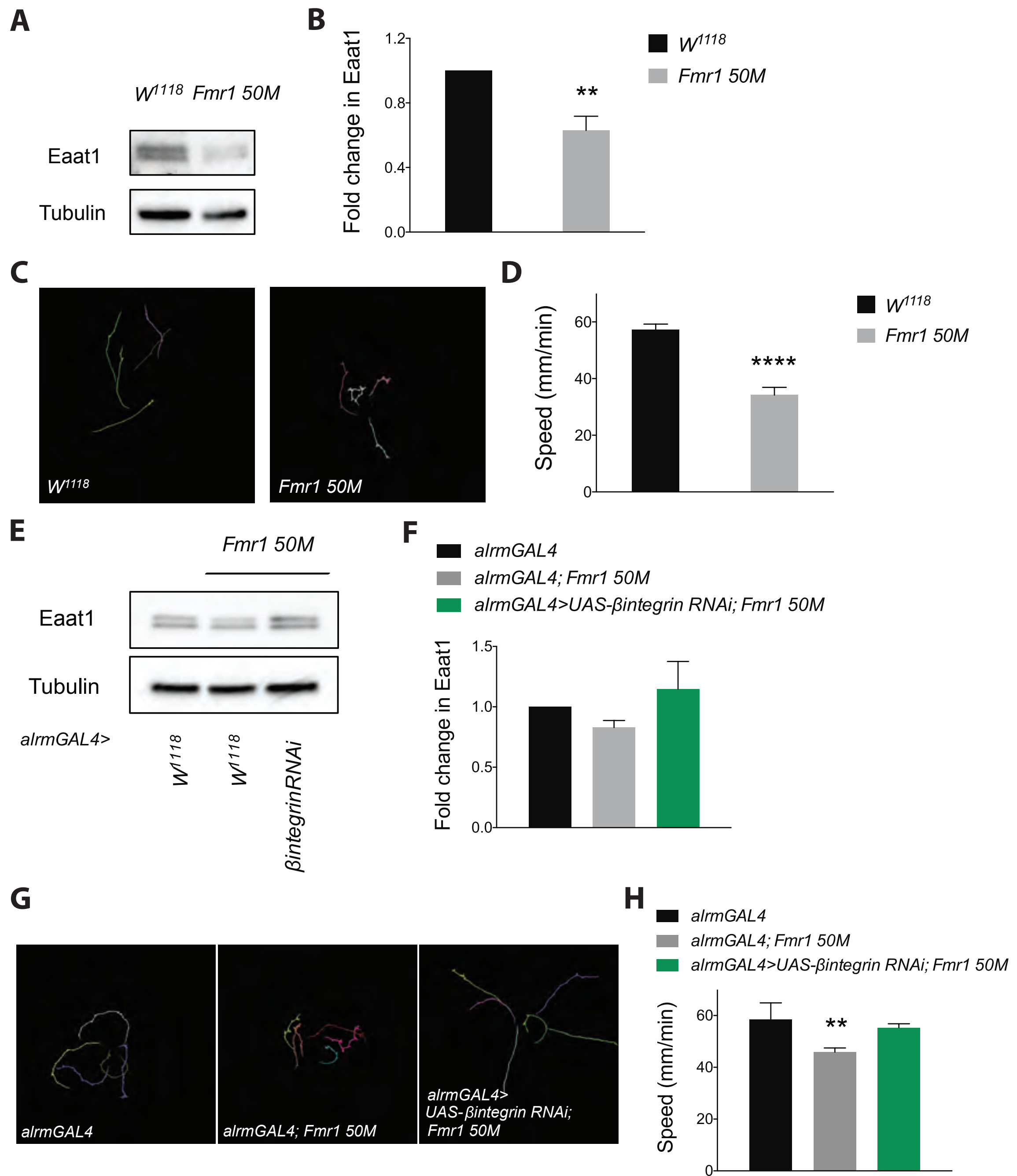


Figure A.2: Potential application for a disease (Fragile X syndrome fly model)

A, Western blot samples were prepared from 3rd instar larval CNS from *W^{1118}* and *$Fmr1^{50M}$* . Blots were probed with anti-EAAT1 antibody. B, Quantification of EAAT1 levels from Western blot analysis shown in A (n = 5 experiments). Error bars represent SEM. **P<0.005; Unpaired t test. C, Raw traces of 3rd instar larval locomotion in *W^{1118}* and *$Fmr1^{50M}$*. D, Quantitative of the larval locomotion shown in C shows significant impairment of the locomotion speed in *$Fmr1^{50M}$* compared to *W^{1118}*. Error bars represent SEM. ****P<0.0001; Unpaired t test. E, Western blot samples were prepared from 3rd instar larval CNS from alrmGAL4, alrmGAL4; $Fmr1^{50M}$, and alrmGAL4/UAS-βintegrin; $Fmr1^{50M}$. F, Quantification of EAAT1 levels from Western blot analysis shown in E (n = 4 experiments). Error bars represent SEM. Two-way ANOVA and Dunnett's multiple comparisons test. G, Raw traces of 3rd instar larval locomotion in alrmGAL4, alrmGAL4; $Fmr1^{50M}$, and alrmGAL4/UAS-βintegrin; $Fmr1^{50M}$. H, Quantitative of the larval locomotion shown in G shows a significant rescue of the behavioral defect in alrmGAL4; Fmr^{150M} compared to alrmGAL4/UAS-βintegrin; $Fmr1^{50M}$. Error bars represent SEM. **P<0.05; two-way ANOVA and Dunnett's multiple comparisons test.

Table 2.1: List of genes that scored as suppressors in the screen

Gene symbol	CG number	VDRC ID	Library	off-target	Number of files
Ten-a	CG42338	105037	KK	0	23
by	CG9379	22823	GD	0	24
CG17839	CG17839	100149	KK	1	23
drl	CG17348	100039	KK	1	28
14-3-3epsilon	CG31196	108129	KK	0	17
CG17124	CG17124	19078	GD	1	25
Tsp47F	CG9033	44288	GD	0	30
Klp31E	CG5300	34983	GD	0	25
Lmpt	CG42679	42892	GD	0	25
Tsp42Eq	CG12832	30329	GD	2	19
P5cr	CG6009	46892	GD	0	24
plexB	CG17245	27220	GD	0	10
Hem	CG5837	103380	KK	0	26
CG32792	CG32792	47047	GD	0	22
CG1801	CG1801	8655	GD	0	26
CG9864	CG9864	103970	KK	1	29
tsl	CG6705	14429	GD	0	19
CG33253	CG33253	6178	GD	1	32
a	CG6741	16826	GD	410	26
in	CG16993	103407	KK	0	32
Smvt	CG2191	102662	KK	0	24
SerT	CG4545	11346	GD	0	21
CG2616	CG2616	102630	KK	0	20
CG4797	CG4797	10598	GD	0	26
NepYr	CG5811	103973	KK	0	19
pros	CG17228	101477	KK	0	21
klg	CG6669	102502	KK	0	18
fry	CG32045	40309	GD	633	14
tomboy40	CG8330	105557	KK	0	22
E23	CG3327	105055	KK	1	26
CG8116	CG8116	39218	NA	NA	17
CG12818	CG12818	31898	GD	0	16
scramb2	CG1893	104647	KK	1	29
ninaC	CG5125	110702	KK	1	17
prc	CG5700	100357	KK	1	13
GluRIIE	CG31201	49547	GD	0	29
Tbh	CG1543	51667	GD	0	43
PGRP-LD	CG33717	5038	GD	1	37
Zip3	CG6898	37358	GD	2	39
kek5	CG12199	47768	GD	5	35

fdl	CG8824	4637	GD	0	34
babo	CG8224	3825	GD	0	21
Nplp4	CG15361	104662	KK	0	22
Pdfr	CG13758	106381	KK	0	13
Snmp1	CG7000	104210	KK	0	19
mgl	CG42611	103661	KK	0	13
mthl8	CG32475	100246	KK	0	17
ppk25	CG33349	101808	KK	0	14
PGRP-SC2	CG14745	104578	KK	0	28
brp	CG42344	104630	KK	0	12
CG4465	CG4465	100202	KK	0	15
Ir31a	CG31718	100345	KK	0	10
trk	CG5619	51240	GD	0	10
Sld5	CG14549	43588	GD	0	12
CG9095	CG9095	23158	GD	2	21
tipE	CG1232	4482	GD	1	26
HLH106	CG8522	37640	GD	0	25
SsRbeta	CG5474	12101	GD	0	17
phyl	CG10108	35469	GD	0	29
sdk	CG5227	9437	GD	0	21
bcd	CG1034	48966	GD	0	20
trn	CG11280	107883	KK	0	34
CG31103	CG31103	2657	GD	0	12
Nup358	CG11856	38583	GD	3	12
kek2	CG4977	42450	GD	1	19
CG13743	CG13743	40974	GD	540	26
CG12341	CG12341	7391	GD	0	23
CG42514	CG42514	45643	GD	0	10
l(2)01289	CG9432	107350	KK	1	24
chas	CG32556	109841	KK	1	23
CG14299	CG14299	110420	KK	0	16
Drak	CG32666	107263	KK	1	22
CG10486	CG10486	107903	KK	0	17
Ir67c	CG32058	107921	KK	0	20
ppk21	CG12048	107892	KK	0	15
Tpc2	CG2857	107215	KK	0	20
pst	CG8588	107243	KK	0	10
Gp150	CG5820	100134	KK	1	11
cos	CG1708	108914	KK	0	19
KCNQ	CG33135	106655	KK	0	18
CG1703	CG1703	105998	KK	0	21
Snap24	CG9474	108209	KK	0	33

bou	CG14430	107102	KK	0	22
Ptp99A	CG11516	27207	GD	0	20
CG5888	CG5888	12413	GD	0	17
Gbp	CG15917	108755	KK	0	27
lox2	CG4402	108217	KK	0	21
btl	CG32134	110277	KK	0	16
mfas	CG3359	103621	KK	1	25
CG14238	CG14238	2673	GD	0	26
gcl	CG8411	28897	GD	1	17
rab3-GAP	CG7061	106905	KK	0	11
Pdk1	CG1210	109812	KK	0	13
shf	CG3135	14803	GD	11	16
Rala	CG2849	105296	KK	0	12
CG13278	CG13278	109927	KK	0	15
CG8142	CG8142	108452	KK	1	15
jagn	CG10978	108991	KK	0	11
boss	CG8285	4365	GD	0	11
CG9903	CG9903	42689	GD	0	28

Table 2.2: List of genes that scored as enhancers in the screen

Gene symbol	CG number	VDRC ID	Library	off-target	Number of files
alphaPS5	CG5372	100120	KK	0	27
Ipp	CG12843	101044	KK	0	15
CSN7	CG2038	40690	GD	0	28
Eaat1	CG3747	109401	KK	0	18
ATPsyn-Cf6	CG4412	107826	KK	0	15
Pkc98E	CG31743	105821	KK	0	16
CG31743	CG12484	104814	KK	0	12
CG12484	CG9012	103383	KK	0	16
Ir10a	CG34143	100181	KK	0	22
slo	CG30265	106618	KK	1	14
CG30265	CG10693	104421	KK	0	14
CG33270	CG33270	109109	KK	4	19
spin	CG8428	105462	KK	0	15
CG6845	CG6845	107719	KK	0	10
Gprk2	CG17998	101463	KK	0	11
nAcRbeta-21C	CG11822	42742	GD	0	11
ATP7	CG1886	108159	KK	0	11
TBPH	CG10327	104401	KK	0	17
Ccn	CG32183	101518	KK	0	12
betaTub60D	CG3401	102052	KK	0	12
Dyb	CG8529	104485	KK	1	12
RYamide	CG40733	109267	KK	0	12
kz	CG3228	104253	KK	0	19
Nf-YA	CG3891	106132	KK	0	19
Tip60	CG6121	110617	KK	0	19
CCHa1	CG14358	104974	KK	2	19
spen	CG18497	108828	KK	2	19
spz6	CG9196	100897	KK	0	13
shakB	CG34358	24578	GD	0	13
Gdi	CG4422	108693	KK	1	20
Vha13	CG6213	106536	KK	0	14
CaMKII	CG18069	100265	KK	0	22
Takr99D	CG7887	43329	GD	0	15
Cap	CG9802	101501	KK	2	15
Mdh1	CG5362	110604	KK	0	15
Mcm2	CG7538	103619	KK	2	15
rdo	CG15151	107213	KK	5	15
nrm	CG43079	104295	KK	0	23
Girdin	CG12734	103615	KK	0	23
Ccn	CG32183	101520	KK	1	16

gfA	CG32538	100756	KK	0	16
CG8468	CG8468	6452	GD	0	24
sog	CG9224	105853	KK	3	16
T48	CG5507	100334	KK	0	32
CG5398	CG5398	109717	KK	0	16
CG14646	CG14646	103770	KK	0	25
CG11147	CG11147	101601	KK	0	17
mam	CG8118	102091	KK	0	17
nimC4	CG16876	101915	KK	0	17
CG8908	CG8908	100472	KK	1	17
CG9104	CG9104	110579	KK	0	17
CG8925	CG8925	101128	KK	0	17
Lac	CG12369	107450	KK	0	26
jub	CG11063	101993	KK	0	18
CG16718	CG16718	108953	KK	0	27
Synd	CG33094	104580	KK	0	19
pnr	CG3978	101522	KK	0	19
Ir7b	CG15326	100498	KK	1	19
ppk6	CG11209	101091	KK	1	19
CG34120	CG34120	101700	KK	0	19
Tapdelta	CG9035	8759	GD	0	10
Mpcp	CG4994	101316	KK	1	10
Osi4	CG10303	102619	KK	0	10
Vha55	CG17369	46554	GD	1	10
VhaM9.7-a	CG1268	104315	KK	0	10
dome	CG14226	36355	GD	0	10
kon	CG10275	106680	KK	1	10
Snmp2	CG7422	101136	KK	0	20
CG8026	CG8026	105681	KK	0	10
Bap	CG12532	7721	GD	0	10
qtc	CG14039	17349	GD	13	10
vlc	CG8390	46230	GD	0	10
CG33298	CG33298	42776	GD	2	10
Nup44A	CG8722	106489	KK	2	10
CG1090	CG1090	26783	GD	0	10
AdoR	CG9753	1385	GD	0	10
Npc1b	CG12092	108054	KK	0	10
Osi10	CG15593	105915	KK	0	10
Atg18	CG7986	105366	KK	0	10
Pvf1	CG7103	102699	KK	0	10
endos	CG6513	106825	KK	0	10
CG30345	CG30345	103652	KK	0	10

NtR	CG6698	108234	KK	0	10
Roc1a	CG16982	106315	KK	0	10
Caps	CG33653	110055	KK	0	31
Toll-7	CG8595	39176	GD	2	21
cm	CG3035	110746	KK	0	21

Table 3: Summary of statistical analysises

Figure 3.3B		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-tensin RNAi*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-βintegrin RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-talin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	0.5 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-tensin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-talin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-FAK RNAi*	****
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-βintegrin RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-talin RNAi*	*
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-talin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-tensin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-talin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-FAK RNAi*	****
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-βintegrin RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4>UAS-tensin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-talin RNAi*	ns
	alrmGAL4>UAS-βintegrin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
	alrmGAL4>UAS-talin RNAi vs. *alrmGAL4>UAS-FAK RNAi*	ns
Figure 3.3C		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP	ns
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP vs. alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP	**
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi	**
	alrmGAL4>UAS-βintegrin RNAi; UAS-mCD8GFP vs. alrmGAL4>UAS-βintegrin RNAi; UAS-tensin RNAi	ns
Figure 3.4B		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	W^{1118} *vs. UAS-tensin RNAi*	ns
	W^{1118} *vs. UAS-βintegrin RNAi*	ns
	W^{1118} *vs. UAS-talin RNAi*	ns
	W^{1118} *vs. UAS-FAK RNAi*	ns
	UAS-tensin RNAi vs. UAS-βintegrin RNAi	ns

	UAS-tensin RNAi vs. UAS-talin RNAi	ns
	UAS-tensin RNAi vs. UAS-FAK RNAi	ns
	UAS-βintegrin RNAi vs. UAS-talin RNAi	ns
	UAS-βintegrin RNAi vs. UAS-FAK RNAi	ns
	UAS-talin RNAi vs. UAS-FAK RNAi	ns
	1.5 mg/ml PTX	
	W^{1118} *vs. UAS-tensin RNAi*	ns
	W^{1118} *vs. UAS-βintegrin RNAi*	ns
	W^{1118} *vs. UAS-talin RNAi*	ns
	W^{1118} *vs. UAS-FAK RNAi*	ns
	UAS-tensin RNAi vs. UAS-βintegrin RNAi	ns
	UAS-tensin RNAi vs. UAS-talin RNAi	ns
	UAS-tensin RNAi vs. UAS-FAK RNAi	ns
	UAS-βintegrin RNAi vs. UAS-talin RNAi	ns
	UAS-βintegrin RNAi vs. UAS-FAK RNAi	ns
	UAS-talin RNAi vs. UAS-FAK RNAi	ns
Figure 3.4C		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-tensin RNAi	ns
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi	ns
	alrmGAL4 vs. alrmGAL4>UAS-talin RNAi	ns
	alrmGAL4 vs. alrmGAL4>UAS-FAK RNAi	ns
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-βintegrin RNAi	ns
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-talin RNAi	ns
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-talin RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
	alrmGAL4>UAS-talin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-tensin RNAi	****
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi	****
	alrmGAL4 vs. alrmGAL4>UAS-talin RNAi	****
	alrmGAL4 vs. alrmGAL4>UAS-FAK RNAi	****
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-βintegrin RNAi	ns
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-talin RNAi	ns
	alrmGAL4>UAS-tensin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-talin RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
	alrmGAL4>UAS-talin RNAi vs. alrmGAL4>UAS-FAK RNAi	ns
Figure 3.5		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX:*W1118 vs.* 0.0 mg/ml PTX:*by33c*	****
	0.0 mg/ml PTX:*W1118 vs.* 1.5 mg/ml PTX:*W1118*	****
	0.0 mg/ml PTX:*W1118 vs.* 1.5 mg/ml PTX:*by33c*	****
	0.0 mg/ml PTX:*by33c vs.* 1.5 mg/ml PTX:*W1118*	****
	0.0 mg/ml PTX:*by33c vs.* 1.5 mg/ml PTX:*by33c*	****
	1.5 mg/ml PTX:*W1118 vs.* 1.5 mg/ml PTX:*by33c*	****
Figure 3.6B		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX:*alrmGal4/+ vs.* 0.0 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi*	ns
	0.0 mg/ml PTX:*alrmGal4/+ vs.* 1.5 mg/ml PTX:*alrmGal4/+*	****
	0.0 mg/ml PTX:*alrmGal4/+ vs.* 1.5 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi*	****
	0.0 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi vs.* 1.5 mg/ml PTX:*alrmGal4/+*	****
	0.0 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi vs.* 1.5 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi*	****

	1.5 mg/ml PTX:*alrmGal4/+ vs.* 1.5 mg/ml PTX:*alrmGal4/UAS-Mmp1 RNAi*	****
Figure 3.6C		
Test used	two-way ANOVA; Dunnett's multiple comparisons	
Post hoc test		Summary
	1.5 mg/ml PTX *vs.* W^{1118} acetone	****
	1.5 mg/ml PTX *vs.* W^{1118} 50μM inhibitor	****
	1.5 mg/ml PTX vs. 1.5 mg/ml PTX + 50μM inhibitor	*
Figure 3.6C		
Test used	two-way ANOVA; Dunnett's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX:*elavGAL4 vs.* 0.0 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi*	ns
	0.0 mg/ml PTX:*elavGAL4 vs.* 1.5 mg/ml PTX:*elavGAL4*	****
	0.0 mg/ml PTX:*elavGAL4 vs.* 1.5 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi*	****
	0.0 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi vs.* 1.5 mg/ml PTX:*elavGAL4*	****
	0.0 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi vs.* 1.5 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi*	****
	1.5 mg/ml PTX:*elavGAL4 vs.* 1.5 mg/ml PTX:*elavGAL4>UAS-Mmp1 RNAi*	ns
Figure 3.8A		
Test used	two-way ANOVA; Bonferroni's multiple comparisons	
Post hoc test		Summary
	alrmGAL4 - *alrmGAL4>UAS-βintegrin RNAi*	
	0.0 mg/ml PTX	ns
	50 mm inhibitor	ns
	1.5 mg/ml PTX	*
	1.5 mg/ml PTX + 50 mM inhibitor	ns
Figure 3.8B		
Test used	two-way ANOVA; Dunnett's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-Mmp1*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi; UAS-Mmp1*	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-Mmp1*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi*	****
	alrmGAL4 vs. *alrmGAL4>UAS-βintegrin RNAi; UAS-Mmp1*	****
Figure 3.8C		
Test used	two-way ANOVA; Bonferroni's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi*	ns
	alrmGAL4 vs. *alrmGAL4>UAS-Mmp1; UAS-talin RNAi*	ns
	alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi vs. *alrmGAL4>UAS-Mmp1; UAS-talin RNAi*	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. *alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi*	*
	alrmGAL4 vs. *alrmGAL4>UAS-Mmp1; UAS-talin RNAi*	ns
	alrmGAL4>UAS-mCD8GFP; UAS-talin RNAi vs. *alrmGAL4>UAS-Mmp1; UAS-talin RNAi*	*
Figure 3.10D		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX:*alrmGAL4* vs. 0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1*	*
	0.0 mg/ml PTX:*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4*	****
	0.0 mg/ml PTX:*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	****
	0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1* vs. 1.5 mg/ml PTX :*alrmGAL4*	****
	0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	****
	1.5 mg/ml PTX :*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	ns
Figure 3.12A		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary

	0.0 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi	ns
	alrmGAL4 vs. alrmGAL4>UAS-tensin RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-tensin RNAi	ns
	1.5 mg/ml PTX	
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi	****
	alrmGAL4 vs. alrmGAL4>UAS-tensin RNAi	****
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-tensin RNAi	ns
	1.5 mg/ml PTX + 500 mM DHK	
	alrmGAL4 vs. alrmGAL4>UAS-βintegrin RNAi	ns
	alrmGAL4 vs. alrmGAL4>UAS-tensin RNAi	ns
	alrmGAL4>UAS-βintegrin RNAi vs. alrmGAL4>UAS-tensin RNAi	ns
Figure 3.12A		
Test used	two-way ANOVA; Bonferroni's multiple comparisons	
Post hoc test		Summary
	alrmGAL4 - alrmGAL4>UAS-Mmp1 RNAi	
	0.0 mg/ml PTX	ns
	1.5 mg/ml PTX	*
	1.5 mg/ml PTX + 50 mM DHK	ns
Figure 3.12A		
Test used	two-way ANOVA; Tukey's multiple comparisons	
Post hoc test		Summary
	0.0 mg/ml PTX:*alrmGAL4* vs. 0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1*	*
	0.0 mg/ml PTX:*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4*	****
	0.0 mg/ml PTX:*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	****
	0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1* vs. 1.5 mg/ml PTX :*alrmGAL4*	****
	0.0 mg/ml PTX:*alrmGAL4>UAS-Eaat1* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	****
	1.5 mg/ml PTX :*alrmGAL4* vs. 1.5 mg/ml PTX :*alrmGAL4>UAS-Eaat1*	ns

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