Astrocyte-Neuron Interactions Regulate Nervous System Assembly and Function: A Dissertation

Allie Muthukumar

University of Massachusetts Medical School Worcester

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ASTROCYTE-NEURON INTERACTIONS REGULATE NERVOUS SYSTEM ASSEMBLY AND FUNCTION
ASTROCYTE-NEURON INTERACTIONS REGULATE NERVOUS SYSTEM
ASSEMBLY AND FUNCTION

A Dissertation Presented
By

ALLIE MUTHUKUMAR

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Biomedical Sciences, Worcester
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JANUARY, 8 2015

NEUROBIOLOGY
ASTROCYTE-NEURON INTERACTIONS REGULATE NERVOUS SYSTEM ASSEMBLY AND FUNCTION

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ALLIE MUTHUKUMAR

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Abstract

Astrocytes densely infiltrate the brain and intimately associate with synaptic structures. In the past 20 years, they have emerged as critical regulators of both synapse assembly and synapse function. During development, astrocytes modulate the formation of new synapses, and later, control refinement of synaptic connections in response to activity dependent cues. In a mature nervous system, astrocytes modulate synapse function through a variety of mechanisms. These include ion buffering, neurotransmitter uptake and the release of molecules that activate synaptic receptors. Through such roles, astrocytes shape the structure and function of neuronal circuits. However, how astrocytes and synapses reciprocally communicate during circuit assembly remains an unanswered question in the field.

The vast majority of our understanding of astrocyte biology has come from studies conducted in mammals, where it is challenging to dissect molecular mechanisms with cell type specificity. *Drosophila melanogaster* is a less established model system for studying astrocyte-neuron interactions, but its vast array of genetic tools and rapid life cycle promises great potential for precisely targeted manipulations. My thesis work has utilized *Drosophila melanogaster* to investigate the reciprocal nature of astrocyte-synapse communication. First, I characterized *Drosophila* late metamorphosis as a developmental stage in which astrocyte-synapse associations can be studied. My work demonstrates that during this time, when the adult *Drosophila* nervous system is being assembled, synapse formation relies on the coordinated infiltration of astrocyte membranes into the neuropil. Next, I show that in a reciprocal manner, neural activity can shape astrocyte biology during this time as well and impart long lasting effects on neuronal circuit function. In particular expression of the astrocyte GABA transporter (GAT) is modulated in an activity-dependent manner via astrocytic GABA$_B$R1/2 receptor signaling. Inhibiting astrocytic GABA$_B$R1/2 signaling strongly suppresses hyperexcitability in a *Drosophila* seizure model,
arguing this pathway is important for modulating excitatory/inhibitory balance in vivo. Finally, utilizing the ease of the *Drosophila* system, I performed a reverse genetic screen to identify additional astrocyte factors involved in modulating excitatory-inhibitory neuronal balance.
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<td>AL</td>
<td>antennal lobe</td>
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<tr>
<td>alrm</td>
<td>astrocytic leucine-rich repeat molecule</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>dLGN</td>
<td>dorsal lateral geniculate nucleus</td>
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<td>eas</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>Gpc4</td>
<td>Glypican 4</td>
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<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
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<td>ionotopic glutamate receptor</td>
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<td>Kir</td>
<td>inward rectifier potassium channel</td>
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<td>long-term depression</td>
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<td>MB</td>
<td>mushroom body</td>
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<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
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<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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<td>mIPSC</td>
<td>miniature inhibitory postsynaptic currents</td>
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<td>Neuropil</td>
<td>region densely populated by neurites and synapses</td>
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<td>NMDA</td>
<td>(N)-Methyl-D-aspartate</td>
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<td>P2X</td>
<td>ionotopic purinergic receptor</td>
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<td>Pertussis toxin</td>
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<td>superior posterior slope</td>
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<td>Tetanus toxin</td>
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<td>Trpa1</td>
<td>transient receptor potential cation channel</td>
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<td>TSP</td>
<td>Thrombospondin</td>
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Preface

The screen described in chapter 4 was performed by Allie Muthukumar and Sukhee Cho. Sukhee Cho also contributed to the collection of data presented in figure 4.2 and assisted with sample preparations of the data presented in figure 4.5.
CHAPTER I
Introduction
The central nervous system (CNS) relies on appropriate communication between diverse networks of cells. In particular, a delicate interplay between neurons and glia is required for proper nervous system function. For a long time, glia were considered to be passive support cells that merited little attention. Now, we know that glia perform essential roles and actively interact with neurons. One subtype of glia, oligodendrocytes, wrap around axons and regulate action potential conduction (Nave and Trapp, 2008). Another subtype of glia, microglia, are the resident immune cells of the CNS. Their phagocytic properties are essential for the clearance of neuronal debris following injury, as well as for appropriate synapse pruning in a healthy brain (Davalos et al., 2005; Doherty et al., 2009; Schafer et al., 2012). A third class of glia, astrocytes, vastly infiltrates the CNS interacting with neurons, blood vessels, epithelium, and extracellular matrix, thereby influencing the cellular and molecular makeup of the environment. Astrocytes are implicated in numerous biological processes, including synaptogenesis, synapse function, neurogenesis, neuronal wiring, metabolic control, synaptic pruning and glial scar formation (Araque et al., 2014; Ashton et al., 2012; Christopherson et al., 2005; Rodriguez et al., 2014; Silver and Miller, 2004; Tasdemir-Yilmaz and Freeman, 2014; Wilhelmsson et al., 2012; Ziegenfuss et al., 2012). In particular, their involvement in synapse development and function has generated a tremendous amount of intrigue and interest in the neurobiology field.

The intimate integration of astrocytes within neural networks is immediately evident from their cellular architecture. They possess elaborate tufted membrane arborizations that weave throughout the neuropil space. Interestingly, individual
astrocytes occupy unique spatial domains that do not overlap with the territories of neighboring astrocytes. Consequently, astrocytes tile with each other to fully cover neuropil areas (Bushong et al., 2002; Halassa et al., 2007; Stork et al., 2014). While each astrocyte demands a unique spatial territory, its function is not necessarily imparted in a cell-autonomous fashion. Membranes belonging to neighboring astrocytes are connected through gap junctions, coupling individual cells (Adermark and Lovinger, 2008; Houades et al., 2008; Meme et al., 2009). Together, the astrocyte network forms an intricate web-like syncytium that infiltrates the neuropil and intimately associates with synaptic structures. For example, in the rat hippocampus, approximately 60% of synapses closely associate with astrocyte membranes (Ventura and Harris, 1999). Intriguingly, these associations are dynamic. The fine astrocyte membrane processes that enwrap dendritic spines display rapid rearrangements that correlate with dendritic spine remodeling (Bernardinelli et al., 2014; Haber et al., 2006). In accordance with such close structural associations, astrocyte and synapse functions are also intimately related. Astrocytes express numerous neurotransmitter receptors that render them directly responsive to neurotransmitter signaling, and in turn, astrocytes fulfill a number of important roles that complement synapse biology (Araque et al., 1999; Zhang et al., 2014). For example, through the buffering of ions, astrocytes provide homeostatic regulation that ensures appropriate neuronal activity (Rangroo Thrane et al., 2013). Additionally, astrocytes are essential for clearing neurotransmitters from extracellular space. Through the regulated activity of neurotransmitter transporters, astrocytes mediate the termination of a synaptic signal thereby modulating synaptic efficacy and tone (Tanaka et al., 1997). There is also
mounting evidence that through the release of substrates, astrocytes can modulate synapse assembly as well as synaptic transmission (Christopherson et al., 2005; Pascual et al., 2005). Thus, it has become evident both synapse function and development are reliant on astrocyte biology.

**Astrocyte regulation of synapse function**

Astrocytes are influential regulators of synapse function. There is increasing evidence that astrocytes can modulate synaptic transmission and thereby sculpt neural circuit function. The mechanisms by which astrocytes impart their mark are diverse, ranging from the active release of substrates that bind synaptic receptors to homeostatic mechanisms such as K⁺ buffering that determines the ionic environment of a synapse. Below I discuss some of the key aspects of astrocyte-synapse interactions that pertain to synapse function in a mature nervous system.

**Neurotransmitter uptake**

An important role for astrocytes is the regulated clearance of neurotransmitters from extracellular regions. Astrocytes express transporters for several of the brain’s neurotransmitters, including glutamate and GABA. These transporters are densely expressed on the surface of astrocyte membranes and are therefore well positioned to monitor NT uptake at both synaptic and extrasynaptic regions (Minelli et al., 1996; Rothstein et al., 1994; Zhang et al., 2014; Zhou and Sutherland, 2004).
Through the clearance of neurotransmitters, astrocytes modulate the temporal and spatial dynamics of neurotransmitter signaling. This regulation is a key process in controlling synaptic strength and efficacy as well as influencing neurotransmitter tone across larger areas (Huang and Bergles, 2004; Pannasch et al., 2014; Pita-Almenar et al., 2012). For example, inhibiting glutamate uptake leads to excess extracellular glutamate levels that can alter post-synaptic efficacy. In the rat hippocampus, N-Methyl-D-aspartate (NMDA) receptor mediated post-synaptic currents were prolonged when glutamate transport activity was inhibited using pharmacological blockers (Arnth-Jensen et al., 2002). Elevated glutamate levels also mediate long term depression (LTD) by modulating postsynaptic metabotropic glutamate receptor (mGluR) activity (Huang et al., 2004; Reichelt and Knöpfel, 2002). Repetitive, high frequency pre-synaptic activity can induce slow excitatory post-synaptic currents that are mediated by mGluR activity and facilitate LTD. In rat cerebellum, inhibiting glutamate transport increased mGluR activity and promoted mGluR mediated LTD (Brasnjo and Otis, 2001). Additionally, genetic removal of astrocyte glutamate transporters lead to excitotoxic degeneration and neuronal hyperexcitability (Rothstein et al., 1996; Tanaka et al., 1997). Deletion of the astrocyte glutamate transporter, GLT-1, in mice resulted in spontaneous seizures and eventual death due to increased extracellular glutamate levels (Tanaka et al., 1997). Parallel to glutamate transporters, astrocyte GABA transporters (GATs) also mediate synapse efficacy and tone. Reduced astrocyte GAT levels in the mouse hippocampus resulted in reduced inhibitory postsynaptic current (IPSC) amplitudes, due to elevated
GABA levels and subsequent desensitization of GABA receptors (Shigetomi et al., 2012).

Thus, the regulated activity and expression of astrocyte neurotransmitter transporters is an important mechanism by which astrocytes shape synapse function. Intriguingly, astrocyte glutamate transporter expression is dynamically regulated in response to neural activity. Through astrocyte metabotropic glutamate receptor signaling events, glutamate transporter expression is either upregulated or downregulated in response to increased or decreased synaptic activity respectively (Benediktsson et al., 2012; Devaraju et al., 2013; Yang et al., 2009). Furthermore, this response is transcriptionally regulated via the transcription factor, nuclear factor-κB (NF-κB) (Ghosh et al., 2011). Thus, it seems astrocyte glutamate transporter expression is tightly regulated to complement neurotransmitter release. While most studies have focused on the regulation of glutamate transporters, little attention has been given to other neurotransmitter transporter subtypes. GABA is the primary inhibitory neurotransmitter in the brain, yet we know very little about the regulation of astrocyte GATs. Shigetomi et al demonstrated that near membrane calcium elevations, mediated by the calcium channel TrpA1, could modulate astrocyte GAT expression in the rat hippocampus. However, if astrocyte GAT expression is sensitive to GABA release is not known. Understanding the mechanisms by which astrocytes handle different neurotransmitter types would be telling of how astrocytes integrate with neuronal networks.

Buffering of K⁺
Maintaining ion homeostasis is imperative for proper nervous system function. Another way astrocytes influence synapse function is through the buffering of K+. The nervous system is markedly sensitive to changes in extracellular K+ concentrations and therefore relies on astrocytes to buffer K+ and tightly regulate extracellular K+ concentrations (Kofuji and Newman, 2004). Even a small amount of K+ efflux from neurons can dramatically alter extracellular K+ concentrations, which can dramatically change a neuron’s resting membrane potential and affect the activation of voltage gated channels, electrogenic transport of neurotransmitters and synaptic transmission (Kofuji and Newman, 2004; Noori, 2011).

Astrocytes express a number of different transporters and channels that render them permeable to K+ and contribute to their buffering capabilities. For example, the astrocytic Na+-K+-ATPase pump as well as the Na+-K+-Cl- co-transporter (NKCC) have been shown to mediate K+ uptake and relieve the extracellular environment of excess K+(Wang et al., 2012). Astrocyte Kir4.1 K+ channels are another important molecular mediator of astrocytic K+ buffering. In cooperation with gap junction channels, Kir4.1 K+ channels mediate the coordinated transport of K+ between astrocytes in the CNS in so called spatial buffering (Butt and Kalsi, 2006; Zhang et al., 2014). In this model, some astrocytes take up K+ via Kir4.1 channels from areas of high K+ and then redistribute K+ to other astrocytes in the nervous system in areas of low K+ through the gap junction-coupled astrocyte syncytium. Indeed, K+ clearance is impaired when astrocyte gap junction function is disrupted (Wallraff et al., 2006).
Disruption of astrocyte mediated K⁺ homeostasis can alter neuronal firing (Melom and Littleton, 2013; Sibille et al., 2014). In mouse models of Huntington’s disease (HD), decreased astrocyte Kir4.1 channel expression elevated extracellular K⁺ levels in the striatum. The authors showed that comparable elevations in wildtype mice increased neuronal excitability in a manner that resembled neuronal excitability seen in HD mice. Importantly, viral delivery of Kir4.1 channels to astrocytes in HD animals, restored extracellular K⁺ levels, reduced neuron excitability and reduced motor dysfunction (Tong et al., 2014). Likewise, Thrane et al demonstrated that compromised astrocyte K⁺ buffering could alter the activity of neuronal channels and transporters to alter neuronal firing properties. In these studies, increased extracellular K⁺ levels were associated with overactivation of the neuronal NKCC transporter, which led to the accumulation of intraneuronal Cl⁻, which led to neuronal depolarization that ultimately resulted in decreased inhibitory signals (Rangroo Thrane et al., 2013).

**Astrocyte Transmitter release:**

Another mechanism by which astrocytes modulate neural activity is by triggering the release of regulatory factors, or “glio”transmitters, that can modulate the dynamics of synaptic transmission (Andersson et al., 2007; Araque et al., 2014; Henneberger et al., 2010; Jourdain et al., 2007; Liu et al., 2004). These factors, which include ATP, D-serine and glutamate, activate neuronal receptors that mediate pre-synaptic firing or post-synaptic responses. For example, in rat hippocampus, astrocyte derived glutamate can bind post-synaptic kainate glutamate receptors to modulate post-synaptic currents.
Similarly, ATP that is converted to adenosine can modulate pre-synaptic release probability by binding to presynaptic adenosine receptors. By binding A1 adenosine receptors, pre-synaptic release probability can be decreased, while by binding A2A adenosine receptors pre-synaptic release probability can be increased (Panatier et al., 2011; Pascual et al., 2005). The contexts that determine which modulatory effects are induced when and why are not yet understood.

Whether or not astrocytes directly release “glio”transmitters is a topic of much debate in the field. It is evident “glio”transmitters modulate neural activity, but the source of these transmitters is not clear; there is limited evidence that astrocytes directly release transmitters in vivo. An alternative model to astrocyte release of transmitters is that astrocytes signal to a different cell type that subsequently secretes the transmitter. In most studies, changes in neural activity are linked to broad disruption of astrocyte function that is rescued using pharmacological methods that affect both astrocytes and neurons. For example, in Gourdon et al, authors demonstrate that norepinephrine modulates miniature excitatory postsynaptic current (mEPSC) amplitudes by triggering astrocyte release of ATP to activate neuronal P2X receptors (Gordon et al., 2005). While application of norepinephrine modulated mEPSC amplitudes in wildtype tissue, application of norepinephrine had no effect on mEPSCs following severe toxin-induced disruption of astrocyte metabolic function. The mEPSC amplitude modulation could be rescued by application of P2X agonists, suggesting astrocyte disruption resulted in the loss of endogenous ATP production. While such experiments imply that astrocytes have a role in regulating synapse function, they do not demonstrate that astrocytes themselves
release transmitters. Because many of the receptors that respond to “glio” transmitters are expressed by neurons and glia, cell type specific manipulations performed in vivo would help delineate the contributions of different cell types.

In accord with a lack of direct evidence for transmitter release, the mechanisms by which astrocytes are proposed to release transmitters are unclear and controversial. It is thought that transmitters could be released via vesicles, volume regulated anion channels, neurotransmitter transporters and gap junction channels. However, here also, there is limited in vivo evidence of such processes taking place and therefore requires a great deal of investigation in the future (Allen, 2014; Hamilton and Attwell, 2010; Henneberger et al., 2010; Stehberg et al., 2012). Clarifying how substrates are released by astrocytes in vivo is an important next step in the field. This will help delineate the direct role of astrocytes in modulation of neuronal activity by transmitters. It will also shed light on the molecular mechanisms by which astrocytes are recruited to regulate transmitters.

Membrane dynamics

Astrocytes extend fine membrane processes that closely associate with synaptic structures. The spatial properties of these associations have direct consequences on synaptic function. For example, the amount of synaptic material encased by astrocyte membranes can determine synapse volume and thereby influence the spatial and temporal dynamics of neurotransmitter signaling. Additionally, the efficiencies of astrocyte neurotransmitter uptake as well as “glio” transmitter response are influenced by the spatial distribution of astrocyte and synapse membranes. For example, when astrocyte
membranes were seen invading the synaptic cleft, thereby altering synaptic cleft volume and the spatial relationship between astrocyte and synapse membranes, rates of astrocyte glutamate uptake increased. Subsequently, these mice displayed reduced excitatory synaptic strength (Pannasch et al., 2014).

Understanding how adhesion is modulated between astrocytes and neurons is an important step in understanding astrocyte impact on synapse function. Recently, astrocyte gap junction channels have emerged as important modulators of astrocyte dynamics (Pannasch et al., 2014). While they have been implicated in membrane re-distribution, the molecular signals involved in this process are unclear. Additionally, it should be noted that astrocyte-neuron interactions are sensitive to their surrounding environments and to regulation by other cell types as well. For example, Shao et al demonstrated epidermal cells are important regulators of glia-neuron adhesion. Disruption of glial morphology, due to impaired epidermal-astrocyte signaling, resulted in misguided astrocyte-neuron contacts that lead to ectopic synaptic structures (Shao et al., 2013). The significance of astrocyte membrane dynamics in shaping synapse function has been greatly overlooked by the field. It is an emerging topic with prospects of furthering our understanding of how astrocytes modulate neural activity.

**Astrocyte regulation of synapse development**

Astrocytes impart their influence on neuron biology even at developmental stages. Astrocytes have proven to be key regulators of circuit assembly, capable of both positively and negatively regulating synapse numbers.
Curiosity was sparked when seminal work by Ullian et al. demonstrated both synapse number and synapse efficacy were dependent on the presence of astrocytes in vitro. Retinal ganglion cells (RGCs) cultured in the presence of astrocyte-conditioned medium formed significantly more synapses than RGCs cultured alone. Ultrastructure analysis showed that seven-fold more synapses formed in the presence of astrocytes. Similarly, confocal analysis showed that the number of synaptic puncta, counted as the co-localization of pre- and post-synaptic proteins, was dramatically increased in the presence of astrocytes. Interestingly, total amounts of synaptic protein were unchanged in culture, suggesting astrocytes influence localization of synaptic proteins and promote the coordinated assembly of pre and post-synaptic specializations. In addition to structure, synapse function was also affected. The presence of astrocytes enhanced synaptic efficacy through both pre- and post-synaptic mechanisms. Also, when astrocytes were removed from culture following synapse formation, synapse numbers decreased, suggesting astrocytes play an important role in synapse stability. Although these experiments were performed in vitro and required follow up investigations in vivo, the study shed light on several intriguing aspects of astrocyte-neuron communication that carved out directions for future studies (Ullian et al., 2001b).

Since the studies by Ullian et al., several astrocyte factors that regulate excitatory synapse formation have been identified. As of yet, there is no astrocyte factor that is sufficient to promote the formation of a mature functional synapse. Rather, it seems that several astrocyte factors cooperate to orchestrate synapse formation. For example, Thrombospondins (TSP 1 and 2) and Hevin are extracellular matrix proteins that promote
the assembly of glutamatergic synapse structure (Christopherson et al., 2005; Kucukdereli et al., 2011). RGCs cultured with purified TSP1 or Hevin formed significantly more synaptic structures that are ultrastructurally normal, but postsynaptically silent. Consistently, mice deficient in TSP1 and TSP2 had 25% fewer synaptic structures in the cerebral cortex at P21; a developmental stage at which synapse numbers have plateaued in the mouse cortex. Similarly, Hevin knockout mice showed a 35% decrease in synaptic structures in the superior colliculus at P14, a developmental stage at which synapse formation is nearing its end. Other pro-synaptogenic astrocyte factors include Glypicans (Gpc4 and Gpc6), a family of heparan sulphate proteoglycans (Allen et al., 2012). In contrast to the roles of TSP and Hevin, astrocyte glypicans promote synapse function. Application of purified Gpc4 to cultured RGCs was sufficient to increase the frequency and amplitude of post-synaptic responses. This regulation is achieved through the recruitment of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors to the synapse. Accordingly, mEPSC amplitudes were significantly decreased in the hippocampus of Gpc4 knockout animals during synapse formation. Immunohistochemistry also revealed a decrease in the number of functional excitatory synapses, marked by the triple co-localization of the markers for pre-synaptic structure (VGLUT), post-synaptic structure (MAGUK) and post-synaptic function (GluA1). While the number of VGLUT and MAGUK co-localized puncta did not change, there was a significant reduction in the localization of all three markers (Allen et al., 2012).
Astrocytes can also negatively regulate synapse numbers during development. One way they do this is by phagocytosing synapses. This feature of astrocytes is required for the elimination of synapses during developmental pruning. In *Drosophila*, the phagocytic receptor, Draper, is required in astrocytes to mediate astrocytic engulfment of synapses during disassembly of the larval nervous system (Tasdemir-Yilmaz and Freeman, 2014). Similarly, the mammalian ortholog of Draper, MEGF10, and another phagocytic receptor, MERTK, mediate phagocytic properties of mammalian astrocytes. Through MEGF10 and MERTK pathways, astrocytes help refine neural circuits in the developing CNS by phagocytosing weak synaptic inputs (Chung et al., 2013). Impaired phagocytosis by astrocytes resulted in an excess number of synaptic inputs that abnormally innervated their target neurons in the developing mouse dorsal lateral geniculate nucleus (dLGN). Astrocytes also indirectly regulate synapse elimination by regulating microglia-mediated phagocytosis of synapses (Bialas and Stevens, 2013). Microglia are major contributors to neural circuit refinement. Via mediation by the neuronal protein C1q, microglia engulf weak synaptic inputs during developmental pruning (Schafer et al., 2012). The release of transforming growth factor-β (TGF-β) by astrocytes is an important regulator of neuronal C1q expression, thus modulating microglia synapse elimination (Bialas and Stevens, 2013). Another mechanism by which astrocytes can negatively regulate synapse numbers is by inhibiting synapse formation. Astrocyte release of SPARC (secreted protein acidic and rich in cysteine), a matricellular protein, negatively regulates synapse formation. Mice deficient in SPARC had about 70% more synapses in the superior colliculus at P14, a developmental stage at which
synapses are forming (Kucukdereli et al., 2011). Astrocyte SPARC is thought to impart its effects in multiple ways. One model is that SPARC antagonizes the pro-synaptogenic protein Hevin by competing for a common unknown receptor (Kucukdereli et al., 2011). SPARC has also been shown to reduce synapse numbers by decreasing surface levels of AMPARs (Jones et al., 2011).

It is important to note the roles of these astrocyte factors *in vivo* have been studied using whole animal knockout of target genes. As of yet, synapse numbers have not been assayed *in vivo* after astrocyte specific knockout of an astrocytic synaptogenic factor. Because many of these factors are broadly expressed in the animal, conditional knockouts will better reveal how astrocytes specifically contribute to synapse formation. The requirement of astrocytes for synapse formation has been challenging to test *in vivo*. Tsai et al approached this question by selectively eliminating astrocytes in small regions in the developing mouse spinal cord. However, a caveat to this approach is that because several synaptogenic factors are secreted molecules, the remaining astrocytes in neighboring regions may impart synaptogenic effects onto areas lacking astrocytes.

While the field has made great strides in identifying astrocyte factors that regulate synapse numbers, the molecular mechanisms by which astrocytes interact with neurons to impart their influence is not well understood. The pro-synaptogenic factor TSP1 interacts with its neuronal binding partner α2δ1, which is localized to the synapse (Eroglu et al., 2009). However, the downstream signaling cascades that lead to regulation of synapse numbers are elusive. More strikingly, the neuronal targets of most other astrocyte synaptogenic factors are not even known. Additionally, we do not understand the cues
that trigger astrocytes to impart their regulatory functions. Are astrocytes innately hardwired to release particular factors at specific times during development? Or, do astrocytes actively respond to neuronal cues? Such questions are particularly interesting in light of the fact astrocytes have distinct regulatory effects on each class of synapse. Our understanding of astrocyte regulation of synapse formation comes almost exclusively from the study of excitatory synapses. But there is emerging evidence that astrocytes are also capable of regulating GABAergic synapse formation. The presence of astrocyte-conditioned media increased the number of inhibitory synaptic structures and the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal neurons. TSP1, an astrocyte factor known to promote glutamatergic synapse formation, did not have an effect on GABAergic synapses (Hughes et al., 2010). Thus, the astrocyte factors that regulate excitatory versus inhibitory synapses are distinct. Additionally, selective effects on synapse formation of particular synapse types were observed following astrocyte elimination in a small region of the developing mouse spinal cord (Tsai et al., 2012). Following astrocyte elimination, the numbers of synapse structures were assessed in the region lacking astrocytes. The formation of cholinergic and VGlut2 glutamatergic synapses was unaffected while the numbers of VGlut1 glutamatergic synapses decreased and GABAergic synapses increased. This demonstrates in vivo astrocytes can selectively control synapse formation based on the class of synapse. Understanding how astrocytes specifically interact with distinct neuronal subtypes is an impeding hurdle in the field that requires attention.
Thesis summary

Astrocytes are critical regulators of synapse function and development. Our understanding of astrocyte biology predominantly comes from studies conducted in mammalian model systems, where it is challenging to probe molecular interactions with cell type specificity. The genetic tractability and short life cycle of *Drosophila melanogaster*, a species of fly, provides great potential to advance the glia biology field. Using *Drosophila*, one can easily manipulate specific cell populations with spatial and temporal precision (Brand and Perrimon, 1993; Potter et al., 2010). Additionally, the short breeding time (~10 days) of the fly allows for rapid analysis of both developmental and adult studies. These features also enable opportunities to perform reverse and forward genetic screens to identify novel molecular regulators of astrocyte function.

The *Drosophila* CNS contains both neuronal and glial networks. CNS tissue can be grossly categorized into two distinct regions: cortex and neuropil. Neuronal cell bodies reside in the cortex, while neurites and synapses project into neuropil areas. The *Drosophila* glia population is composed of multiple subtypes that reside in distinct regions of the brain and impart unique properties (Awasaki et al., 2008; Doherty et al., 2009). For example, surface glia reside on the brain surface, surrounding neuronal cell bodies and encapsulating the CNS. Cortex glia infiltrate the cortex and wrap their membranes around neuronal cell bodies. Ensheathing glia surround neuropil structures and display immune responsive properties, similar to microglia. Via signaling through the phagocytic receptor Draper, ensheathing glia are essential for clearing debris following neuronal injury. Finally, astrocytes reside on the periphery of neuropil
structures and project highly ramified membrane processes into neuropil areas that intimately associate with synaptic structures. *Drosophila* astrocytes morphologically and functionally resemble their mammalian counterparts in several critical ways. Astrocytes from both species tile, thus organizing themselves within the neuropil in the same manner (Stork et al., 2014). This suggests intriguing parallels in the ways astrocytes grow and undergo morphogenesis. Also, like mammalian astrocytes, *Drosophila* astrocytes express neurotransmitter transporters and modulate neurotransmitter uptake (Neckameyer and Cooper, 1998; Stacey et al., 2010; Stork et al., 2014).

While the fly offers many experimental advantages, several basic principles regarding the relationship between astrocytes and synapses are not yet established. For example, the relationship between astrocytes and synapses during development is not well defined in the fly. Like in mammals, *Drosophila* astrocytes display phagocytic properties during synapse pruning, but whether *Drosophila* astrocytes also promote synapse formation, a hallmark feature of mammalian astrocytes, is not yet known (Tasdemir-Yilmaz and Freeman, 2014). My thesis work investigates reciprocal signaling between astrocytes and synapses in *Drosophila* during circuit assembly. First, my work describes the coordinated development of astrocytes and synapses during late metamorphosis, the developmental stage at which the adult nervous system is assembled. Next, utilizing this developmental window, I investigated how astrocyte-synapse interactions during development can modulate neuronal output. In particular, my work explored the signaling events that underlie astrocyte GABA transporter expression and its roles in balancing excitatory and inhibitory neuronal signaling. Finally, utilizing the ease
of the *Drosophila* system, I performed a reverse genetic screen to identify new astrocyte factors important for mediating excitatory-inhibitory neuronal balance.
CHAPTER II

Coordinated development of astrocytes and synapses during *Drosophila* late metamorphosis
Introduction

How astrocytes and neurons communicate during development to shape nervous system output is not well understood. Astrocytes are known to be important regulators of synapse number, but the molecular mechanisms by which this is accomplished are still elusive. Furthermore, little is known about the ways in which feedback from neural activity mediates astrocyte development. The genetic tractability and short life cycle of *Drosophila melanogaster* presents an opportune environment to investigate reciprocal astrocyte-synapse interactions. However, examination of *Drosophila* astrocytes at CNS synapses has been limited (Tasdemir-Yilmaz and Freeman, 2014). Several basic questions regarding the relationship between *Drosophila* astrocytes and CNS synapses remain. For example, are *Drosophila* astrocytes required for proper synapse formation?

Here, we turned to late pupa stages of development to investigate how astrocytes and synapses coordinately develop during assembly of the adult *Drosophila* CNS. Distinct from mammals, *Drosophila* undergo two rounds of nervous system assembly. The first round of neurogenesis occurs during embryo stages, which gives rise to embryo and larva nervous systems. Following larval stages, the animal undergoes metamorphosis where the animal develops within a puparium for ~100 hours. During this time, when the animal is referred to as a pupa, the larval nervous system is largely dismantled and a second round of nervous system assembly takes place to give rise to the adult nervous system. Most developmental studies in *Drosophila* have focused on embryo and larval stages. However, these stages are brief, and leave narrow windows of time to study a developing circuit. Moreover, these early stage nervous systems disassemble during
metamorphosis, which prevents one from examining the long-term effects of developmental processes. In contrast, pupa provide a larger window of time to investigate a developing nervous system and importantly, they progress into adult animals where one can study the behavioral consequences of developmental manipulations.

The precise timing of when synapses form in the adult nervous system is not known. Neurites that are specific to the adult nervous system wire the brain and reach their targets (without yet forming synapses) by 48 h after puparium formation (APF) (Jefferis et al., 2004). Strikingly, there has been no account of when synapses develop in the time between neuronal wiring and animal eclosion. Interestingly, astrocytes also exist in an immature state at 48 h APF. Only astrocyte cell bodies that lack elaborate membrane arborizations are present at this time. Thus, it can be deduced that synapses and astrocytes develop during the time span of late metamorphosis. However, the temporal regulation of these processes and whether or not the two are linked has not been examined. Here, we demonstrate that during late metamorphosis, as the adult *Drosophila* brain is forming, synapse formation is tightly coupled with astrocyte development and depends on the presence of astrocytes.

**Results**

**Astrocytes invade the developing adult neuropil coordinately with synaptogenesis**

During *Drosophila* metamorphosis the larval nervous system is dismantled and adult neural circuitry is constructed. The majority of pruning of larval neurites is complete by
~48 h after puparium formation (APF) (Jefferis et al., 2004; Marin et al., 2005; Zhu et al., 2003). To define the relative timing of astrocyte infiltration and synapse formation during development of the adult Drosophila neuropil, we assayed astrocyte morphology and synapse formation in the central brain at 48, 60, 72, 84, and 96 h APF, and adult stages. Astrocyte membranes were labeled using the astrocyte driver alrm-GAL4 to drive membrane-tethered GFP (UAS-mCD8::GFP) and co-stained for the presynaptic active zone marker Bruchpilot (nc82 antibody) to label the neuropil (Figure 2.1a). We have focused primarily on the antennal lobe (AL) region of the brain. This brain region, based on our analysis, appears to be characteristic of astrocyte infiltration and neuropil development throughout the central brain (Figure 2.2). Astrocyte cell bodies were present at the interface of the neuropil and the cortex at 48 h APF, although astrocyte membranes had not yet invaded the neuropil. By 60 h APF, short, thick astrocytic membrane processes radiating from astrocyte cell bodies could be seen infiltrating neuropil regions. At 72 h APF, infiltration had progressed significantly, and astrocyte processes were found throughout the brain neuropil. Although secondary branches off the main processes could be seen, the fine branching that characterizes the bushy tufted morphology of mature astrocytes was not yet observed (Figure 2.1a, 2.3). At 84 h APF astrocytes had densely infiltrated neuropil areas of the brain, and displayed fine branching and tufted morphology (Figure 2.1a, 2.3). A comparable morphology was observed at 96 h APF and in adult stages. Thus, the initial phases of astrocyte infiltration into the neuropil occurred between 60 and 84 h APF (Figure 2.1a, 2.2, 2.3).
Figure 2.1

(a) Immunostaining for astrocytes and neural progenitors. Images show astrocytes (GFP/NK82) at the anterior lobe at different time points: 48 h APF, 60 h APF, 72 h APF, 84 h APF, 96 h APF, and adult. The scale bars are 50 μm.

(b) Electron microscopy images of the anterior lobe at different time points: 48 h APF, 60 h APF, 72 h APF, 84 h APF, 96 h APF, and adult. Arrows indicate synapses and other structures.

(c) Graphs showing the number of synapses per 100 μm² at 48 h APF, 60 h APF, 72 h APF, 84 h APF, 96 h APF, and adult for AL, MB, and SPSL regions.
Figure 2.1: Astrocyte infiltration and synaptogenesis are temporally correlated during late metamorphosis

(a) Confocal section through the AL region showing astrocyte infiltration at several timepoints during metamorphosis. Astrocyte membranes are labeled by UAS-mCD8::GFP expression using the alrm-GAL4 driver (green), and neuropil is labeled by nc82 antibody staining (red). Scale bar = 10µm. (b) Ultrastructure of AL neuropil at several timepoints during metamorphosis, highlighting the progression in synapse development. Arrows point to pre-synaptic sites and asterisks mark post-synaptic structures. Synaptic structures are prominent starting at 72 h APF. Scale bar = 0.5µm. (c) Quantification of the number of synaptic structures in the AL (n ≥ 20 sections for each timepoint), MB (n ≥ 6 sections for each timepoint), and SPSL (n ≥ 9 sections for each timepoint) during late metamorphosis. Error bars, s.e.m.
Figure 2.2

(a)

48 h APF
60 h APF
72 h APF
84 h APF
96 h APF
Adult

Astrocyte>GFPCre/NC82

(b)

Astrocyte>GFPCre
NC82
Merge
Figure 2.2: Astrocytes infiltrate the neuropil throughout the central brain during late metamorphosis

(a) Confocal section through central brain showing astrocyte infiltration during late metamorphosis. Astrocyte membranes are labeled by UAS-mCD8::GFP expression using the alrm-GAL4 driver (green), and neuropil is labeled by nc82 antibody staining (red). Scale bar = 50µm. (b) Confocal section through the AL region showing astrocyte morphology at adult stages. Astrocyte membranes are labeled by UAS-mCD8::GFP expression using the alrm-GAL4 driver (green), and neuropil is labeled by nc82 antibody staining (red). Cell bodies, marked by arrow heads, reside along the periphery of neuropil regions while astrocyte processes are present within the neuropil. Scale bar = 10µm.
Figure 2.3

Astrocytes-GFP

60 h APF

72 h APF

84 h APF

96 h APF
Figure 2.3: Initial phases of astrocyte infiltration

Confocal section through the central brain, zoomed in on only a few cells, in order to highlight the change in astrocyte morphology during the initial phases of infiltration. Astrocyte membranes are labeled by UAS-mCD8::GFP expression using the alm-GAL4 driver (green). Scale bar = 10μm.
We next sought to determine when synaptic structures could first be observed within the neuropil, and how this might be coordinated with astrocyte infiltration. While nc82 can label presynaptic structures, identification of a synapse by antibody stains is typically accomplished by demonstrating co-localization of pre- and post-synaptic markers within a critical distance (Allen et al., 2012; Christopherson et al., 2005; Eroglu et al., 2009; Ullian et al., 2001b). Despite our best efforts we were unable to identify a post-synaptic marker that revealed punctate staining within the Drosophila pupal or adult neuropil. We therefore turned to transmission electron microscopy (TEM) as a means to identify synapses in the developing neuropil by ultrastructural criteria: we scored for the presence of a post-synaptic density in opposition to clusters of pre-synaptic vesicles. We focused our analysis on the AL, mushroom body (MB), and the superior posterior slope (SPSL) neuropil regions of the brain at 48, 60, 72, 84, 96 h APF, and in the adult.

The progressive infiltration of the neuropil with astrocyte membranes coincided with the formation of morphologically identifiable synapses in the Drosophila pupal brain (Figure 2.1b,c). At 48 h APF the neuropil was devoid of structures resembling synapses. At 60 h APF we observed the widespread appearance of immature synaptic structures, which were characterized by poorly defined post-synaptic densities that lacked pre-synaptic vesicles. By 72 h APF mature synapses were present, and continued to increase in numbers until 84 h APF, after which synaptic density and morphology remained largely unchanged. From these data we conclude that the major wave of synaptogenesis occurs in the Drosophila brain between 60 and 84 h APF. Thus, synapse formation and the infiltration of astrocyte membranes into the neuropil and
synaptogenesis are temporally coordinated during late metamorphosis (*i.e.* both occur between 60 and 84 h APF).

**Astrocytes display compensatory growth and requirement in the adult CNS**

The observation that *Drosophila* astrocyte infiltration into the neuropil during pupal stages occurs coordinately with synapse formation raises the intriguing possibility that astrocytes might regulate synapse formation in a manner similar to mammalian astrocytes (Christopherson et al., 2005; Hughes et al., 2010; Ullian et al., 2001a). To address this possibility *in vivo*, we ablated astrocytes during late metamorphosis in order to assay for changes in synaptic numbers. Ablation of astrocytes was achieved genetically by expressing the pro-apoptotic gene, *head involution defective* (*hid*), in a conditional manner (Grether et al., 1995). Briefly, *UAS-hid* was expressed under the control of the *alm-GAL4* driver in the presence of *tub-GAL80<sup>ts</sup>*. GAL80<sup>ts</sup> is a temperature sensitive inhibitor of GAL4 useful for conditional activation of Gal4/UAS: at 18°C, GAL80<sup>ts</sup> suppresses GAL4 activity; at 25°C GAL80<sup>ts</sup> activity is partially inhibited allowing for low-level Gal4/UAS activation; and at 30°C GAL80<sup>ts</sup> activity is strongly inhibited and Gal4/UAS activation is maximal. In order to ablate astrocytes only during late metamorphosis, we reared animals at 18°C and shifted to 25°C or 30°C at ~40 h APF and allowed animals to remain at these temperatures until eclosion (Figure 2.4a).

Astrocytes were visualized by anti-GAT immunofluorescence (*a* specific marker for astrocytes) (Figure 2.4b). To assess the degree of ablation under each condition, cells that were positive for both anti-GAT and anti-Repo (*a* pan-glial nuclear marker)
Figure 2.4

(a) Temperature (°C) diagram with stages of development: Embryo, Larva, Pupa (metamorphosis), and Adult. Two ablation levels are indicated: Severe and Moderate. Synaptogenesis is shown in the green box.

(b) Images of the central brain and thoracic ganglion showing control and treated conditions.

(c) Images of astrocytes, neuropil, and their merge.

(d) Graph showing the number of astrocytes in different conditions: Control, Astro-Hid (25°C), and Astro-Hid (30°C).

(e) Graph showing the number of astrocytes in different conditions: Control, Astro-Hid (30°C).

(f) Graph showing the percentage of total astrocytes in different conditions: Control, Astro-Hid (25°C), and Astro-Hid (30°C).
Figure 2.4: Genetic ablation of astrocytes during synaptogenesis

(a) Temperature shift scheme for astrocyte ablations. The alrm-GAL4 driver and tub-GAL80ts were used to conditionally express UAS-hid in astrocytes specifically during late metamorphosis. Varying degrees of GAL80 activity and UAS-hid expression were achieved by varying the incubation temperature during late metamorphosis. Low-level Hid expression was achieved at 25°C and maximal Hid expression was achieved at 30°C. (b) Confocal slice of central brain and ventral nerve cord of adult animals immunostained with anti-GAT antibody to visualize astrocytes. Astrocyte staining is moderately reduced following Hid expression in astrocytes at 25°C, and robustly reduced after Hid expression in astrocytes at 30°C, indicating moderate and severe ablation conditions, respectively. Scale bar = 10µm. (c) Confocal sections through AL of adult animals where astrocytes are labeled by anti-GAT antibody staining (green) and neuropil is labeled by anti-HRP antibody staining (red). Astrocyte processes can fully cover neuropil space when ablations are performed at 25°C. Large regions of neuropil are left unoccupied by astrocyte processes when ablations are performed at 30°C. Scale bar = 10µm. (d) Number of astrocytes remaining in the AL, MB, SOG, and TGab of the adult CNS following moderate (25°C) astrocyte ablations (n = 5 brains for control and Astro>Hid AL, MB, SOG, and TGab). (e) Number of astrocytes remaining in the AL, MB, SOG, and TGab of the adult CNS following severe (30°C) astrocyte ablations (n = 7 brains, control AL; n = 8 brains, Astro>Hid AL; n = 5 brains, control and Astro>Hid MB; n = 5 brains, control and Astro>Hid SOG; n = 5 brains, control TGab; n = 7 brains, Astro>Hid TGab). Fewer astrocytes remain when ablations are performed at 30°C, compared to 25°C; demonstrating the varying degrees of astrocyte ablation achieved by the two temperature conditions. (f) Fates of animals undergoing ablation procedure (n ≥ 30 flies for each condition). The majority of animals struggle to eclose when undergoing severe astrocyte ablations (30°C). *P≤0.05, **P≤0.01, ***P≤0.001, unpaired Student’s t-test. Error bars, s.e.m.
Figure 2.5
Figure 2.5: Identification of astrocytes after ablation procedures

(a) Confocal section through AL of adult animals where astrocytes are labeled by anti-GAT antibody staining (green), neuropil is labeled by anti-HRP antibody staining (red), and glial nuclei are labeled with anti-Repo staining (blue). Repo² nuclei belonging to GAT⁺ cells were identified as individual astrocytes within neuropil regions of interest. Scale bar = 10μm. (b) High magnification images of Repo⁺ nuclei belonging to GAT⁺ cells in our various regions of interest and ablation conditions. Arrows point to examples of cells that are both Repo⁺ and GAT⁺ (astrocytes). Arrow heads point to Repo⁺ nuclei that do not belong to GAT⁺ cells. Scale bar = 5μm.
immunofluorescence were counted in multiple central nervous system regions including the AL, MB, subesophageal ganglion (SOG), and the abdominal segments of the thoracic ganglion (TGab), (Figure 2.5 2.4b,d,e). After a shift to 25°C (moderate ablation), astrocyte numbers decreased from 22.4 ± 1.21 (n=5) to 11.8 ± 1.56 (n= 5) in the AL, from 10.2 ± 0.49 (n= 5) to 5.8 ± 0.73 (n= 5) in the MB, from 31.8 ± 1.7 (n=5) to 20.6 ± 1.9 (n= 5) in the SOG, and from 23.6 ± 1.29 (n=5) to 8.2 ± 0.73 (n= 5) in the TGab (Figure 2.4d). Surprisingly, in spite of the reduction in cell numbers by ~50% in all regions, the remaining ~50% of astrocytes remaining after ablation infiltrated the vast majority of the neuropil with membrane processes (Figure 2.4c). This observation indicates astrocytes can exhibit significant plasticity in their morphology during development. Additionally, we note that despite the significant reductions in astrocyte cell numbers in the CNS, 100% of the pupae shifted to 25°C survived to adulthood and did not display any obvious behavioral defects (Figure 2.4f). These observations argue that astrocytes as a population are highly plastic, and apparently generated in sufficiently high numbers to accommodate significant reductions in the astrocyte population without in turn causing dramatic changes in development, animal survival, or overt behavior.

After a shift to 30°C (severe ablation) astrocyte numbers decreased more dramatically: from 24.75 ± 0.82 (n=8) to 4.5 ± 0.62 (n= 8) in the AL, from 10 ± 0.32 (n= 5) to 4.2 ± 0.58 (n= 5) in the MB, from 32.4 ± 3.04 (n=5) to 7.2 ± 1.3 (n= 5) in the SOG, and from 24.67 ± 1.7 (n=5) to 3.4 ± 0.57 (n= 7) in the TGab (Fig. 2b,e). Under these conditions, the remaining astrocyte processes appeared more sparsely distributed and the remaining small numbers of astrocytes were unable to fully cover neuropil space (Figure
Several areas were observed in which the neuropil completely lacked astrocyte infiltration. Moreover, 41% of pupae undergoing severe ablations failed to eclose, 32% partially emerged from their pupal cases but died before fully eclosing, and only 27% survived to adulthood (Figure 2.4f). The latter collection of surviving animals were highly uncoordinated, could not walk or fly, and lived for at most only a few days. Taken together, these ablation studies indicate that the fly CNS is very robust and can tolerate a significant loss of astrocytes. Reducing the number of astrocytes in the CNS by ~50% during synaptogenesis did not noticeably affect animal survival or result in obvious behavioral defects. Only the ablation of ~75% or more of Drosophila astrocytes during pupal stages greatly reduced survival and resulted in severe defects in motor activity and premature death.

**Drosophila astrocytes are required for synaptogenesis in vivo**

We next sought to determine whether elimination of astrocytes led to changes in synapse formation. Gross synapse morphology, as defined above by TEM, did not appear altered in astrocyte-ablated animals (Figure 2.6a). Post-synaptic density (PSD) length, a characteristic feature of synaptic structure, was unchanged after astrocyte ablation (Figure 2.6b). Similarly, we found that the percentage of synapses that display T-bar morphology, a characteristic pre-synaptic structure of Drosophila synapses, was unaltered (Figure 2.6c). However, astrocyte ablation did have a dramatic effect on the total number of synapses present in those animals that survived to adult stages. In astrocyte ablated animals, synaptic density was reduced 32% in the AL, from 37.2 ± 1.4
(n=23 sections) to $25.3 \pm 0.88$ (n=27 sections) synapses per 100\(\mu\)m\(^2\); 36% in the MB, from $48.3 \pm 3.8$ (n=7 sections) to $30.1 \pm 3.5$ (n=11 sections) synapses per 100\(\mu\)m\(^2\); 47% in the SPSL, from $30.6 \pm 1.4$ (n=8 sections) to $16.1 \pm 3.0$ (n=8 sections) synapses per 100\(\mu\)m\(^2\) (Figure 2.6b). While no other obvious changes to neuropil structure could be assessed, astrocyte ablated tissue was marked by a high frequency of ruptured mitochondria (Figure 2.7), which might result from a prolonged absence of astrocytes. However, we cannot definitively state whether or not it is a direct result of astrocyte loss.

We quantified synapse numbers at 84 h APF, the time point at which total synapse numbers reached $\sim$90% in control animals (Figure 2.1c). We found that even at this earlier time point, synapse numbers were significantly reduced in the absence of astrocytes (Figure 2.6a,d, 2.7, 2.8). Interestingly, we noticed several immature synaptic structures (similar in morphology to what was observed at 60 h APF) in astrocyte-ablated animals. Whether these structures are delayed in development and eventually mature, or fail to mature is unclear (Figure 2.6a, 2.9). We examined whether neuronal architecture or survival were grossly affected in these animals. To our surprise, gross morphology of the adult Drosophila brain appeared unaffected by severe astrocyte depletion. For example, after nc82 staining of the adult brain, AL glomerular organization appeared normal at the light level: glomerular borders were sharply defined, and identifiable glomeruli were appropriately sized (Figure 2.6e). To explore whether neuronal cell numbers were reduced in the absence of astrocytes, Neuronal projections belonging to second order antennal lobe projection neurons (PNs), marked by GH146-QF/QUAS-mCD8::GFP, exhibited normal morphology with dendrites projecting into
Figure 2.6

(a) Comparison of Adult and 84 h APF in Control and Astro-Hid conditions.

(b) PSD length (µm²) in Control and Astro-Hid conditions.

(c) Percentage of synapses per 100 µm² in AL, MB, and SPSL.

(d) Number of synapses per 100 µm² in AL, MB, and SPSL for Adult and 84 h APF.

(e) Images showing NC82, Projection Neurons-GFP, and PDF neurons in Control and Astro-Hid conditions.

(f) Number of RNs in Astro-Hid conditions.

(g) Number of PDF neurons in Astro-Hid conditions.
Figure 2.6: Synapse number, but not gross neural architecture, is altered when astrocytes are ablated during late metamorphosis

(a) Ultrastructure of synapses in the AL at adult and 84 h APF animals following 30°C astrocyte ablation. Morphology of mature synaptic structures is unaltered by ablation. Arrows point to pre-synaptic sites and asterisks mark post-synaptic structures. Scale bar = 0.5µm. (b) Quantification of post-synaptic density (PSD) length in AL, MB, and SPSL regions of the adult brain after 30°C astrocyte ablation (n = 80 PSDs from 12 sections, AL control; n = 81 PSDs from 12 sections, AL Astro>Hid; n = 47 PSDs from 7 sections, MB control; n = 50 PSDs from 7 sections, MB Astro>Hid; n = 58 PSDs from 7 sections, SPSL control; n = 63 PSDs from 7 sections, SPSL Astro>Hid. (c) Quantification of the percentage of synapses with T-bar morphology in the AL, MB, and SPSL regions of the adult brain after 30°C astrocyte ablation (n = 23 sections, AL control; n = 24 sections, AL Astro>Hid; n = 7 sections, MB control; n = 11 sections, MB Astro>Hid; n = 8 sections, SPSL control; n = 8 sections, SPSL Astro>Hid. (d) Quantification of the number of synaptic structures in the AL, MB, and SPSL regions of adult and 84 h APF animals following 30°C astrocyte ablation (n = 17 sections, AL adult control; n = 22 sections, AL adult Astro>Hid; n = 19 sections, AL 84 h APF control; n = 20 sections, AL 84 h APF Astro>Hid; n = 17 sections, MB adult control; n = 22 sections, MB adult Astro>Hid; n = 19 sections, MB 84 h APF control; n = 20 sections, MB 84 h APF Astro>Hid; n = 17 sections, SPSL adult control; n = 22 sections, SPSL adult Astro>Hid; n = 19 sections, SPSL 84 h APF control; n = 20 sections, SPSL 84 h APF Astro>Hid. (e) Brain architecture is grossly unaltered following astrocyte ablations performed at 30°C. Projected confocal z-stacks showing (i) glomeruli structure in brains stained with nc82 antibody (red), (ii) morphology of PNs marked by GH146-QF/QUAS-mCD8::GFP, (iii) morphology of axonal projections and arborizations of PNs, (iv) morphology of PDF neuron axonal projections in the central brain, (v) morphology of PDF neuron dendritic arborizations in the optic lobe. Scale bar = 10µm for (i) and (iii); 50µm for (ii); 25µm for (iv) and (v). (f) Quantification of the number of PNs per hemisphere (n = 6 brains, control and Astro>Hid) and (g) PDF+ neurons per hemisphere (n = 6 brains, control; n = 5 brains, Astro>Hid) following 30°C astrocyte ablation. ***P≤0.001, unpaired Student’s t-test for (b-d), and (f-g). Error bars, s.e.m.
Figure 2.7
Figure 2.7: Ultrastructure of neuropil after astrocyte ablation

Low magnification image of AL neuropil tissue ultrastructure following the 30°C astrocyte ablation procedure. Asterisks mark mitochondrial structures. Many mitochondrial structures looked unhealthy and ruptured in adult tissue following astrocyte ablations. However, this trend was not observed at earlier stages. Arrows point to structures that we suspect are astrocyte membranes. These structures become extremely difficult to identify following astrocyte ablations. Scale bar = 1µm.
Figure 2.8
Figure 2.8: Constitutive ablation of astrocytes during late metamorphosis

Confocal section through AL of animals ~60 and ~84 h APF that were undergoing the 30°C astrocyte ablation procedure. Astrocytes are successfully ablated during development. Astrocytes are labeled by anti-GAT staining (green) and neuropil is labeled by anti-HRP staining (red). Scale bar = 10µm.
Figure 2.9
Figure 2.9: Astrocyte ablations result in higher frequency of immature synaptic structures at 84 h APF

(a) Quantification of the number of immature synaptic structures in the AL of 84 h APF animals (n = 19 sections, Control; n = 20 sections, Hid) (b) Stacked representation of the number of synapses with the number of immature synaptic structures in the AL of 84 h APF animals (n = 19 sections, Control; n = 20 sections, Hid). (c) Sum of the number of synapses and immature synaptic structures in the AL of 84 h APF animals and adult animals (n = 19 sections, Control 84 h APF; n = 20 sections, Astro> Hid 84 h APF; n = 23 sections, Control Adult; n = 27 sections, Astro>Hid Adult. ***P≤0.001, unpaired Student’s t-test. Error bars, s.e.m.
glomeruli and axons wiring to the MB and lateral horn (Figure 2.6e). Pigment dispersing factor (PDF+) neurons, lateral neurons representing another population of higher order circadian neurons, exhibited normal morphology. To explore whether neuronal cell numbers were reduced in the absence of astrocytes we counted the number of PNs and PDF+ neurons in the brain. The number of PNs and PDF neurons were found to be comparable in control and astrocyte-ablated animals (Figure 2.6f). Furthermore, immunofluorescence by anti-Draper, which labels membranes of cortex and ensheathing glia in the adult brain, showed that morphology of these glial subtypes was not noticeably altered after astrocyte ablation (Figure 2.10). These data argue that ablation of astrocytes during late metamorphosis does not interfere with gross brain architecture, neurite morphology, or neuronal survival. Rather, our data argue for a more direct involvement of Drosophila astrocytes in regulating synapse numbers.

Discussion

During assembly of the Drosophila adult brain, the formation of synaptic structures coincides with astrocyte membrane invasion into the neuropil. Both these processes occur between 60-84 h APF and are coordinated. Appropriate synapse formation depends on the presence of astrocytes, identifying an important parallel between Drosophila and mammalian astrocytes. These data present late metamorphosis as a potentially useful developmental stage to explore the coordinated development of astrocytes and synapses. For example, neurons can be probed specifically between 60-84 h APF to investigate their influence on astrocytes during synaptogenesis. Because this
Figure 2.10
Figure 2.10: Morphology of cortex glia and ensheathing glia are grossly unaltered following astrocyte ablations

Confocal section through region surrounding AL, showing cortex and ensheathing glia morphology by anti-Draper stain, and location of glial nuclei by anti-Repo stain. Scale bar = 10µm
developmental period is followed by adulthood, a stage that is not rapidly nearing deterioration, one can delineate how astrocyte-neuron interactions during synaptogenesis impart long lasting effects on a nervous system.

**Astrocyte infiltration into neuropil coincides with the formation of *Drosophila* adult CNS synapses.**

Based on the up-regulation of selected presynaptic markers, previous studies have argued that synaptogenesis occurs during late metamorphosis (Jefferis et al., 2004). Whether these observations reflected actual assembly of pre- and post-synaptic compartments and the appearance of mature synaptic structures was not known. We directly examined synapse formation by electron microscopy in multiple parts of the developing adult *Drosophila* central brain and found that the majority of synaptic structures formed between 60-84 h APF. To our knowledge, this is the first detailed ultrastructural analysis of when mature synaptic structures form in the developing adult *Drosophila* CNS. Interestingly, infiltration of astrocyte processes into the adult brain was tightly coordinated with the formation of adult brain synapses.

**The formation of *Drosophila* adult CNS synapses requires the presence of astrocytes**

The coincident timing of astrocyte invasion and synapse formation raised the possibility that *Drosophila* astrocytes and CNS synapses might be reciprocally interdependent for formation during development. Indeed, we found genetic ablation of ~75% of an animal’s astrocytes during the synaptogenic window resulted in a 30-50% (depending on
brain region) reduction in synapses throughout the pupal and adult brains. This loss of synapses was accompanied by dramatic defects in adult behavior such as severe defects in motor function, although we cannot determine whether these result from deficits in synapses, astrocytes, or both.

Consistent with previous studies, our ablation experiments demonstrated astrocytes have compensatory growth properties. Following severe astrocyte ablation, in which ~75% of an animal’s astrocytes are eliminated, remaining astrocytes expanded their territories and sparsely distributed their membranes. This is similar to what was observed in Stork et al when the majority of astrocytes was depleted from the Drosophila larval ventral nerve cord. In contrast, after genetic ablation of ~50% of an animal’s astrocytes, the remaining astrocytes were able to expand their territories and fully cover neuropil areas. Coincidently, neither survival nor overt behavior was impaired in these animals. Whether this is directly attributable to the ability of astrocytes to fully cover neuropil space needs further investigation. Additionally, it must be noted that behavior was observed rather crudely and more sensitive assays are required to assess the effects of moderate astrocyte ablation on circuit function.

Why was there only a partial loss of synapses in the adult when astrocytes were severely ablated? One possibility is that many CNS synapses in Drosophila may form in the absence of input from astrocytes. Astrocyte-secreted factors in mammals are critically important for both excitatory and inhibitory synapse formation in vivo, however no mutants affecting pro-synaptogenic astrocyte-derived molecules have been described in which more than 35% of CNS synapses are eliminated in vivo (Allen et al., 2012;
Christopherson et al., 2005; Kucukdereli et al., 2011). It also remains possible that ablation of *Drosophila* astrocytes preferentially affects specific types of synapses (e.g. cholinergic, glutamatergic, or GABAergic), which could not be determined by our EM analysis. Perhaps in the absence of astrocytes one particular subtype of synapse is completely eliminated. While it appears that other subtypes of *Drosophila* glia do not grow into neuropil regions and occupy the domains normally covered by astrocytes, other glial subtypes could functionally compensate for the loss of pro-synaptogenic astrocytic cues at a distance. Finally, our EM analysis was performed on the few animals that survived to adult stages, and these likely retained more astrocytes than those animals that expired at earlier developmental stages. As such, the observation that only 30-50% of synapses were eliminated could be explained by our inability to remove all astrocytes from the CNS and generate adult animals. It is possible that a small number of astrocytes can still have a pro-synaptogenic effect on their surroundings, especially since many astrocyte-secreted factors have been shown to potently promote synapse formation. Nevertheless, our *in vivo* demonstration of a requirement for *Drosophila* astrocytes in synaptogenesis is consistent with pro-synaptogenic roles for astrocytes in mammals (Allen et al., 2012; Christopherson et al., 2005; Kucukdereli et al., 2011; Ullian et al., 2001b), and our data reveal that astrocytic control of synapse formation is a conserved feature of mammalian and invertebrate CNS development.
CHAPTER III

Activity-dependent regulation of astrocyte GAT levels during synaptogenesis
Introduction

Brain circuits are comprised of complex ensembles of excitatory and inhibitory neurons and glial cells. Neurons and glia are intimately associated from very early developmental stages and the proper assembly of functional neural circuits is thought to require extensive neuron-glia signaling (Allen et al., 2012; Christopherson et al., 2005; Eroglu et al., 2009; Schafer et al., 2012; Tasdemir-Yilmaz and Freeman, 2014; Ullian et al., 2001a). Defining precisely how neurons and glia communicate during development to ensure proper neural circuit assembly remains a major challenge for the field. Astrocytes have emerged as critical regulators of neuronal development, particularly with respect to promoting synapse formation (Allen et al., 2012; Christopherson et al., 2005; Ullian et al., 2001a).

Reciprocal mechanisms by which synapses might signal to astrocytes to regulate their development remain more mysterious, despite the fact that astrocytes regulate key aspects of neural circuit function, including the balance of excitatory and inhibitory neurotransmission. Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and can be rapidly cleared by astrocytes via uptake through glutamate transporters (Lehre et al., 1995; Su et al., 2003). The principle inhibitory neurotransmitter Gamma-aminobutyric acid (GABA) serves as a brake to dampen excitatory signaling when appropriate. GABA inhibitory activity is mediated by GABA receptors (GABA-Rs) either locally at synapses where it hyperpolarizes the postsynaptic cell, or at extra-synaptic sites where it provides widespread tonic inhibition of neuronal firing (Isaacson et al., 1993; Rossi and Hamann, 1998). Debilitating diseases are caused
by imbalances in excitatory and inhibitory firing. For instance in epilepsy, mis-regulated GABA levels are believed to lead to hyperexcitability and ultimately cause seizures (Briggs and Galanopoulou, 2011; Cope et al., 2009; Dudek and Staley, 2007).

GABAergic signaling can be fine-tuned at multiple levels, including changes in GABA-R density or alterations in GABA-R subtype composition (Jacob et al., 2008). However, uptake of GABA by astrocytic GABA transporters (GATs) is also an important regulatory mechanism. GABA removal and degradation at synapses is critical for proper termination of GABAergic signaling, while uptake at non-synaptic sites can influence GABA tone across larger areas in the brain (Sarup et al., 2003; Schousboe, 2003).

Reduced GAT levels in mouse hippocampal astrocytes resulted in increased tonic inhibitory currents and reduced IPSC amplitudes in hippocampal interneurons, likely due to elevated GABA levels and subsequent desensitization of GABA receptors (Shigetomi et al., 2012). Similarly, blockade of astrocytic GAT in the rat hippocampus contributes to increased extracellular GABA concentrations and increased tonic GABA receptor mediated currents in dentate granule cells (Kersanté et al., 2013).

Despite the importance of astrocytic GATs in modulating GABAergic signaling, surprisingly little is known about how GAT levels are established during development or dynamically regulated in the mature brain. Rat astrocytic GAT is detectable at birth, but does not appear to take on adult patterns of expression in the cerebral cortex until postnatal week three (Vitellaro-Zuccarello et al., 2003). Interestingly, the timing of these postnatal changes coincides with periods of astrocyte morphogenesis and synaptic refinement (Freeman, 2010). This raises the intriguing possibility that initial GABAergic
synaptic activity might contribute to shaping the spatiotemporal pattern of astrocytic GAT expression.

GABAergic signaling is also a major component of neural circuit activity in the *Drosophila* nervous system (Enell et al., 2007; Küppers et al., 2003; Neckameyer and Cooper, 1998). There is a single *Drosophila* ortholog of the mammalian GABA transporters belonging to the SLC6 family, termed GAT, which is expressed in CNS astrocytes but not neurons, suggesting that astrocytes are the primary cell type responsible for GABA clearance (Neckameyer and Cooper, 1998; Stork et al., 2014). Consistent with this notion, GAT depletion from astrocytes causes profound defects in animal behavior (Stork et al., 2014).

Here we explore synapse-astrocyte interactions that underlie GAT activation and modulation in *Drosophila* astrocytes and roles for GAT in balancing excitatory and inhibitory signaling *in vivo*. Coincident with synaptogenesis during development of the adult nervous system, astrocytes exhibit an increase in GAT, which we show is modulated by GABAergic neuronal activity and astrocytic GABABR1/2 receptor signaling, suggesting that astrocytes regulate GAT levels by direct measurement of extracellular GABA. Finally, we demonstrate that inhibiting astrocytic GABABR1/2 signaling strongly suppresses seizure activity in bang-sensitive mutants with hyperexcitable neurons, arguing this pathway is critical for modulating excitatory/inhibitory balance *in vivo*.

**Results**
GABAergic neuron signaling regulates GAT levels

Astrocytic uptake of GABA through GATs is thought to be a key mechanism used to balance excitation and inhibition in the CNS (Madsen et al., 2010; Sarup et al., 2003; Schousboe, 2003). *Drosophila* larval astrocytes express the sole *Drosophila* ortholog of the Na⁺ and Cl⁻ dependent GABA transporter (GAT) of the SLC6 family (Neckameyer and Cooper, 1998), and its depletion results in uncoordinated animals that exhibit severely reduced motility in larvae and adults (Stork et al., 2014), which argues for an important role for *Drosophila* astrocytes in modulating GABA CNS tone. Intrinsic and extrinsic mechanisms that regulate how astrocytes acquire the appropriate molecular and morphological phenotypes remain poorly defined. Given that glutamatergic neuronal signaling regulates levels of the astrocyte glutamate transporter GLT-1, and thereby astrocyte control of glutamate tone (Benediktsson et al., 2012; Devaraju et al., 2013; Tanaka et al., 1997; Yang et al., 2009), we explored potential roles for GABAergic signaling in regulating levels of astrocytic GATs.

We first confirmed the specificity of our GAT antibody by performing Western blot analysis following knockdown of *gat* in astrocytes using *UAS-gat RNAi*. In control animals we observed a ~50 kDa band corresponding to GAT, which was eliminated in *gat* knockdown animals (Figure 3.1a,b). Immunofluorescent stains using this antibody also showed robust localization of GAT to adult astrocyte membranes (Figure 3.1c). GAT immunofluorescence was dramatically reduced after *gat* knockdown in astrocytes, but unchanged following *gat* knockdown in neurons (Figure 3.1c, 3.2). To determine the time course of GAT expression during development of the adult nervous system, we
Figure 3.1

(a) Astroastro-gAT (b) fold change in GAT

(c) AstroGFP, GAT, Merge

(d) h APF

(e) fold change in GAT

(f) GABA neuron>SYT::GFP, GAT, Merge
Figure 3.1: GAT is exclusively expressed in astrocytes and activated during synaptogenesis

(a) UAS-gat RNAi was expressed in astrocytes using the alrm-GAL4 driver. Western blots performed on larval CNS and adult brain lysates were probed with anti-GAT antibody to confirm specific knockdown of GAT in astrocytes. GAT runs at approximately 50kDa. (b) Quantification of GAT levels from Western blot analysis shown in (a) (n = 3 experiments, 3rd instar larva; n = 3 experiments, adult).

(c) The alrm-GAL4 driver was used to co-express UAS-mCD8::GFP and UAS-gat RNAi, or express UAS-mCD8::GFP alone. Adult brains were stained with anti-GAT antibody. Confocal section through AL shows that GAT (red) localizes specifically to astrocyte membranes (green). Scale bar = 10µm.

(d) Western blot performed on WT brain lysates from several stages of metamorphosis was probed with anti-GAT antibody. Quantification of GAT levels from Western blot analysis shown in (d) (n = 3 experiments).

(e) Adult brains expressing UAS-syt::eGFP using the gad-GAL4 driver were stained with anti-GAT antibody. GABAergic pre-synaptic sites (GABA neuron>Syt::eGFP) and GAT protein are present through out the central brain. High magnification images of the AL and MB regions show that GABAergic pre-synaptic sites and GAT proteins are in close association. Scale bar = 20µm. *P≤0.05, ***P≤0.001, unpaired Student’s t-test for (b) and 1-way ANOVA with Tukey’s post hoc test for (e). Error bars, s.e.m.
Figure 3.2
Figure 3.2: GAT is not expressed in neurons

The pan-neuronal driver, *elav-GAL4*, was used to express *UAS-gat RNAi*. Adult brains were stained with anti-GAT and anti-Elav antibodies. Confocal section through region surrounding AL shows that GAT⁺(red) cells are not Elav⁺ (green), and that *gat* knockdown in neurons has no effect on GAT expression. Scale bar = 10µm
performed Western blot analysis of GAT expression in dissected pupal brains during late metamorphosis (Figure 3.1d,e). Interestingly, we found that GAT levels increased during late stages of metamorphosis and displayed the most notable increase around 84 h APF, a time point at which most CNS synapses had formed (e.g. Figure 2.1c). To explore the spatial relationship between astrocyte GAT proteins and GABAergic synapses we examined GAT immunostaining while labeling GABAergic presynaptic sites using the GABA neuron specific driver, *gad-GAL4*, to express *UAS-syt::eGFP*. As expected, the two markers did not co-localize but were in close association throughout the central brain (Figure 3.1f).

To determine whether GABAergic neurons regulate astrocytic GAT levels, we used multiple approaches. First, we ablated GABA neurons during metamorphosis and examined GAT expression. The *gad-GAL4* driver was used to express *UAS-mCD8::mcherry* and *UAS-hid* in a conditional manner with *tub-GAL80ts*. GABAergic neurons were ablated specifically during metamorphosis by shifting animals to 30°C (thereby activating *Gal4/UAS*) at 0 h APF (Figure 3.3). Brains were then dissected and analyzed at 84 h APF. Ablation of GABA neurons was confirmed by the reduction in the number of *mCD8::mcherry* expressing cell bodies (Figure 3.4a,b). The number of GABA neurons in the medial-ventral region of the brain decreased from 64.8 ± 1.2 (n = 5 brains) to 8.60 ± 2.4 (n = 5 brains) following Hid induction. Interestingly, under these conditions we observed a significant reduction in GAT expression throughout the brain by immunofluorescent stains (Figure 3.4a,c). For instance, GAT levels were reduced by 33% and 37% in the AL and SOG, respectively. Quantification of astrocyte numbers
Figure 3.3
**Figure 3.3:** Temperature shift scheme for GABA neuron ablations

The gad-GAL4 driver and tub-GAL80ts were used to conditionally express UAS-hid in GABA neurons specifically during metamorphosis.
Figure 3.4

a

GABA neurons | GAT | Merge

Control

Central brain

GABA neuron-Hid

AL

GABA neuron-Hid

b

Number of GABA neurons

Control | GABA neuron-Hid

80 60 40 20 0

***

c

Red content in GAT

AL | SOG

0.0 0.2 0.4 0.6 0.8

***

***

d

Number of GABA neurons

Control | GABA neuron-Hid

AL | SOG

0 10 20 30

***
Figure 3.4: GAT expression is sensitive to GABA neurons

(a) Confocal sections showing anti-GAT immunostaining (green) in the central brain and AL region after ablation of GABA neurons. GABA neurons are labeled by UAS-mCD8::mcherry expression using the gad-GAL4 driver (red). Significant reduction in GAT levels is seen throughout the central brain in correspondence with a reduction in GABA neurons. Localization of GAT appears unaltered by GABA neuron ablations, as highlighted in images from the AL. Animals were 84 h APF upon preparation. Scale bar = 20µm. (b) Quantification of the number of GABA neurons in the ventral medial region of the central brain (n = 5 brains for control and GABA neuron>Hid). (c) Quantification of the mean GAT intensity in the AL and SOG regions (n = 8 brains for control and GABA neuron>Hid for AL and SOG). (d) Quantification of the number of astrocytes in the AL and SOG regions (n = 4 brains, control AL; n = 5 brains, GABA neuron>Hid AL, n = 4 brains, control SOG; n = 5 brains, GABA neuron>Hid SOG). ***P < 0.001, unpaired Student’s t-test for (b) and (d), paired Student’s t-test for (c). Error bars, s.e.m.
revealed that ablation of GABA neurons did not result in astrocyte death (Figure 3.4d), arguing against the notion that decreases in GAT were the result of astrocyte loss. CNS-wide decreases in GAT levels in GABA neuron-ablated animals were confirmed by Western blot analysis performed on 84 h APF brains (Figure 3.5b,c). Finally, we found no significant change in \textit{gat} transcripts after GABA neurons were ablated using Hid (Figure 3.5d), suggesting that regulation of GAT levels by GABAergic neurons may be post-transcriptional.

We next asked whether astrocytic GAT levels were regulated by GABAergic neuronal activity using multiple genetic tools to silence or activate GABAergic neurons. Synaptic vesicle release was blocked in GABA neurons by either expressing temperature induced dominant negative Shibire (Shi\textsuperscript{NS}), or Tetanus Toxin light chain (TNT), which blocks synaptic release by cleaving Synaptobrevin. Inhibition of GABA neuron activity by Shi\textsuperscript{NS}, specifically during late metamorphosis, resulted in a 33% decrease in GAT expression (Figure 3.5a,b,c). Astrocyte morphology as well as the distribution of GABA release sites appeared grossly unaffected (Figure 3.6). Similarly, expression of TNT resulted in a 33% decrease in GAT levels (Figure 3.5b,c). As was the case with GABAergic neuronal ablation, \textit{gat} mRNA levels were unchanged in response to expression of TNT in GABA neurons (Figure 3.5d). We next assayed GAT expression after blocking action potential conduction by expressing the human inwardly rectifying K\textsuperscript{+} channel, Kir2.1. This resulted in a 24% decrease in GAT levels (Figure 3.5a,b,c). We next explored whether increased GABAergic neuronal activity could increase astrocytic GAT levels using the temperature sensitive cationic channel TrpA1 that enables
Figure 3.5

(a) Developmental timeline with temperature changes and life stages.

(b) Expression of GABA neurons (84 hrs APF) with Western blots for GAT and Tubulin.

(c) Graph showing fold change in GAT expression.

(d) Relative mRNA expression in GABA neurons (84 hrs APF).

(e) Expression of GABA neurons (adult specific) with Western blots for GAT and Tubulin.

(f) Fold change in GAT expression with specific treatments.
Figure 3.5: GAT expression is fine tuned in response to GABA release specifically during synaptogenesis

(a) Temperature shift scheme for conditional manipulation of GABA neuron activity. The temperature sensitive constructs, UAS-shi\textsuperscript{ts} and UAS-trpA1, as well as UAS-Kir2.1 with tub-GAL80\textsuperscript{ts}, were activated in a conditional manner using the gad-GAL4 driver. Dominant negative shi\textsuperscript{ts} expression, Kir2.1 expression, or TrpA1 activation was induced at 30°C. (b) Western blots performed on brains 84 h APF following various neuronal manipulations using the gad-GAL4 driver. Blots were probed with anti-GAT antibody. GABA neurons were “inactivated” with expression of UAS-hid, UAS-shi\textsuperscript{ts}, UAS-TNT, and UAS-Kir2.1. Alternatively, GABA neurons were “activated” using UAS-trpA1. (c) Quantification of GAT levels from Western blot analysis shown in (b) (n = 4 experiments, Hid; n = 3 experiments, Shi\textsuperscript{ts}; n = 5 experiments, TNT; n = 3 experiments, Kir2.1; n = 3 experiments, TrpA1). GAT levels are significantly reduced when GABA neurons are “inactivated.” This is in contrast to unaltered GAT levels when GABA neuronal activity is silenced by expression of UAS-hid (n = 3) or UAS-TNT (n = 3) using the gad-GAL4 driver. (d) Relative gat mRNA levels are unaltered when GABA neuronal activity is silenced by expression of UAS-hid (n = 3) or UAS-TNT (n = 3) using the gad-GAL4 driver. (e) Western blots performed on adult brains following adult specific silencing of GABA neuronal activity by expression of UAS-shi\textsuperscript{ts} or UAS-kir2.1 using the gad-GAL4 driver. (f) Quantification of GAT levels from Western blot analysis shown in (e) (n = 3 experiments, Shi\textsuperscript{ts}; n = 3 experiments, Kir2.1). **P≤0.01, ***P≤0.001, paired Student’s t-test for (c) and (f), unpaired Student’s t-test for (d). Error bars, s.e.m.
Figure 3.6

Control

GABA neuron>Syt::GFP    GAT    Merge

GABA neuron>Syt::GFP
Figure 3.6: Distribution of GABA release sites and astrocyte morphology are grossly unaltered following inhibition of GABA neuron activity during synaptogenesis

The *gad-GAL4* driver was used to either co-express *UAS-syt::eGFP* and *UAS-shi* or express *UAS-syt::eGFP* alone. Dominant negative Shi was conditionally expressed during synaptogenesis, and 84 h APF animals were dissected and stained with anti-GAT antibody. Confocal section through AL shows astrocyte morphology (red) and distribution of GABAergic pre-synaptic sites (GABA neuron>Syt::eGFP). Scale bar = 10µm.
temperature-induced activation of neuronal depolarization. Despite activation of TrpA1 in GABAergic neurons, we did not observe significant changes in astrocytic GAT levels (Figure 3.5a,b,c).

Sensitivity of astrocyte GAT levels to GABAergic signaling could be specific to development during the major wave of CNS synaptogenesis. Alternatively, GABAergic neuronal activity might serve as a mechanism to regulate astrocyte GAT levels throughout animal life. To discriminate between these possibilities we inhibited GABA neuron activity either by blocking synaptic vesicle release with Shi\textsuperscript{ts}, or expressing Kir2.1 (using \textit{tub-Gal80}\textsuperscript{ts}), only during adult stages. Under these conditions we did not see significant changes in GAT levels (Figure 3.5e,f). This suggests that GAT expression can be fine-tuned in response to GABA release only during a specific developmental phase of neural circuit assembly (between ~60-84 h APF).

From the above data we conclude that astrocytic GAT levels are up-regulated in the CNS during the major wave of synaptogenesis, and that GAT levels in astrocytes are sensitive to GABAergic neuronal signaling during development of the adult CNS.

**Astrocyte GABA\(_B\) receptors regulate GAT during development**

Astrocytes are known to express a number of neurotransmitter receptors, and can be directly sensitive to neurotransmitter release (Charles et al., 2003; Hamilton and Attwell, 2010; Porter and McCarthy, 1997; Uwechue et al., 2012). Given our observation that synaptic release from GABAergic neurons could regulate GAT levels in astrocytes, we explored the possibility this might be regulated by GABA receptors. Astrocyte-specific
knockdown of each of the *Drosophila* ionotropic GABA receptor subunits did not appear to have an effect on GAT levels. However, knockdown of the metabotropic GABA receptor subunit GABA$_B$R2 in astrocytes resulted in a 31% decrease in GAT expression at 84 h APF (Figure 3.7a,b). GABA$_B$R2 functions through obligate dimerization with the GABA$_B$R1 subunit (Bettler et al., 2004; Kaupmann et al., 1998; Mezler et al., 2001; Padgett and Slesinger, 2010). We therefore assayed the effects of GABA$_B$R1 depletion from astrocytes by RNAi and also found a 28% decrease in GAT levels (Figure 3.7a,b). GABA$_B$R1 directly binds GABA while GABA$_B$R2 signals through G protein alpha o subunit (G$_{\text{io}}$) in both *Drosophila* and mammals (Dahdal et al., 2010; Padgett and Slesinger, 2010). Consistent with GABA$_B$R2 signaling through G$_{\text{io}}$ in astrocytes, we found that expression of *pertussis toxin* (Ptx), a specific enzymatic inhibitor of G$_{\text{io}}$ in *Drosophila*, resulted in a similar 30% decrease in GAT levels at 84 h APF (Figure 3.7a,b). In accordance with our observation that GABAergic neuron activity-dependent regulation of astrocytic GAT was post-transcriptional, we found that inhibition of GABA$_B$R1/2 signaling via expression of GABA$_B$R2 RNAi or Ptx in astrocytes did not affect the amount of *gat* transcripts in the brain (Figure 3.7c). Likewise, the requirement for GABA$_B$R1/2 signaling appears limited to a developmental time window: when either GABA$_B$R1 or GABA$_B$R2 was knocked down in adult stages only there was no significant change in GAT levels (Figure 3.8, 3.7d,e), while developmental knockdown of GABA$_B$R1 or GABA$_B$R2 resulted in reduced GAT levels that persisted in adult animals (Figure 3.7f,g). Thus, GAT expression is fine-tuned through GABA$_B$R1/2 signaling
Figure 3.7
Figure 3.7: GAT expression is modulated through astrocytic metabotropic GABA receptors

(a) Western blots performed on brains 84 h APF following inhibition of GABA\textsubscript{B}R1/2 signaling in astrocytes using the \textit{almr-GAL4} driver. Blots were probed with anti-GAT antibody. GABA\textsubscript{B}R1/2 signaling was impaired by expressing \textit{UAS-GABABR1 RNAi}, \textit{UAS-GABABR2 RNAi}, or \textit{UAS-ptx}. (b) Quantification of GAT levels from Western blot analysis shown in (a) (n = 5 experiments, GABABR1 RNAi; n = 3 experiments, GABABR2 RNAi; n = 3 experiments, ptx). (c) Relative \textit{gat} mRNA levels are unaltered when astrocytic GABA\textsubscript{B}R1/2 signaling is perturbed by expressing \textit{UAS-GABABR2 RNAi} (n = 3 experiments) or \textit{UAS-ptx} (n = 3 experiments) using the \textit{almr-GAL4} driver. (d) Western blots performed on adult brains following adult specific inhibition of GABA\textsubscript{B}R1/2 signaling in astrocytes. Blots were probed with anti-GAT antibody. (e) Quantification of GAT levels from Western blot analysis shown in (d) (n = 3 experiments). (f) Western blots performed on adult brains following inhibition of GABA\textsubscript{B}R1/2 signaling throughout development in astrocytes using the \textit{almr-GAL4} driver. Blots were probed with anti-GAT antibody. GABA\textsubscript{B}R1/2 signaling was impaired by expressing \textit{UAS-GABABR1 RNAi} or \textit{UAS-GABABR2 RNAi} though out development. (g) Quantification of GAT levels from Western blot analysis shown in (f) (n = 3 experiments, GABABR1 RNAi; n = 4 experiments, GABABR2 RNAi). *P ≤ 0.05, **P ≤ 0.01, paired Student’s t-test for (b), and (g), unpaired Student’s t-test for (c), and 1-way ANOVA with Tukey’s post hoc test for (e). Error bars, s.e.m.
Figure 3.8
Figure 3.8: Temperature shift scheme for adult specific inhibition of GABA$_B$R1/2 signaling in astrocytes

*Tub-GAL80$^{ts}$* with *UAS-GABA$_B$R1 RNAi* or *UAS-GABA$_B$R2 RNAi* were expressed under the control of the *alarm-GAL4* driver. RNAi expression was induced in 1 day old adult animals for 7-10 days.
during synaptogenesis, and this developmental resetting of GAT levels ultimately determines GAT levels in the mature CNS.

**Decreasing astrocyte GAT suppresses seizure induction**

A primary function for astrocytic GAT proteins is balancing excitatory and inhibitory neuronal signaling in the CNS. To explore whether decreasing astrocyte GAT levels by inhibition of the GABA$_B$R1/2 signaling pathway is sufficient to modify the balance of excitation and inhibition in the *Drosophila* CNS, we turned to behavioral studies in bang sensitive mutants, a common model used to study nervous system hyperactivity and seizure (Parker et al., 2011). Bang sensitive mutants undergo seizure activity followed by paralysis when stimulated by mechanical shock due to neuronal hyperexcitability (Pavlidis and Tanouye, 1995). Intriguingly, bang sensitive mutants like *easily shocked* (*eas*) (Pavlidis et al., 1994) are rescued from seizure activity by application of antiepileptic drugs such as gabapentin (Reynolds et al., 2004) or valproate (Kuebler and Tanouye, 2002), both of which have been implicated in increasing extracellular GABA levels (Sills and Brodie, 2001).

We reasoned that if impairment of astrocytic GABA$_B$R1/2 signaling could reduce functional GAT levels, GABA would not be cleared as efficiently from the CNS, and this would in turn suppress bang sensitivity in the eas$^{PC80}$ mutant background phenotype. We therefore crossed RNAi constructs targeting $GABA_B$R2 and the *UAS-pto* construct into the eas$^{PC80}$ mutant background, subjected animals to mechanical shock for 10 seconds to induce seizures followed by paralysis, and then assayed recovery time for 200 seconds.
Consistent with previous reports, we found that while control animals recovered within 10 seconds after treatment, $eas^{PC80}$ mutants underwent robust seizure activity and paralysis with a mean recovery time of ~77 seconds (Figure 3.9a,b). Similar recovery times were observed for all driver- or UAS-alone controls in an $eas^{PC80}$ mutant background. In striking contrast, we found that $eas^{PC80}$ animals expressing astrocytic $GABA_{B}R2\ RNAi$ or Ptx required significantly less time (i.e. ~43 second mean recovery time) to recover from paralysis (Figure 3.9a,b). Consistent with our previous results in control animals, we found that GAT levels were reduced when $GABA_{B}R1/2$ signaling was inhibited in adult $eas^{PC80}$ mutant animals (Figure 3.9c,d). These data provide direct functional support for the notion that $GABA_{B}R1/2$ signaling regulates functional GAT on astrocyte membranes in response to GABAergic neuronal activity, which is in turn important for modulation of excitation and inhibition balance in the nervous system.

**Discussion**

**Astrocyte GAT is regulated by GABAergic neuronal activity**

It is unclear how astrocytes acquire their final morphological and molecular phenotypes, and how much of this is governed by their environment. We provide mechanistic evidence for direct regulation of the astrocytic GABA transporter, GAT, in response to GABAergic neuronal activity. Western blot analysis of GAT expression during adult synaptogenesis revealed that GAT is strongly up-regulated during the major wave of synapse formation and coordinated with astrocyte infiltration of the neuropil. Strong expression of GAT is observed at 84 h APF, a developmental time point at which ~90%
Figure 3.9
Figure 3.9: Regulation of GAT through astrocytic metabotropic GABA receptors can modulate neurotransmission

(a) Flies were vortexed for 10 seconds to provide mechanical stimulation (“bang”) and induce paralysis in bang sensitive mutants. The percent of flies recovering from paralysis is shown as a function of time. Flies without the eas$_{PC80}$ mutation do not display a bang sensitive phenotype, and therefore 100% of these flies display normal behavior within the first 10 s. eas$_{PC80}$ flies and eas$_{PC80}$ flies carrying only the alrm-GAL4 driver or UAS constructs display similar recovery response profiles to each other. GABA$_B$R2 signaling was inhibited in eas$_{PC80}$ flies by expressing UAS-ptx or UAS-GABA$_B$R2 RNAi using the alrm-GAL4 driver. Recovery profiles are shifted toward shorter recovery times when GAT levels are reduced via inhibition of astrocytic GABA$_B$R1/2 signaling in eas$_{PC80}$ flies (n > 100 flies for each genotype). (b) Mean recovery time calculated from data shown in (a). The recovery times of each individual animal were pooled and averaged. (c) Western blot performed on adult brain lysates was probed with anti-GAT antibody. In comparison to eas$_{PC80}$ flies or eas$_{PC80}$ flies carrying the alrm-GAL4 driver, GAT expression was reduced in eas$_{PC80}$ flies expressing UAS-ptx or UAS-GABA$_B$R2 RNAi using the alrm-GAL4 driver. (d) Quantification of GAT levels from western blot analysis shown in (c) (n = 3 experiments). *P≤0.05, **P≤0.001, 1-way ANOVA with Tukey’s post hoc test for (b) and repeated measures ANOVA with Tukey’s post hoc test for (c). Error bars, s.e.m.
of synaptic structures have formed, and astrocytes have densely infiltrated neuropil regions and taken on their mature tufted morphology.

Multiple lines of evidence argue that astrocytic GAT activation is regulated directly by GABA release by GABAergic neurons. In animals at 84 h APF, ablation of GABA neurons reduced GAT levels throughout the brain. Likewise, blockade of synaptic vesicle release (using Shi\textsuperscript{ts} and TNT) or action potential firing (using Kir2.1) in GABA neurons also resulted in reduced astrocyte GAT levels, indicating that GAT is modulated by GABA release. Interestingly, astrocytes are likely capable of directly measuring extracellular GABA levels through metabotropic GABA receptors (GABA\textsubscript{B}R1/2) and adjusting GAT levels accordingly. Depletion of GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2 by RNAi or inhibition of G\textsubscript{o}\textsubscript{o} signaling by Ptx expression specifically in astrocytes resulted in reduced levels of GAT at late pupal stages. Somewhat surprisingly, we see this mechanism of GAT regulation to be present only during development and coincident with synaptogenesis. Adult specific manipulations of GABA neuron activity or GABA\textsubscript{B}R1/2 signaling did not noticeably alter GAT expression. Nevertheless, fine tuning of GAT during development is important for establishing adult levels since knockdown of GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2 during development reduced GAT expression even in adult stages. Direct measurement of GABA by astrocytes through GABA\textsubscript{B}R1/2 signaling would provide a simple mechanism for how astrocytes adjust their levels of GAT expression (and therefore ability to clear extracellular GABA) in response to alterations in GABA release. Adult expression patterns of mammalian astrocytic GAT-3 are established during postnatal stages that also coincide with periods of neuronal circuit
refinement and astrocyte maturation (Vitellaro-Zuccarello et al., 2003). Furthermore, mammalian astrocytes have been found to express GABA<sub>B</sub> receptors (Charles et al., 2003; Oka et al., 2006). We therefore speculate a similar astrocyte-synapse signaling event may therefore also modulate astrocytic GAT levels in mammals, but this awaits exploration.

Each of our manipulations resulting in block of GABAergic neural activity or GABA<sub>B</sub>R1/2 signaling resulted in no more than ~50% reduction in astrocytic GAT levels. On one hand, this could be explained by the nature of the manipulations - perhaps they did not result in a complete loss of GABA neurons, neural activity or GABA<sub>B</sub>R1/2 signaling. Also, because our antibody against GAT does not distinguish between GAT that is inserted in the membrane and GAT that is retained in intracellular pools, the level of functional GAT at the membrane may in fact be less than what is observed in immunostains. Finally, it is important to note that multiple mechanisms likely exist for modulating astrocytic GAT. It is possible that the initiation of GAT expression is hardwired at some level during development to establish a baseline for handling GABA, which might explain the consistent ~50% reduction in expression level we observed in all our manipulations of GABAergic neurons or astrocytic GABA<sub>B</sub> receptor signaling. Subsequent modulation and fine-tuning of GAT levels may then be regulated by activity-dependent plasticity imparted by GABA<sub>B</sub>R1/2 signaling or other physiological mechanisms. Indeed, recent studies by Shigetomi et al. revealed an exciting role for astrocyte TrpA1-dependent calcium events in regulating GAT-3 levels, demonstrating another mechanism by which astrocytic GAT levels are fine-tuned. Given the
significance of GABA signaling in neural circuit function, it is not surprising that multiple pathways exist to modulate GAT levels.

Modulation of GAT levels through GABA\textsubscript{B}R1/2 signaling does not appear to occur at the transcriptional level. Our real-time quantitative PCR data revealed that gat mRNA levels are unchanged in dissected brains when GABAergic activity is blocked or when GABA\textsubscript{B}R1/2 signaling is inhibited. These data argue post-transcriptional regulation is likely important for GABAergic activity-dependent changes in GAT. For example, translation of GAT mRNAs may be regulated locally, or GAT protein degradation may be regulated in response to GABA\textsubscript{B}R1/2 signaling. Shigetomi et al. also revealed that mammalian astrocytic GAT-3 expression can be regulated by mechanisms involving dynamin-dependent endocytosis (Shigetomi et al., 2012). Thus, post-transcriptional regulation of astrocytic GATs may be an important manner by which astrocytes modulate GABA tone.

**Regulation of GAT in CNS excitatory/inhibitory balance**

Tight regulation of GABA transporter levels and activity is critical in establishing balance between excitatory and inhibitory signaling. We provide strong behavioral evidence in the bang sensitive *Drosophila* mutant eas\textsuperscript{PC80} - a model for seizure activity (Pavlidis et al., 1994) - that GABA\textsubscript{B}R1/2 signaling can modulate extracellular GABA levels by regulating GAT expression and suppress hyperexcitability. Neurons in the eas\textsuperscript{PC80} mutant have an altered phospholipid profile (Pavlidis et al., 1994). Stimulation of eas\textsuperscript{PC80} mutant neurons by sensory input (mechanosensory) or high
frequency stimulation correlates with a seizure phase, which is then followed by conduction failure and paralysis that is resolved by ~2 minutes, whereas control animals are unaffected by these stimulations (Pavlidis and Tanouye, 1995). We found that inhibition of GABA\textsubscript{B}R1/2 signaling by Ptx or \textit{GABA\textsubscript{B}R2 RNAi} expression in astrocytes significantly ameliorated the effects of seizure activity. These data provide \textit{in vivo} evidence supporting a role for astrocytic GABA\textsubscript{B}R1/2 signaling in modulating neural circuit function. The simplest mechanistic interpretation of our results is that inhibition of GABA\textsubscript{B}R1/2 signaling results in decreased GAT, and increased levels of extracellular GABA, which in turn suppresses the effects of neuronal hyperexcitability.

Deciphering the molecular pathways mediating GAT regulation \textit{in vivo} will be critical to understand how the balance between excitatory and inhibitory signals is maintained. Our demonstration that GABA\textsubscript{B}R2 and \textit{G\textsubscript{\alpha}o} signaling can directly modulate GAT levels \textit{in vivo} provides an exciting first insight into how astrocytes adjust GAT levels in response to GABA release.
CHAPTER IV

Screening for novel astrocyte modulators of neural activity
Introduction

My thesis work presented in Chapter 3 demonstrated that astrocyte GABA transporters (GATs) are important modulators of neuronal hyperexcitability. Utilizing a Drosophila genetic model of seizure activity ($eas^{PC80}$), we showed neuronal hyperexcitability could be reduced by lowering GAT expression levels. This sparked our curiosity about other astrocyte proteins that may also mediate balance between excitatory and inhibitory signals. Utilizing $eas^{PC80}$ flies, we performed a reverse genetic screen to identify additional astrocyte genes important for modulating hyperexcitability. We identified 141 astrocytic genes that reduced hyperexcitability when silenced and 20 astrocytic genes that increased hyperexcitability when silenced. Amongst our list of candidates, is the gene tensin, which encodes a focal adhesion protein that mediates a variety of cellular processes, including cell adhesion, cytoskeletal rearrangements and gene transcription (Millard et al., 2011). Our preliminary studies suggest astrocyte Tensin interacts with other focal adhesion components to mediate excitatory-inhibitory neuronal balance.

Results

Reverse genetic screen

A role for astrocyte GAT in modulating neuronal hyperexcitability made us wonder what other astrocyte genes have a role in mediating excitatory-inhibitory neuronal balance. We performed a reverse genetic screen to identify astrocyte genes important for modulating the hyperexcitability displayed by $eas^{PC80}$ animals. Upon mechanical stimulation, excitatory neurons in $eas^{PC80}$ flies are overactivated and induce
network hyperexcitability that results in seizures followed by paralysis (Pavlidis et al., 1994). The amount of time required by $eas^{PC80}$ flies to recover from paralysis can be used as a readout for the degree of hyperexcititation experienced by the animal. Utilizing this paradigm, we screened for astrocyte genes that enable $eas^{PC80}$ flies to recover from paralysis more quickly (like astrocyte GAT) or more slowly. In the $eas^{PC80}$ genetic background, the alrmGAL4 driver was used to systematically express a collection of UAS-regulated RNAi constructs only in astrocytes, thus, generating a collection of “candidate lines” in which $eas^{PC80}$ animals have target genes silenced only in astrocytes. In this manner, we screened 2281 genes. Our targets included the vast majority of Drosophila transmembrane proteins as well as a comprehensive collection of major signaling molecules found in the fly. For each candidate line, we subjected ~10 animals to mechanical stimulation to induce seizures and paralysis. The percentage of animals recovered from paralysis was then monitored at 1, 1.5, 2, 3, and 4 minutes after stimulation. Because $eas^{PC80}$ animals recover from paralysis by 3 minutes, we considered the candidate lines in which 100% of the animals recovered by 1.5 minutes as “suppressors” of hyperexcitability and the candidate lines in which 100% of the animals failed to recover by 4 minutes as “enhancers” of hyperexcitability. For candidates that were recovered as suppressors or enhancers, we performed two additional rounds of screening, raising the number of animals tested to ~30 for that particular candidate line, to confirm the phenotype.

At 1.5 minutes, 100% recovery was observed in 141, or 6.17%, of candidate lines. At 4 minutes, 100% recovery failed to occur in 350, or 15.5%, of candidate lines. To
focus only on the strongest enhancer phenotypes, we converged on the lines that showed less than 75% recovery at 4 minutes, which resulted in 20 candidate lines. Thus, from our primary screen, we obtained 141 astrocyte genes that suppressed hyperexcitability when silenced by RNAi and 20 astrocyte genes that enhanced hyperexcitability when silenced by RNAi (Figure 4.1a,b).

**Astrocyte tensin associates with focal adhesions to modulate neuronal hyperexcitability**

From our collection of suppressors, we were particularly interested in the protein Tensin. Tensin associates with focal adhesions, which are specialized protein complexes that link the plasma membrane to actin cytoskeleton (Haynie, 2014; Torgler et al., 2004; Wozniak et al., 2004). Because focal adhesions can act as a bridge between extracellular and intracellular environments, we postulated that Tensin was ideally positioned to mediate astrocyte interactions with its environment.

Because only a small number of animals (~30) were tested while screening, and because RNAi expression can sometimes affect off-target genes resulting in false positive results, it was important to further validate our finding that astrocyte tensin plays a role in modulating neuronal hyperexcitability. Still utilizing the $eas^{PC80}$ seizure model, a comprehensive bang-sensitivity behavior analysis was performed as previously described (Chapter 3)(Figure 4.2a,b). Unlike the screen, in which a small number of animals were monitored at distant intervals, we examined a greater number of animals at 10 second intervals. The mean recovery time of $eas^{PC80}$, as well as $eas^{PC80}$ with $alrmGAL4$ flies
## Figure 4.1

### a

<table>
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<th>Time (min)</th>
<th>Number of candidate lines with 100% recovery</th>
<th>Percentage of candidate lines with 100% recovery</th>
<th>Number of candidate lines with ≤75% recovery</th>
<th>Percentage of candidate lines with ≤75% recovery</th>
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<td>20</td>
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</tr>
</tbody>
</table>

### b

- **transporter or channel (37)**
- **membrane protein receptors (21)**
- **unknown (13)**
- **membrane dynamics (12)**
- **regulation of phosphorylation (10)**
- **cytoskeletal rearrangements (8)**
- **cell adhesion (7)**
- **cell metabolism (7)**
- **regulation of transcription (7)**
- **glycan biology (5)**
- **ion binding (5)**
- **protein binding (5)**
- **lateral inhibition (2)**
- **DNA replication (1)**
- **DNA repair (1)**

- **transporter or channel (6)**
- **protein binding (3)**
- **cell metabolism (3)**
- **lateral inhibition (3)**
- **ion binding (1)**
- **membrane protein receptors (1)**
- **regulation of phosphorylation (1)**
- **regulation of transcription (1)**
- **cell adhesion (1)**
- **unknown (1)**

**total = 141**

**total = 20**
Figure 4.1: Summary of screen results

(a) Summary of number of candidate lines displaying 100% recovery and ≤75% recovery. Suppressors belong to category outlined in red and enhancers belong to category outlined in blue. (b) Biological processes associated with suppressors and enhancers.
Figure 4.2
Figure 4.2: Astrocyte Tensin modulates hyperexcitability in eas\textsuperscript{PC80} flies

(a) Flies were vortexed for 10 seconds to provide mechanical stimulation ("bang") and induce paralysis in bang sensitive mutants. The percent of flies standing is shown as a function of time. Flies without the eas\textsuperscript{PC80} mutation do not display a bang sensitive phenotype, and therefore 100% of these flies display normal behavior within the first 10 s. eas\textsuperscript{PC80} flies and eas\textsuperscript{PC80} flies carrying only the alrm-GAL4 driver display similar recovery response profiles to each other. Recovery profiles shifted toward shorter recovery times when tensin was knocked down in astrocytes using two different RNAi target sequences (VDRC and TRiP). (b) Mean recovery time calculated from data shown in (a). ***$P \leq 0.001$, 1-way ANOVA with Tukey’s post hoc test for (b). Error bars, s.e.m.
were 75.3 ± 2.9 sec (n = 206 flies) and 64.4 ± 2.3 sec (n = 336 flies) respectively. In contrast, the mean recovery time of eas\textsuperscript{PC80} animals expressing UAS-tensin RNAi in astrocytes was significantly reduced. Two independent RNAi lines containing distinct target sequences were tested; one resulted in a mean recovery time of 35.5 ± 2.7 sec (n = 71 flies; VDRC) and the other resulted in a mean recovery time of 37.7 ± 3.9 sec (n = 35 flies; TRiP). Knockdown of astrocytic tensin without the eas\textsuperscript{PC80} mutant background behaved as wildtype animals (Figure 4.2a,b). We note the mean recovery times of eas\textsuperscript{PC80} and eas\textsuperscript{PC80} with alrmGAL4 were comparable but statistically different, suggesting the alrmGAL4 driver may exert some minor affects on behavior. Nevertheless, the mean recovery time of animals expressing UAS-tensin RNAi in astrocytes was dramatically lower and significantly different than the mean recovery time of eas\textsuperscript{PC80} with alrmGAL4 flies.

To test if astrocyte Tensin is important for modulating neural activity in a context that is independent of eas\textsuperscript{PC80}, we used another approach to induce hyperexcitability. This time we turned to the larval nervous system and used pharmacological tools to alter network firing. Picrotoxin (PTX), an ionotropic GABA receptor antagonist, is a drug widely used to induce network hyperexcitability. Picrotoxin can be bath applied to tissue or orally administered to animals to effectively block inhibitory neuronal signals and induce network hyperexcitability (Bateup et al., 2013; Stilwell et al., 2006). Drosophila larvae that are fed PTX display uncoordinated motor movements and eventually undergo sustained muscle contractions that impair their locomotion; a phenotype seen in other Drosophila larva models of hyperexcitability as well (Melom and Littleton, 2013). Thus,
using locomotion speed after PTX exposure as a readout for hyperexcitability, we tested if loss of astrocyte Tensin could rescue this phenotype.

The speed of third-instar larvae was assayed following PTX feeding for 9 hours. In accordance with previous reports, PTX feeding impaired larval locomotion velocity in a dose-dependent manner (Stilwell et al., 2006). While larvae feeding on food without PTX moved at 64.9 ± 3.4 mm/min (n = 18 larvae), larvae feeding on food containing 0.5mg/ml and 1.5mg/ml PTX moved at 22.8 ± 2.8 mm/min (n = 16 larvae) and 8.5 ± 1.8 mm/min (n = 20 larvae) respectively. Of the larvae fed PTX, larvae deficient in astrocyte Tensin moved significantly faster than control animals (Figure 4.3a). Animals expressing UAS-tensin RNAi in astrocytes moved ~2 times faster than control animals after 0.5mg/ml PTX feeding [46.5 ± 2.1 mm/min (n = 16 larvae)] and ~5 times faster than control animals after 1.5mg/ml PTX feeding [41.6 ± 3.5 mm/min (n = 15 larvae)]. Locomotion speed was unaltered in animals expressing UAS-tensin RNAi without PTX treatment [66.6 ± 1.4 mm/min (n = 20 larvae)]. Also, astrocyte membranes and neuropil structure looked grossly normal following astrocyte tensin knockdown (Figure 4.3b). Levels of astrocyte GAT, a modulator of hyperexcitability (Chapter 3), were also unaltered in astrocyte Tensin knockdown animals (Figure 4.3c). To further test the role of Tensin in rescuing the effects of PTX, we tested the response of tensin null (by33c) animals to PTX treatment (Torgler et al., 2004). Here also we saw significant rescue of hyperexcitability (Figure 4.3d). Tensin null animals moved ~1.5 times faster than control animals after 0.5mg/ml PTX feeding [w+: 18.7 ± 1.0 mm/min (n = 21 larvae); by33c: 29.9 ± 1.3 mm/min (n = 18 larvae)] and ~2.5 times faster than control animals after 1.5mg/ml
Figure 4.3

(a) Graph showing speed (mm/min) vs. picrotoxin concentration (mg/ml) for different conditions:
- almgAL4 control
- almgAL4 > UAS-tensin RNAi
- almgAL4 > UAS-βtubegin RNAi
- almgAL4 > UAS-talin RNAi
- almgAL4 > UAS-FAK RNAi

(b) Images illustrating different conditions:
- Merge
- almgAL4 > GFP
- NC82

(c) Bar graph showing fold change in GAT with and without tensin RNAi

(d) Bar graph showing speed (mm/min) vs. picrotoxin concentration (mg/ml) for:
- w' control
- by33c

(e) Bar graph showing fold change in GAT for:
- w' control
- by33c
Figure 4.3: Tensin associates with focal adhesions to modulate Picrotoxin-induced hyperexcitability

(a) Locomotion speeds of third instar larvae were assessed after feeding the larvae Picrotoxin (PTX) for 9 hours. While locomotion speeds of control animals decreased with PTX feeding, locomotion speeds decreased to a significantly lesser extent following knockdown of focal adhesion transcripts. (b) Confocal section through larval ventral nerve cord of third instar larva. Astrocyte membranes are labeled by UAS-mCD8::GFP expression using the alrm-GAL4 driver (green), neuropil is labeled by nc82 antibody staining (red) and glial nuclei are labeled by anti-Repo antibody (blue). Scale bar = 10µm. (c) Quantification of western blots performed on larval CNS lysates that were probed with anti-GAT antibody (n = 3 experiments). GAT levels were unaltered when tensin was knocked down in astrocytes. (d) Locomotion speeds of tensin null (by33c) third instar larvae were assessed after feeding the larvae PTX for 9 hours. Tensin null larvae moved significantly faster after PTX feeding than control larva (w'). (c) Quantification of western blots performed on larval CNS lysates that were probed with anti-GAT antibody (n = 3 experiments). GAT levels were unaltered in animals heterozygous for by33c as well as in animals homozygous for by33c. *P≤0.05, **P≤0.01, ***P≤0.001, 2-way ANOVA with Tukey’s post hoc test for (a) and (b). Error bars, s.e.m.
PTX feeding \([w^-: 7.9 \pm 1.0 \text{ mm/min} (n = 32 \text{ larvae}); by33c: 19.7 \pm 1.7 \text{ mm/min} (n = 18 \text{ larvae})]\). It should be noted tensin null animals did display slight locomotion defects to begin with \([w^-: 58.2 \pm 3.7 \text{ mm/min} (n = 20 \text{ larvae}); by33c: 49.3 \pm 2.3 \text{ mm/min} (n = 21 \text{ larvae})]\). This is not surprising as Tensin is widely expressed in the nervous system and regulates adhesion between cells(Torgler et al., 2004). Similarly to what was observed when Tensin was knocked down in astrocytes, GAT levels were unaltered in tensin null animals (Figure 4.3d).

The role of Tensin at focal adhesions is well established(Haynie, 2014; Torgler et al., 2004; Wozniak et al., 2004). Therefore, we were curious if other focal adhesion components that interact with Tensin could also modulate the effects of PTX. First we tested the role of \(\beta\)-integrin, a core focal adhesion protein and a binding partner of tensin(Legate et al., 2009; Torgler et al., 2004). Following \(\beta\)-integrin knockdown in astrocytes, animals moved \(~1.8\) times faster than control animals after 0.5mg/ml PTX feeding \([40.1 \pm 3.0 \text{ mm/min} (n = 20 \text{ larvae})]\) and \(~3.6\) times faster than control animals after 1.5mg/ml PTX feeding \([30.3 \pm 2.2 \text{ mm/min} (n = 23 \text{ larvae})]\). Talin, another important regulator of focal adhesions, is recruited during the early stages of focal adhesion assembly and is required for initiating contact between focal adhesions and the cytoskeleton(Brown et al., 2002). After knockdown of talin in astrocytes, animals moved \(~1.9\) times faster than control animals after 0.5mg/ml PTX feeding \([42.4 \pm 2.1 \text{ mm/min} (n = 20 \text{ larvae})]\) and \(~4.5\) times faster than control animals after 1.5mg/ml PTX feeding \([37.9 \pm 2.2 \text{ mm/min} (n = 20 \text{ larvae})]\). In a similar manner, Focal adhesion kinase (FAK), a signaling molecule widely associated with focal adhesion regulation, also showed
rescue (Millard et al., 2011). After astrocytic knockdown of FAK, animals moved ~1.8 times faster than control animals after 0.5mg/ml PTX feeding [41.3 ± 3.3 mm/min (n = 13 larvae)] and ~4.7 times faster than control animals after 1.5mg/ml PTX feeding [40.0 ± 2.7 mm/min (n = 18 larvae)]. Together, these data suggest an important role for astrocyte focal adhesions in modulating network activity.

**Disrupting Tensin in only a few astrocytes is sufficient to modulate hyperexcitability**

Intriguingly, disrupting Tensin in only a few astrocytes is sufficient to reduce PTX-induced hyperexcitability. UAS-tensin RNAi was sparsely expressed in only a few astrocytes using an alrm-FRT-QF-FRT-GAL4 construct (Stork et al., 2014). Without flippase (FLP) activity, the alrm promoter drives QF expression, but with flippase activity the QF coding sequence is excised and the alrm promoter drives GAL4 expression and thus activates UAS-target genes. In combination with repo-FLP, a flippase under the control of a pan-glial promoter, we sporadically expressed UAS-tensin RNAi in only a few astrocytes. Expression of UAS-mcd8::GFP served as a control (Figure 4.4a). Similar to before, the velocity of third-instar larvae was assayed following PTX feeding for 9 hours (Figure 4.4b). Animals expressing UAS-tensin RNAi in a few astrocytes moved ~4 times faster than control animals after 1.5mg/ml PTX feeding [GFP: 8.4 ± 1.5 mm/min (n = 17 larvae); tensin RNAi: 33.2 ± 1.3 mm/min (n = 48 larvae)]. Similar effects were observed when β-integrin, Talin, or FAK were sparsely knocked down in larval astrocytes. Relative to controls, animals moved ~3 times faster after sparse β-integrin knockdown [25.0 ± 1.7 mm/min (n = 17 larvae)]; ~3.2 times faster after
Figure 4.4: Disruption of focal adhesions in only a few astrocytes is sufficient to modulate hyperexcitability

(a) Projected confocal z-stacks of third instar larva CNS showing sparse astrocyte labeling using the *alrm-FRT-QF-FRT-GAL4* construct. Neuropil is labeled by anti-HRP antibody (red), glial nuclei are labeled by anti-Repo antibody (blue), astrocytes are sporadically labeled by *UAS-mCD8::GFP* expression (green), Scale bar = 20µm. (b) Locomotion speeds of third instar larvae expressing various UAS transgenes in only a few astrocytes were assessed after feeding the larvae Picrotoxin (PTX) for 9 hours. (c) The numbers of astrocytes expressing UAS transgenes in the larval ventral nerve cord (VNC) were comparable across genotypes. For 0.0 mg/ml PTX: *UAS-GFP* n = 18; *UAS-tensin RNAi* n = 7; *UAS-β-integrin RNAi* n = 4; *UAS-talin RNAi* n = 10; *UAS-FAK RNAi* n = 11. For 1.5 mg/ml PTX: *UAS-GFP* n = 13; *UAS-tensin RNAi* n = 60; *UAS-β-integrin RNAi* n = 7; *UAS-talin RNAi* n = 9; *UAS-FAK RNAi* n = 9. ***P ≤ 0.001, 2-way ANOVA with Tukey’s post hoc test for (b) and (c). Error bars, s.e.m.
sparse Talin knockdown [26.8 ± 1.9 mm/min (n = 16 larvae)]; and ~2.4 times faster after sparse FAK knockdown 20.1 ± 1.6 mm/min (n = 16 larvae). Similar numbers of astrocytes expressed UAS transgenes in the various genotypes (Figure 4.4c).

Because sparse knockdown of Tensin did not rescue hyperexcitability to the same extent that knockdown in all astrocytes did, we were curious if either the number or particular position of affected astrocytes correlated with the degree of rescue. To address this we kept track of individual animals during our experiments so that positions and numbers of astrocytes expressing UAS-tensin RNAi could be correlated to locomotion speed for each animal. To our surprise, clone number did not correlate with locomotion velocity (Figure 4.5a). Next, we examined whether disrupting Tensin in ventrally positioned astrocytes versus dorsally positioned astrocytes could bias locomotion velocity. Sensory and motor circuits are spatially segregated in the larval ventral nerve cord(Kohsaka et al., 2012). Sensory neuron axons, or sensory neuron output, project to ventral regions of the ventral nerve cord while motor neuron dendrites, or motor neuron input, project to dorsal regions of the ventral nerve cord. Thus, we hypothesized that Tensin may have differential influence over sensory and motor circuits. However, here also, we did not observe a correlation between speed and dorsal-ventral positioning of Tensin deficient astrocytes (Figure 4.5b,c,d,e). Lastly, we checked if the presence of Tensin deficient astrocytes in the central brain directed locomotion speed and here also, we found no correlation to exist (Figure 4.5f).

Discussion
Figure 4.5

(a) Number of astrocytes expressing UAS tensin RNAi
(b) Number of dorsal astrocytes expressing UAS tensin RNAi
(c) Number of ventral astrocytes expressing UAS tensin RNAi
(d) Percentage of dorsal astrocytes expressing UAS tensin RNAi
(e) Percentage of ventral astrocytes expressing UAS tensin RNAi
(f) Astrocytes expressing UAS tensin RNAi

Present in central brain
Absent in central brain

Speed mm/min
Figure 4.5: locomotion speed following PTX-induced hyperexcitability does not correlate with numbers of positions of astrocytes

Following 9 hours of PTX feeding, locomotion speed did not correlate with (a) the number of astrocytes expressing *UAS-tensin RNAi* in the VNC (b) the number of dorsally positioned astrocytes expressing *UAS-tensin RNAi* in the VNC (c) the number of ventrally positioned astrocytes expressing *UAS-tensin RNAi* in the VNC (d) the percentage of the dorsally positioned astrocytes expressing *UAS-tensin RNAi* in the VNC (e) the percentage of the ventrally positioned astrocytes expressing *UAS-tensin RNAi* in the VNC (f) the presence of *UAS-tensin RNAi* expressing astrocytes in the central brain. Correlation was tested using Pearson’s correlation (R squared ≤ 0.05 for all)
In this study we performed a reverse genetic screen to identify astrocyte genes important for modulating neuronal hyperexcitability. Our primary screen identified 141 suppressors and 20 enhancers. We confirmed that the focal adhesion protein Tensin, a candidate from our collection of suppressors, modulates network hyperexcitability in two independent contexts. At adult stages, loss of astrocyte tensin reduced hyperexcitability in \( eas^{PC80} \) flies, a genetic model for seizures. Likewise, at larval stages, drug (PTX) – induced hyperexcitability was reduced in animals deficient in astrocyte tensin. In addition to Tensin, other focal adhesion proteins (\( \beta \)-integrin, Talin, FAK) also displayed similar phenotypes, suggesting regulation of astrocyte focal adhesions is important for modulating neuronal hyperexcitability. Intriguingly, disrupting focal adhesions in only a few astrocytes was sufficient to induce global changes in behavior.

How are Tensin and focal adhesions regulating hyperexcitability? Focal adhesions are dynamic protein complexes that form bridges between the ECM and a cell’s intracellular environment; they span the plasma membrane and link to the cytoskeleton. Through the regulated recruitment of different proteins, focal adhesions initiate numerous different downstream signaling cascades(Davis et al., 1991; Millard et al., 2011). For example, through pathways that mediate cytoskeletal rearrangements, focal adhesions can regulate migration and adhesion(Chen et al., 2000; Wozniak et al., 2004). Through pathways that regulate gene expression, focal adhesions mediate cell proliferation and survival(Fonar et al., 2011; Shibue and Weinberg, 2009). Because focal adhesions act as hubs for many intracellular signaling events, there are several plausible ways focal adhesions could play a role in modulating neural activity. For example, by
mediating cell adhesion, astrocyte membrane associations with the synapse could be altered. This is a sound hypothesis, considering Tensin has been implicated in mediating cell adhesion. However, this does not mean Tensin necessarily acts the same way in astrocytes in this particular context. Because focal adhesions can regulate gene expression, it is also possible astrocyte focal adhesions are critical for regulating expression of channels and transporters important for maintaining K⁺ homeostasis. At this point, one can only speculate how focal adhesions modulate hyperexcitability. Much work is required in the future to determine the relevant function of astrocyte tensin at focal adhesions.

An intriguing result was that knockdown of Tensin in only a few astrocytes was sufficient to mediate PTX-induced hyperexcitability. This may result from modulation of local networks close to the impaired astrocyte, which induces homeostatic changes in global network firing. Alternatively, astrocyte Tensin may be required for long-range regulation of neurons. For example, astrocyte Tensin might mediate the release of substrates that act at a distance. Interestingly, we did not observe a correlation between the number of astrocytes lacking Tensin in the CNS and the degree of modulation. In some cases, animals that had only one astrocyte lacking Tensin counteracted hyperexcitability better than animals that lacked Tensin in several astrocytes. In a similar manner, the degree of modulation did not correlate with whether Tensin was knocked down in astrocytes associating with sensory circuits or in astrocytes associating with motor circuits. However, it is possible such correlations would be missed due to the manner in which the experiments were performed. Because Tensin function was
impaired using RNAi and clones were sporadically induced using the repo-FLP construct, tensin transcripts were knocked down for different times and at potentially different strengths in each astrocyte. Therefore, Tensin was not necessarily more impaired in animals with more astrocytes expressing UAS-tensin-RNAi. It should also be noted that the significance of counting cell numbers is sometimes dwindled given the inconsistent shape and volume of astrocytes. A given single astrocyte may occupy the equivalent volume of multiple small astrocytes. Similarly, given the ramified processes of astrocytes, it is difficult to assess where an astrocyte imparts its regulation. How does one characterize the position of an astrocyte that has a dorsally positioned cell body with branches that infiltrate into ventral regions of the ventral nerve cord? In our analysis, we used cell body position as our criteria, but perhaps the entire space of an astrocyte should be taken into account in future studies. Nevertheless, the fact that just a few astrocytes are sufficient to dramatically alter circuit function reveals an intriguing aspect of astrocyte biology.
CHAPTER V

Conclusion and future directions
In the past 20 years astrocytes have emerged as pertinent regulators of synapse biology. Astrocytes are mediators of both synapse formation and synapse function. During the formation of the nervous system, astrocytes mediate the formation of new synapses and, later, the pruning or retention of synapses in response to activity dependent cues. In the fully-formed nervous system, they contribute to signaling and excitatory-inhibitory balance at the synapse through a variety of mechanisms including ion buffering, neurotransmitter uptake and mediation of “glio”transmitter effects. Through these vital roles, astrocytes critically shape the structure and output of neural circuits.

The vast majority of our understanding of astrocyte biology has come from studies conducted in mammals, where it is challenging and time consuming to dissect molecular mechanisms with cell type specificity. While *Drosophila* is a less established model system for studying astrocyte-neuron interactions, its vast array of genetic tools and rapid life cycle promises great potential for precisely targeted manipulations. My thesis work has utilized *Drosophila melanogaster* to investigate reciprocal astrocyte-synapse interactions during nervous system development. First, I characterized *Drosophila* late metamorphosis as a developmental stage in which astrocyte-synapse associations can be studied. Next, I utilized this developmental window to investigate how astrocytes and neurons communicate during development to influence an establishing circuit. My work demonstrates astrocyte GABA transporter levels are fine tuned through activity dependent mechanisms to modulate balance between excitatory and inhibitory signals during development, which have long lasting effects that persist into adulthood. Finally, utilizing the ease of the *Drosophila* system, I performed a
reverse genetic screen to identify novel astrocyte factors involved in mediating excitatory inhibitory neuronal balance.

**Coordinated development of astrocytes and synapses during *Drosophila* metamorphosis**

My thesis work demonstrates that during late metamorphosis, when the *Drosophila* adult nervous system is assembling, synapse formation coincides with astrocyte membrane infiltration into the neuropil. The major waves of synaptogenesis and astrocyte infiltration occur between 60-84 h APF. We found that genetic ablation of astrocytes specifically during this synaptogenic period resulted in a significant reduction in synapse numbers, thus demonstrating astrocytes are required for proper synapse formation *in vivo*. These studies revealed an important parallel between *Drosophila* and mammalian astrocytes and helped promote *Drosophila* as a useful model system to investigate astrocyte-synapse associations. This developmental stage can now be used to probe future questions regarding astrocyte-synapse interactions.

While both positive and negative astrocyte regulators of synapse numbers are known to the field, these regulators have not been investigated in a cell-type specific manner (Allen et al., 2012; Christopherson et al., 2005; Kucukdereli et al., 2011). Rather, the genes of interest were deleted in many different cell types, making it challenging to resolve the particular contribution from a single cell type. For the majority of these factors, it is not known which molecular signaling pathways are initiated or even what their neuronal targets are. Additionally, the molecular mechanisms by which these factors
act are not well understood. Thus, the specific contribution of astrocytes in mediating synaptogenesis \textit{in vivo} is still emerging.

\textit{Drosophila} metamorphosis could be used to identify cell type-specific \textit{in vivo} molecular regulators of astrocyte-synapse associations. For example, astrocyte genes enriched during late metamorphosis may be regulators of synaptogenesis. An important next step will be to determine the molecular profiles of astrocytes at various time points during late metamorphosis in efforts to identify potential astrocyte synaptogenic factors. Techniques such as Translating Ribosome Affinity Purification (TRAP) could be used, where cell type-specific expression of an affinity tagged ribosomal subunit protein (EGFP::L10a) enables purification of translating mRNA transcripts from a specific cell type (Heiman et al., 2008; Thomas et al., 2012). Purified mRNA is subsequently sequenced using microarray or RNA sequencing methods. Translating mRNA enriched in astrocytes at \textasciitilde50 or \textasciitilde60 h APF could provide strong candidates for astrocyte factors that initiate synapse formation. Likewise, translating mRNA enriched at \textasciitilde78 h APF would provide mid to late synaptogenic candidates, while translating mRNA enriched at \textasciitilde96 h APF or even later adult stages could reveal genes required for synapse maintenance. Reciprocally, profiling of neurons would provide insight into the molecular mechanisms by which neurons interact with astrocytes during synapse formation. For example, one could look for complementary enrichment of substrate-ligand pairs in astrocytes and neurons, respectively. Additionally, how astrocyte molecular profiles are influenced by neural activity could be studied. One could profile developing astrocytes after manipulating neural activity in specific subsets of neurons. This would help address
questions regarding how astrocytes impart distinct regulatory functions based on synapse class, a topic of ambiguity in the field.

Of course, profiling cells alone does not ensure enriched mRNA transcripts encode proteins that regulate synapse formation. Profiling experiments would provide candidate genes for which their function in synapse formation would need to be tested. In our studies, synapse integrity was evaluated using ultrastructure analysis, but performing EM studies on a long list of candidate genes would not be a practical or efficient way to test their effects on synapse formation. Efforts are being made in the field to generate better tools to visualize synapses in the adult *Drosophila* CNS (Chen et al., 2014; Mosca and Luo, 2014). Recent work by Mosca *et al* used a genetic reporter to label endogenous active zone sites in small distinct populations of neurons in order to characterize synapse organization in the adult *Drosophila* brain. With the advent of such tools, candidate genes from profiling studies could be conveniently screened to determine their effects on synapse organization.

**Activity-dependent regulation of astrocyte GAT levels during synaptogenesis**

Whether neuronal signals are required to shape astrocyte biology in a manner that ultimately affects a developing circuit has been an unexplored topic in the glial field. My thesis work demonstrates that during synaptogenesis, astrocyte GAT expression is regulated in an activity-dependent manner via astrocyte metabotropic GABA receptor signaling. Furthermore, my work demonstrates that the ability of astrocytes to fine-tune
GAT levels during development is important for establishing balance between excitatory and inhibitory neuronal signals and mediating hyperexcitability even at adult stages.

Genetic ablation of GABA neurons as well as genetic inhibition of GABA release resulted in reduced astrocyte GAT levels throughout the brain, demonstrating astrocyte GAT expression is sensitive to GABA release. Our studies suggest astrocytes detect changes in GABA levels by directly binding GABA and signaling through metabotropic GABA receptors (GABA_B1/2). This mechanism of regulation is similar to that of astrocyte glutamate transporters that are also sensitive to glutamate release and astrocyte metabotropic glutamate receptor signaling (Benediktsson et al., 2012; Yang et al., 2009). Though these processes seem similar, there are important differences in the molecular details of their regulation. While neural activity regulates astrocyte glutamate transporter transcript levels, GABA transporter transcript levels were unaltered in response to perturbations in GABA release. Thus, while the mechanisms by which astrocytes detect neurotransmitter levels are similar for different neurotransmitter types (via metabotropic neurotransmitter receptors), the downstream signaling events that are initiated are quite different. This makes sense, considering metabotropic GABA receptors initiate a different set of downstream signaling cascades than metabotropic glutamate receptors (Dahdal et al., 2010; Willard and Koochekpour, 2013; Yang et al., 2009). While the metabotropic glutamate receptors that mediate glutamate transporter expression are G protein alpha q subunit (G_{aq}) associated and upregulate cAMP to trigger the release of internal Ca^{2+} stores, metabotropic GABA receptors are G protein alpha o/i subunit (G_{a0i}) associated and downregulate cAMP activity. An important future direction will be to
determine the precise mechanisms by which astrocytes post-transcriptionally mediate GAT levels following GABA signaling. These mechanisms may include modulation of GAT mRNA translation or mediate of GAT protein degradation.

Another future direction is to examine how astrocyte GAT expression responds to localized changes in GABA release. In our experiments, GABAergic signaling was blocked in all GABA neurons, which densely infiltrate the entirety of the central brain. However, at what distances astrocytes detect changes in GABA release and regulate GAT levels to modulate GABA tone is not known. To address this, one could assess astrocyte GAT expression after manipulating GABAergic activity in a clonal manner. If astrocyte GAT levels are altered specifically in patches that correspond to areas where neural activity was also manipulated, it would suggest astrocytes respond to changes in GABA release in a localized manner. On the other hand, if GAT is reduced globally, it would suggest signaling between astrocytes and GABA release sites are more long ranged. Of course, it is also possible that GAT levels are regulated in a non-cell autonomous fashion. In this case, an individual astrocyte may be able to directly detect GABA levels only at local ranges, but through non-cell autonomous signaling events influence GAT expression in neighboring astrocytes. How astrocytes strike a balance between regulating specific synapses and acting as a global syncytium that maintains homeostasis remains an unresolved question.

**Astrocytes mediate excitatory and inhibitory neuronal balance.**
Astrocytes are important mediators of excitatory and inhibitory neuronal balance. Astrocytes oversee this balance through a variety of mechanisms that modulate neural activity. In particular, the regulated activity and expression of astrocyte neurotransmitter transporters have emerged as important players in combating neuronal hyperexcitability (Madsen et al., 2010; Sarup et al., 2003; Tanaka et al., 1997). However, despite their medical significance, our understanding of how astrocyte neurotransmitter transporters are regulated is still limited – especially in the case of GATs. My thesis work demonstrated that impairing GABA<sub>B</sub>R1/2 signaling during synaptogenesis mitigates neuronal hyperexcitability by reducing astrocyte GAT expression levels. This provided \textit{in vivo} evidence that astrocyte metabotropic GABA receptors, by regulating GATs, are important mediators of excitatory and inhibitory balance.

Intrigued by the roles of GAT and GABA<sub>B</sub>R1/2, we performed a reverse genetic screen to identify other astrocyte factors important for mediating excitatory and inhibitory neuronal balance. For this, we turned to \textit{eas<sup>PC80</sup>} flies, a genetic model of hyperexcitability. In \textit{eas<sup>PC80</sup>} animals, mechanical stimulation triggers overactivation of excitatory neurons, which leads to network hyperexcitability and eventual paralysis (Kroll and Tanouye, 2013; Pavlidis et al., 1994). The amount of time required by an animal to recover from paralysis can be used as a readout for the degree of hyperexcitability experienced by the animal. Utilizing this paradigm, we assayed hyperexcitability of \textit{eas<sup>PC80</sup>} animals after specific knockdown of target genes in astrocytes only. From this, we identified 141 astrocyte genes that suppress hyperexcitability when silenced and 20 astrocyte genes that enhance hyperexcitability.
when silenced. An overwhelming portion of our hits included membrane transporters and channels, which may be telling of the weight astrocyte transporters and channels carry in mediating electrical and chemical homeostasis. Likewise, membrane receptors also constituted a large portion of hits. This is not surprising as transmembrane receptors are ideally positioned to relay molecular signals between astrocytes and their surrounding environment (i.e. neurons, ECM). Overall, the screen has generated a large collection of candidate genes to pursue in the future.

My work has started to investigate the role of astrocyte Tensin associated focal adhesions in regulating hyperexcitability; identifying a previously unknown modulator of neural activity. Focal adhesions are protein clusters that link plasma membrane to cytoskeleton networks and serve as hubs for numerous intracellular signaling cascades. The role of tensin in focal adhesions has been mostly attributed to mediating cell adhesion (Chen et al., 2000; Chiang et al., 2005). However, these data arise from in vitro studies conducted in cell lines from outside the nervous system. Whether astrocyte Tensin modulates cell adhesion in a similar manner in vivo remains to be seen. In our studies, confocal analysis of astrocyte membrane morphology looked grossly normal following tensin knockdown in astrocytes, suggesting cell adhesion is not dramatically affected. However, the distribution of fine membrane processes close to synaptic structures, which cannot be resolved at the confocal level, may be altered. Such changes in membrane positioning around a synapse could explain the ability of tensin to modulate neural activity, for astrocyte membrane rearrangements around a synapse can alter the rates of neurotransmitter uptake (Pannasch et al., 2014). It is also plausible that astrocyte
focal adhesions regulate the expression of channels and transporters that mediate K$^+$ buffering. At this point, one can only speculate the mechanisms by which astrocyte focal adhesions modulate neural activity. Nevertheless, my work has exposed previously unexplored aspects of astrocyte biology that require future investigations.

**Concluding Remarks**

Astrocytes intimately integrate with neuronal networks to facilitate appropriate neuronal output. My thesis work has contributed to our understanding of how astrocytes and synapses communicate to shape the assembly and function of establishing circuits. My work demonstrated that *Drosophila* late metamorphosis is a promising model system to investigate astrocyte-synapse interactions and that during this time, astrocytes and synapses reciprocally communicate to mediate circuit output. While *Drosophila* astrocytes are critical for establishing proper synapse numbers, in turn, neural activity regulates astrocyte GABA transporter levels in a manner that ultimately influences circuit function and animal behavior. Additionally, my work has shed light on several avenues of investigation to pursue in the future. Our genetic screen has identified a large collection of astrocyte genes with potential roles in modulating neural activity that await future investigation.
Materials and Methods
Drosophila strains

The following Drosophila strains were used: Canton S, w^1118 Canton S, alrm-GAL4(Doherty et al., 2009), gad-GAL4(Ng et al., 2002) (gift from Gero Miesenböck), elav-GAL4(Luo et al., 1994), GH146-QF(Potter et al., 2010), UAS-mCD8::GFP(Lee et al., 1999), UAS-syt::eGFP(Zhang et al., 2002), UAS-mCD8::mcherry, UAS-hid(Grether et al., 1995), UAS-shi^G(Kitamoto, 2001), UAS-TNT(Sweeney et al., 1995) flies (active TNT-E and inactive IMPTNT-VA, gifts from John Carlson and Scott Waddell), UAS-Kir2.1(Baines et al., 2001), UAS-TrpAI(Hamada et al., 2008), UAS-px(Ferris et al., 2006) (gift from Vivian Budnik), UAS-GABA_B1_RNAi (TRiP.JF02989), UAS-GABA_B2_RNAi (VDRC transformant 1784), UAS-GAT_RNAi (VDRC transformant 13359), QUAS-mCD8::GFP(Potter et al., 2010), tub-GAL80^(McGuire et al., 2003), eas_P80 (gift from Barry Ganetzky and Mark Tanouye), UAS-tensin_RNAi (VDRC transformant 22823), UAS-tensin_RNAi (TRiP.HMS01743), by33c (gift from Nick Brown), UAS-β integrin_RNAi (VDRC transformant 103704), UAS-talin_RNAi (VDRC transformant 40399), UAS-FAK_RNAi (VDRC transformant 108608).

Temperature shift schemes

In order to assess control conditions, GAL4 driver lines were crossed to w^1118 Canton S flies. For all experiments, control and experimental animals underwent identical temperature shifts. Due to variations in developmental speed at different temperatures, the following incubation periods were determined based upon assessment of morphological features corresponding to the developmental stages of interest.
Astrocyte ablations: alm-GAL4 flies were crossed to UAS-hid, tub-GAL80ts flies. Crosses were set up at 18°C, and 0 h APF pupae were collected and incubated at 18°C for another 48 hr. For studies conducted in the adult, the staged pupae were shifted to 25°C or 30°C until animals were one day old. For studies conducted on animals ~60 or ~84 h APF, the staged pupae were shifted to 30°C for 24 h or 48 h respectively. To assess loss of astrocytes, brains were immunostained with anti-GAT and anti-Repo antibodies. Repo positive nuclei that belonged to GAT positive cells were counted as astrocytes. When astrocytes were counted within the AL, SOG, and TGab, these neuropil regions of interest were identified by anti-HRP staining. When astrocytes were counted within the MB, anti-Fasciclin II staining was used to identify the boundaries of the MB.

GABA neuron ablations: gad-GAL4, UAS-mCD8::mcherry flies were crossed to UAS-hid, tub-GAL80ts flies. Crosses were raised at 18°C. Animals were collected at 0 h APF and transferred to 30°C for 72 h, at which point they displayed morphological features of 84 h APF. Loss of GABA neurons was assessed by counting the number of mcherry labeled cell bodies within a specified 100 x 110 x 50 µm³ region in the ventral-medial region of the central brain.

Conditional manipulation of GABA neuron activity: gad-GAL4 flies were crossed to either UAS-shi ts, UAS-Kir2.1 with tub-GAL80ts, or UAS-trpA1 flies. Crosses were raised at 18°C, and 0 h APF pupae were collected and incubated at 18°C for another 48 h. Staged pupae were then shifted to 30°C for 48 h, at which point they displayed morphological features of 84 h APF. For adult specific inhibition of GABA neuron
activity, crosses were raised at 18°C, and 1 day old adults were shifted to 30°C for 7 days for *shir* experiments and for 3 days for *Kir2.1* experiments.

**Real Time PCR**

Pupal heads (84 h APF) were dissected in Jan’s saline (1.8mM Ca²⁺) and immediately frozen on dry ice. Total RNA was extracted using Trizol reagent. RNA pellets were resuspended in diethylpyrocarbonate (DEPC) treated water and RNA concentration was measured using a Nanodrop 2000c spectrometer (Thermo Scientific). RNA was DNase treated (DNase I, Amplification Grade, Invitrogen) and then reverse-transcribed using Superscript III First-Strand Synthesis System (Invitrogen).

Relative quantification of gene expression was performed using TaqMan probes and an ABI Prism 7000 Sequence Detection System. Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen) was used with the following primers and probes: gat F-primer, GGTGGTCTCCGTATCTGCTCTT; gat R-primer, GAGATTGGAAATATTCGCTGGG; gat-probe, 6FAM-TTTGGGAGCGGCGAGCTCTTCA-BHQ1; Rpl32 F-primer, GGCCCAAGATCGTGAAGAAG; Rpl32 R-primer, TAAGCTGTCGCACAAATGGC; Rpl32 probe, 6FAM-AGCAGTCTCACTGGCAAATGGC-BHQ1. Assay efficiencies were experimentally determined using a 5-point dilution series of cDNA spanning a 100-fold range in concentration (gat, 101%; Rpl32, 95%). 0.025 µg cDNA template was used per reaction. Statistical analysis was performed on $2^{-\Delta Ct}$ values.
**Immunohistochemistry**

Pupal brains were dissected in PTX (0.3% Tx-100, 1XPBS) and fixed in 4% formaldehyde for 20 minutes. For adult brains and adult CNS preparations, heads or whole flies respectively were fixed in 4% formaldehyde for 17 minutes, washed 4 x 3 min with PTX, dissected in PTX and then fixed in 4% formaldehyde again for 7 minutes. For larval stains, the CNS of wandering third instar larva were dissected in PBS and fixed in 4% formaldehyde with PTX for 17 minutes. For all preparations, the final fixation was followed by 5 quick washes with PTX and then 3 quick washes with PBT (0.1% BSA, 0.3% Tx-100, 1XPBS). Tissues were then blocked in PBT for 30 min at room temperature, and then probed with appropriate primary antibodies for 2 nights at 4°C. Samples were washed 6 x 10 minutes with PBT, probed with appropriate secondary antibodies overnight at 4°C, washed 6 x 10 minutes with PBT and then stored in Vectashield anti-fade reagent (Vector Laboratories). Antibodies were used at the following dilutions: 1:50 mouse nc82 (Developmental Studies Hybridoma Bank); 1:5000 rabbit anti-GAT; 1:10 mouse anti-PDF (Developmental Studies Hybridoma Bank); 1:500 mouse anti-GFP (Chemicon); 1:150 rat anti-Fasciclin II (Developmental Studies Hybridoma Bank); 1:100 mouse anti-Elav (Developmental Studies Hybridoma Bank); 1:500 rabbit anti-Draper; 1:10 mouse anti-Repo (Developmental Studies Hybridoma Bank); 1:10 guinie pig anti-Repo; 1:500 goat Cy3 conjugated anti-HRP; 1:200 donkey FITC conjugated rabbit IgG; 1:200 donkey Cy3 conjugated rabbit IgG; 1:200 donkey FITC conjugated mouse IgG; 1:200 donkey Cy3 conjugated mouse IgG; 1:200 donkey Cy5 conjugated mouse IgG (all Jackson Immunoresearch).
Confocal microscopy

Tissues were mounted in Vectashield anti-fade reagent and imaged using a 3i Everest spinning disk confocal microscope. Whole brain images for Supplementary Fig 1 were taken using a Zeiss EC Plan-Neofluar 10X objective (NA=0.3). Whole brain images for Fig 4f were taken using a Plan-Apochromat 20X objective. All other images were taken using a Zeiss Plan-Apochromat 40X oil objective (NA=1.3). Images were stitched together when regions of interest did not fit within the field of view (Fig. 2a).

Immunoblotting

*Drosophila* brains were dissected in 1X PBS and collected by light centrifugation. Brains were homogenized in SDS loading buffer (60mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% b-mercaptoethanol, 0.01% bromophenol blue), and centrifuged at 16000g for 10 minutes to clear the homogenate. Supernatants were then collected and boiled for 5 minutes. Samples were resolved by SDS-PAGE (BioRad), and transferred to nitrocellulose membranes (BioRad). Membranes were blocked in blocking buffer (5% Non-Fat Dry Milk, 0.01% Tween-20, 1X PBS) and then immunoblotted with appropriate antibodies. All antibodies were diluted in blocking buffer. Primary antibody incubations were performed overnight at 4°C, followed by 5 x 10 min washes in wash buffer (0.01% Tween-20, 1X PBS), appropriate HRP conjugated secondary antibody incubation for 1.5 hours at room temperature, 5 x 10 min washes in wash buffer, and then chemiluminescence detection (ECL Plus Amersham). Immunoblots were stripped by
rocking in mild stripping buffer (0.2M glycine, 0.1% SDS, 1% Tween, pH 2.2) at room temperature for 10 min, followed by 2 x 5 min washes in 1XPBS, and then 2 x 5 min washes in wash buffer. After this, immunoblots were blocked again and re-probed. Antibodies were used at the following dilutions: 1:10,000 rabbit anti-GAT; 1:50,000 mouse anti-tubulin (Sigma); 1:6000 sheep HRP conjugated anti-mouse IgG (Abcam); 1:6000 goat HRP conjugated anti-rabbit IgG (Abcam).

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

**Behavioral Assays**

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

**Transmission Electron Microscopy**

For all EM experiments, at least two brains were independently prepared and sectioned for every condition/time-point. *Drosophila* heads were incubated in fixation buffer (2.5% glutaraldehyde in 0.1 M Sodium Cacodylate buffer pH 7.2) for 1 hour at 4°C.
Brains were then dissected out in fixation buffer and incubated in fixation buffer overnight at 4°C. Samples were then processed and analyzed at the University of Massachusetts Medical School Electron Microscopy core facility according to standard procedures. Briefly, fixed samples were transferred to fresh fixation buffer and continued to incubate overnight at 4°C. Samples were rinsed 2 x with fixation buffer and treated with 1% osmium tetroxide for 1 hour at room temperature. Samples were washed 2 x with dH2O for 5 minutes and then dehydrated through a graded ethanol series of 20% increments that ended with two changes in 100% ethanol. Samples were infiltrated with two changes of 100% propylene oxide and then with a 1:1 propylene oxide/SPI-Pon 812 resin mixture. The following day, the samples were infiltrated with 3 changes of fresh 100% SPI-Pon 812 resin, after which the samples were polymerized at 68°C in plastic capsules. Thin sections were placed on copper support grids and contrasted with lead citrate and uranyl acetate. Sections were examined using the FEI Tecani 12 BT electron microscope with 80kV accelerating voltage, and images were captured using a Gatan TEM CCD camera. The project described was supported by Award Number S10RR027897 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

**Screen**

Each candidate UAS-RNAi fly line was crossed to easPC80;almGAL4 flies to generate candidate lines. Approximately 10 flies from each candidate line were transferred to
empty food vials and vortexed for 10 seconds to provide mechanical stimulus and induce paralysis. The numbers of flies standing and resuming normal behavior were noted at 1, 1.5, 2, 3 and 4 minutes. If 100% recovery was observed by 1.5 minutes or if less than 100% recovery was observed at 4 minutes, the assay was repeated two more times with additional animals.

**Picrotoxin Behavior Assay**

Picrotoxin was dissolved in acetone (0.1g/ml) to make a stock solution. Stock solution was added to heated liquid fly food to reach desired concentrations and thoroughly mixed together. While still liquid, food was aliquoted into empty food vials and then let to cool and solidify. Young third instar larvae (non-wandering) were transferred to picrotoxin food vial for 9 hours, after which locomotion speed was assayed. For this, Larvae were placed on agar (1%) plates with a transparency film placed over the rim of the agar plate (making sure the film did not touch the agar). The path traveled by each larva was traced by hand on the transparency for 1 min. The distance traveled by each larva was determined using ImageJ software.

**Statistical Analysis**

GraphPad Prism was used to perform two-tailed unpaired Student’s *t*-tests, two-tailed paired Student’s *t*-tests, 1-way ANOVA with Tukey’s post hoc tests, and repeated measures ANOVA with Tukey’s post hoc tests. Data distribution was assumed to be normal but this was not formally tested. No statistical methods were used to pre-
determine sample sizes but our sample sizes are similar to those reported in previous publications. Also, data collection and analysis were not performed blind due to the conditions of the experiments. Data were not collected and processed randomly. Animals were assigned to the various experimental groups based upon genotype. A supplementary methods checklist is available.
References


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