THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

The Role of \textit{Drosophila} Perisynaptic Glial Cells in Glutamate Regulation

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SPRING 2013

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

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ABSTRACT

In the nervous system, an overabundance of the neurotransmitter glutamate, termed excitotoxicity, can lead to neurodegeneration and neurological disease. Our genetic analysis has focused on the roles of glial cells in regulating extracellular glutamate by examining the contributions of two glial proteins: dEAAT1, a glutamate transporter, and Glutamine Synthetase (GS2), an enzyme that converts glutamate to glutamine. Aberrant synaptic function, glutamate excitotoxicity and neurodegeneration may occur when proteins in glial cells, including dEAAT1 and GS2, do not regulate extracellular glutamate properly. Our studies in the Drosophila genetic model system employ in vivo RNA interference (RNAi) methods to reduce (knock down) the function of specific genes expressed in glial cells. Transgenic Drosophila lines in which glial dEAAT1 is knocked down exhibit severe neurodegeneration resulting in paralysis and tremors. On this basis, we have performed a genetic screen for new mutations that can suppress these neurodegenerative phenotypes with the goal of identifying novel molecular mechanisms which may provide protection against neurodegeneration. An exciting new suppressor mutant has been isolated and its genetic characterization is currently under way. In our analysis of GS2, we have generated transgenic lines permitting knockdown of GS2 in glia and have examined the resulting phenotypes. The GS2 knockdown has been instrumental in identifying perisynaptic glial cells involved in tripartite synapses in the Drosophila PNS. Our genetic analysis of these evolutionarily conserved mechanisms is expected to provide new insights into mechanisms of glutamate regulation in neurological health and disease.
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ACKNOWLEDGEMENTS

I would like to thank all of the members of the Ordway/Kawasaki Lab for creating the most productive and enjoyable environment for research. I would especially like to mention Dr. Rick Ordway for being a truly great mentor. He has provided me with opportunities to learn from new experiences, guidance in my writing and research, and a constant source of support. Dr. Fumiko Kawasaki has shown me how to innovatively approach problems and has provided the perfect role model for efficiency and commitment to research. Dr. Janani Iyer showed me the ingenuity involved in molecular biology, the beauty of organization, and the joy of multitasking. Rie Danjo demonstrated the benefits of perfectionist thinking in her approach to research and life. Ximena Checa taught me how to do my first molecular biology experiments and became my first friend in the lab. I'd also like to thank Kerry Cao and all of my friends who have been my companions in writing. I would like to thank my Honors advisor, Dr. Stephen Schaeffer for always being there when I had questions or concerns and for reading my Thesis. Finally, I would like to thank my family, particularly my mother and grandmother, for being the best support system for which anyone could ask.
I would like to make clear that the findings presented in my Thesis would not exist without the significant contributions of fellow lab members. Dr. Richard Ordway played an important role in the design and interpretation of experiments. Dr. Fumiko Kawasaki contributed to the planning and supervision of several aspect of this research. Rie Danjo was instrumental in the immunocytochemistry work. Emily Sun was responsible for the EM experiments. Ximena Checa and Julie Oliver assisted with the UAS-dEAAT1 RNAi screen. Janani Iyer assisted with the supervision of molecular biology for cloning of the GS2- RNAi and GS2-EGFP constructs. Yunzhen Zheng made key contributions to carrying out the current forward genetic screen and immunocytochemistry experiments.
Chapter 1

Introduction

Glial Cells

At the foundation of neural communication lies the unit of the synapse. The axon transforms the electrical signaling of the neuron’s action potentials into a chemical signal. In the presynaptic axon, the depolarization causes an influx of calcium ions that leads to release of neurotransmitters into the synapse. Once released from the presynaptic bouton into the extracellular synaptic cleft, the neurotransmitter acts on postsynaptic cell receptors to induce a postsynaptic potential that can either excite or inhibit the postsynaptic membrane, depending on the specific neurotransmitter. Neurons are central to the function of the nervous system, but do not act without the support of other cells called glia that actually make up ~90% of our brains (Stork, Bernardos & Freeman, 2010).

Often overlooked in past research, glia are now recognized as an important component of neural function. Glia are broadly defined as neural cells that lack the ability to transmit rapid electrical signals in the form of action potentials. Instead, glial cells surround and ensheath the neuron’s somas, axons, and synapses (Allen & Barres, 2009). Glia are categorized on the basis of their morphology, function, and location. These properties exhibit significant variation depending on the organism and change from the developing to adult nervous system (Stork, Bernardos & Freeman, 2010). The experimental system used in the present studies, Drosophila melanogaster, has served as
an important model for genetic analysis of glia (Allen & Barres, 2009). In the adult
*Drosophila* nervous system, glia can be segmented by location into four basic classes:
neuropil, cortex, surface (perineurial and subperineurial) and peripheral (Jackson &
Haydon, 2008). Surface glia surround the central and peripheral nervous system to
contribute components of the blood-brain barrier. Cortex glia surround the soma, while
peripheral glia surround the axons and extend into contact with motor nerve endings
(Jackson & Haydon, 2008). Neuropil glia can be further broken down ensheathing
astrocyte-like glia, and wrapping glia. Astrocyte-like glia have their cell bodies
associated with the synaptic neuropil and glial processes that extend to synapses to clear
extracellular neurotransmitters (Stork, Bernardos & Freeman, 2010).

Glia have a pivotal role in neuronal excitability and behavior, but historically
these cells have not been studied as extensively as neurons. Glial cells in *Drosophila*
have been implicated in many different neural processes such as stress and neuronal
injury, locomotor activity, circadian behavior, axonal and neuromuscular junction
excitability, vision, and even neural plasticity involved in long term memory (Jackson &
Haydon, 2008). The growing interest in glial cells has created a new frontier of research
with so much yet to be understood, especially with relation to the etiology of neurological
disorders. The research conducted throughout my undergraduate career has attempted to
characterize glial cells and their function. A central aspect of the project pertains to the
role of glia in the function of glutamatergic synapses.

**Glutamate Transport**

Glutamate, an amino acid, is the most abundant excitatory neurotransmitter in the
human brain and contributes to depolarization and excitation of the postsynaptic cell.
Despite the major role glutamate plays in normal brain function and development, severe consequences can occur if glutamate is not regulated properly (Sheldon & Robinson, 2007). When glutamate is released into the synapse via exocytosis, its synaptic concentration is increased 1000-fold. Abnormal increases in extracellular glutamate cause prolonged depolarization which in turn causes excessive excitation in the nervous system. Termed glutamate excitotoxicity, this cascade of over-activation can lead to neuronal cell death that can then spreads to surrounding neurons. Defects in glial glutamate uptake have been seen in ALS patient and in mouse models of the disease (Rival et al., 2004).

Since glutamate excitotoxicity causes such severe damage, control of extracellular glutamate concentration is crucial. Glutamate is regulated by perisynaptic glial cells and neurons through the glutamine-glutamate cycle (Figure 1-1).

**Figure 1-1. Glutamate Cycling at Tripartite Synapses**

GLUTAMATE CYCLING AT TRIPARTITE SYNAPSES
1. Synaptic vesicle fusion and GLU exocytosis
2. Glial GLU uptake mediated by dEAAT1
3. Conversion of GLU to GLN by GS2
4. GLN release from glia and uptake by presynaptic terminal
5. Conversion of GLN to GLU within presynaptic terminal
6. GLU uptake by synaptic vesicles

GLU - glutamate (excitatory neurotransmitter)
GLN - glutamine
dEAAT1 - Glial plasma membrane glutamate transporter
GS2 - Glial cytoplasmic Glutamine Synthetase (converts GLU to GLN)
Perisynaptic glia act as support cells that form tripartite synapses with their processes extending around the synaptic cleft. These glial cells uptake glutamate from the extracellular space using excitatory amino acid transporters (EAAT, dEAAT in *Drosophila*) that act as sodium and potassium coupled glutamate transporters. Inside the glial cell, glutamine synthetase (GS) catalyzes the condensation of glutamate and ammonia to form glutamine, which is neither excitatory nor toxic. Glutamine can then be released from the glial cell using system N transporters, taken up by the presynaptic cell using system A neutral amino acid transporters, and reconverted back to glutamine using phosphate-activated glutaminase (PAG) (Kam and Nicoll, 2007). Glutamate can then be repackaged into synaptic vesicles and reused for neurotransmission. Together, these different glial enzymes and transporters function to inhibit glutamate excitotoxicity.

Although all cells synthesize glutamate from intermediates of the tricarboxylic acid (TCA) cycle, this synthesis is not thought to be sufficient for neurotransmitter release, especially considering a lack of neuronal enzymes to replenish the TCA cycle. However, the necessity of glutamate-glutamine recycling is controversial within the field, and the physiological role of the cycle is poorly understood (Kam and Nicoll, 2007). It has been argued that transfer of glutamine from astrocytes to neurons is necessary for proper synaptic function. However, pharmacological inhibition using L-Methionine sulfoximine (MSO) in hippocampal slices to block glutamine synthetase fails to abolish synaptic transmission even with intense activation (Kam and Nicoll, 2007). Despite the overall ambiguity concerning the necessity of the cycle, certain proteins involved in glutamate recycling have been studied extensively.
Mice have two glial glutamate transporters, GLAST and GLT-1, but it has been demonstrated that GLAST plays a more major role in glutamate clearing; knockout of either or both of the genes causes only slight differences in function and development, but knockout of both causes death before birth (Takatsuru, et al. 2007). Decreasing the ability to buffer glutamate in *Drosophila* is neurotoxic (Rival, et al. 2004). The only high affinity glutamate transporter, dEAAT1, which relies on Na+/K+, is selectively found in glial projections into the neuropil near synapses and not near the cell bodies. Using RNA interference (RNAi) to knockdown the function of dEAAT1 causes behavioral deficits that can be rescued by a human glutamate transporter (Rival et al., 2004). The dEAAT1 deficient flies are rather inactive but hyperactive when startled; they are able to walk, but are flight defective. The glutamate accumulation leads to increased oxidative stress in the CNS, which was partially rescued by free radical scavengers and anti-excitotoxic agents (Rival et al., 2004). This study suggests that, like in mammals, extracellular regulation of glutamate is necessary in the insect brain; this finding is significant because glutamate was previously thought to be a minor neurotransmitter in the *Drosophila* brain (Rival et al., 2004). The degeneration is not found within embryos or larvae, which suggests that the adult brain had increased sensitivity to glutamate excitotoxicity and oxidative stress (Rival et al., 2004). dEAAT1 is only found at adult synapses following maturation in glial extensions that closely follow motor axons (Rival et al., 2006). Knockdown of the primary glutamate transporter within glial cells is an important tool that can be utilized to study glutamate regulation.

Glutamine synthetase (GS) has been considered another important component of the glutamate-glutamine cycle. GS is a well conserved protein expressed in all species
investigated that catalyzes the condensation of glutamate and ammonia to form glutamine with the expenditure of an ATP molecule (Lie-Venema et al., 1998). Mammalian systems do not have isozymes, but Drosophila has two isozymes with tissue and developmental stage specificity. GS2 is the Drosophila enzyme that is homologous to the GS in mammals (Caizzi et al., 1990). GS is mainly found in astrocytes but at lower levels in oligodendrocytes and is the only enzyme capable of de novo biosynthesis of glutamine. Knockout mice were created to remove the expression of GS in brain astrocytes. At birth, the Mendelian ratios were preserved with respect to the knockouts and other litter mates, but their health declined after birth. Knockout mice died on postnatal day 2 with starvation as the immediate cause of death despite having no malformations, no seizures, and moving about normally (He et al., 2010). The deficiency created a 14-fold decline in cortical glutamine, but the glutamate levels were unaffected (He et al., 2010).

Drosophila as a Model System

For studying glia, Drosophila has many similarities to more sophisticated mammalian systems that allow for important comparisons. Like mammals, Drosophila possess glial pathways for recycling synaptic neurotransmitters, a subtype of glia that can individually ensheath axons, and a subtype of glia that bear morphological and molecular similarity to astrocytes (Stork, Bernardos & Freeman, 2010). This system allows for the ability to study the mechanisms of glial cell glutamate regulation in vivo using the powerful molecular-genetic tools available within Drosophila. Forward genetic screens that allow for the observable phenotypes to identify genes are significantly more feasible in Drosophila than in mice. In addition, the UAS/GAL4 system is an important tool that allows for targeted gene expression in tissue or cell specific patterns (Brand & Perrimon...
GAL4 dependent target genes can be constructed by subcloning a sequence behind the GAL binding sites referred to as the upstream activating sequence (UAS). In the absence of GAL4, the target gene is silent but can be activated when crossed to flies carrying the driver. The progeny carry both the UAS and the GAL4 transgenes, and the gene product (UAS- gene X) will be expressed in a driver specific pattern. This system is extremely powerful to determine the effect of gene overexpression/ knockdown in specific cells or tissues within the fly.

**Figure 1-2. UAS/GAL4 System**

In order to study glia, labeling of specific glial cells is critical. For *Drosophila*, reversed polarity (repo) is a direct transcriptional target of glial cells missing (Gcm), a marker for cells that become glia. Unlike Gcm, repo is expressed exclusively in glia throughout the entire life cycle; antibodies against REPO uniquely label all glia, while repo-GAL4 is an important tool within the UAS/GAL4 system to drive pan-glial expression. In contrast, dEAAT1-GAL4 drives expression only in the astrocyte-like glia, some cortex glia, and exhibits weak neuronal expression, which is in contrast to the moody-GAL4 driver, which is specific to ensheathing glial (Stork, Bernardos & Freeman, 2010). Having tools to uniquely label and distinguish subtypes of glia are crucial for developing a system to study glial regulation of glutamate transport.
Previous work of our lab has established that the Dorsal Longitudinal Muscle (DLM) can be used as a model for analysis of glutamatergic synapses, as it shares conserved properties with mammalian tripartite synapses and possesses close relations with glia (Kawasaki & Ordway, 2009), (Danjo, Kawasaki, & Ordway, 2011). These cells contain similarities to mammalian cerebellar climbing fibers and Purkinje Cell synapses with high release probability and highly branched axons with small boutons containing one or two active zones (Danjo, Kawasaki, & Ordway, 2011). The accessibility of this synapse is comparable to mouse neuromuscular synapses, but with the added benefit of being glutamatergic rather than cholinergic, creating an ideal model for the study of glial glutamate transport.

The work discussed in this thesis addresses two distinct ideas. First, RNAi mediated knockdown of the glia glutamate transporter, dEAAT1, was used for a mutagenesis based screen to identify suppressors of the glutamate excitotoxic phenotype. This screen is expected to identify novel mechanisms which may provide protection against neurodegeneration and guide development of rational therapies. Second, for the first time in Drosophila, GS2 was knocked down using RNAi transgenic lines to examine the effect of reducing expression of this gene product. The latter work also led to development of important new tools for specific label of perisynaptic glial cells.
Chapter 2

Materials and Methods

Mutagenesis and Screening

All fly stocks for crosses were maintained at room temperature of 23-25 °C. UAS-dEAAT1 RNAi flies were obtained from Dr. Serge Birman (Rival et al. 2006). The remaining lines were from our lab’s stock collection. To isolate modifiers of dEAAT1 RNAi knockdown, UAS-dEAAT1 RNAi (2\textsuperscript{nd} chromosome) males were exposed for 24 h to 25 mM ethyl methanesulfonate (Figure 2-1)

Figure 2-1. Genetic Screen for dEAAT1 Modifiers.

\[
\begin{align*}
\text{F0} & \quad \text{mutagenized} \\
\text{F1} & \quad \text{screen} \\
\text{F2} & \quad \text{phenotype}
\end{align*}
\]

Mutagenized males were mated with females carrying a second, third chromosomal translocation with a visible phenotype of curly wings (Cy), rough eye (Roi), and tubby (Tb) bodies. Mutagenized males carrying the RNAi transgene in trans to the translocation were then crossed to females carrying a glial specific GAL4 driver, Repo-GAL4 in trans to a balancer chromosome, TM6b. From this cross, non-Tb pupae were separated from other progeny and their health assessed in terms of their ability to stand or walk. Stocks were made of identified suppressor lines and the mutation was mapped to a chromosome
by allelic segregation with respect to balancer chromosomes.

**Constructs**

GS2 RNAi and GS2-EGFP fusion protein constructs were cloned using GS2 cDNA (GH03580) obtained from the *Drosophila* Genomics Resource Center. PCR primers were obtained from Integrated DNA Technologies with standard desalting and used in 5 μM working concentrations for PCR. All PCR products were sequenced upon transformation into a vector by the Penn State University nucleic acids facility.

**Table 2-1. Primers Used to Create GS2 Constructs**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS2F_BglII</td>
<td>GAAGATCTATGTCCGCTAGGATCCTGGA</td>
</tr>
<tr>
<td>GS2R_KpnI</td>
<td>GGGGTACCCTATTCGTCCAGGCGAGATGG</td>
</tr>
<tr>
<td>GS2_828R_XbaI</td>
<td>CGTCTAGAAATGTCCCCGAATGCCGCGCAT</td>
</tr>
<tr>
<td>GS2F_XbaI</td>
<td>GCTCTAGAAATGTCCGCTAGGATCCTGGA</td>
</tr>
</tbody>
</table>

For expression of an EGFP-GS2 fusion protein, a UAS transgene was constructed such that the tag is at the N-terminus, as it appeared to be less conserved than the C-terminus (Figure 2-1). The entire ORF was cloned using PFU into a pBlueScriptII SK+ vector using enzymes, KpnI and BglII, whose restriction sites were added to the primer sequences. This vector contained the EGFP coding sequence with a BglII site on its C-terminus that enabled ligating the two fragments without a frameshift. The ligated product was transformed into DH5α competent cells. Following a plasmid preparation
and restriction digest to verify the correct DNA, the EGFP-GS2 insert was shuttled into
the fly transformation vector, pUAST, using NotI and KpnI.

Figure 2-2. Scheme for EGFP-GS2 Construct

EGFP-Gs2

EGFP-LcF in pBluescriptII SK+

EGFP-Gs2 in pBluescriptII SK+

EGFP-LcF in pUAST

EGFP-Gs2 in pUAST

Ligation
Transformation

Ligation
Transformation

Ligation
Transformation

(Template, pBlue-Gs2)
For the GS2 RNAi transgene, primers were selected using the dsRNA Snap Dragon with 3 potential off target gene products of 19 base pairs. The primers cloned 828 bp of the ORF with XbaI sites designed onto each side of the fragment. XbaI was used to cleave both ends of the PCR fragment to shuttle into the cloning vector pGEM-Wiz (Bao & Cagan, 2006). AvrII digests cut and CIP phosphatase were used to create a cloning site on one side of a 45 base pair white intron. After ligation, transformation, and plasmid preparation the same method was repeated, except with an NheI cut site in the vector instead of AvrII. This allowed insertion of the GS2 PCR product to ligate on the other side of the white intron. Clones with inverted GS2 fragments (inverted repeats) were selected and sequenced. The inverted repeat was the shuttled into pUAST using XbaI and BglII. The inverted repeat would create a hairpin, double stranded RNA upon being transcribed.
Transgenic Lines

Final constructs in pUAST were generated as described previously (Kawasaki, Collins, & Ordway, 2002), (Brand & Perrimon 1993). Briefly, DNA was prepared using column purification and then injected into posterior pole of fly embryos carrying a w mutation. Coinjection of the pπ25.7wc contributed transient expression of the P element transposase. The expression vector contained a w+ marker that allowed the F1 generation to be screened for transgene insertions on the basis of the resulting orange eye color. Insertions were then mapped to a chromosome and established in stocks. Expression of the transgenes in the UAS/GAL4 system was driven using a glial specific driver, Repo-GAL4. The transgenic lines were screened for transgene expression was screened using Western blotting.

Western Blot Analysis

Western blot analysis was performed using conventional methods. Head homogenates were used for the work with glial cells. A mouse monoclonal α-GS2 antibody (Millipore) was used at a dilution of 1:1000 to detect the EGFP fusion protein and changes in endogenous GS2 expression following RNAi knockdown. A rabbit polyclonal α-GFP antibody (Invitrogen) was used to verify the specificity of the GS2 antibody with a dilution of 1:1000. Tubulin was the loading control using a monoclonal antiacetylated α-tubulin antibody (Sigma) with a dilution of 1: 2,000,000.

Degenerate PCR

A degenerate PCR method was used to map the chromosomal location of a P-element based transgene. This method was similar to a P-element mapping protocol developed by Georg Dietzel in the B. Dickson lab based on a published protocol in plants (Liu et al.,
This was used to map the insertion of the UAS-dEAAT1 RNAi transgene. The original protocol was modified for UAS P-element insertions with primers designed for the specific UAS construct.

**Table 2-2. Specific Primers to anneal to P3’ Region of pUAST**

<table>
<thead>
<tr>
<th>T1BUAS</th>
<th>GCAGAAGCTTTGGTACTCGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2D</td>
<td>ATTCAAACCCACGGACATG</td>
</tr>
<tr>
<td>T2En</td>
<td>AATCATATCGCTGTCTCACTCA</td>
</tr>
</tbody>
</table>

_The Primers are in a 5’ to 3’ orientation._

**Table 2-3. Arbitrary Degenerate Primers**

<table>
<thead>
<tr>
<th>AD1</th>
<th>NTCGASTWTSGWGTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD2</td>
<td>NGTCGASWGABAWGAA</td>
</tr>
<tr>
<td>AD3</td>
<td>WGTGNAGWANCANAGA</td>
</tr>
</tbody>
</table>

_The Primers are in a 5’ to 3’ orientation. S= G/C, W= A/T, N= A/T/G/C._

The insertions were mapped using thermal asymmetric interlaced PCR. A first round of PCR was used with a 1:100 dilution of a genomic preparation with Taq polymerase. Primers AD1, AD2, or AD3 could be used with specific primer, T1BUAS. A 1:50 dilution of the first PCR would serve as the template for the second PCR. The same AD primer would be used as in the first PCR, but with T2D as the specific primer. Product from the 2nd PCR was ran on a 1 % agarose gel and stained with EtBr. If no product was visible, a third PCR could be used with T2En, but this was usually unnecessary. DNA was then purified from the gel, sequenced, and BLAST was used to find the insertion site within the fly genome.
Table 1. Cycle settings used for TAIL-PCR

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Cycle no.</th>
<th>Thermal settings&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary (1st PCR)</td>
<td>1</td>
<td>93°C, 1 min, 95°C, 1 min&lt;br&gt;94°C, 60 (30) sec; 62°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>94°C, 60 (30) sec; 25°C, 3 min, ramping to 72°C, over 3 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94°C, 30 (10) sec; 68°C, 1 min; 72°C, 2.5 min&lt;br&gt;94°C, 30 (10) sec; 68°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>94°C, 30 (10) sec; 44°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72°C, 5 min</td>
</tr>
<tr>
<td>Secondary (2nd PCR)</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94°C, 30 (10) sec; 64°C, 1 min; 72°C, 2.5 min&lt;br&gt;94°C, 30 (10) sec; 64°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>94°C, 30 (10) sec; 44°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72°C, 5 min</td>
</tr>
<tr>
<td>Tertiary (3rd PCR)</td>
<td>20</td>
<td>94°C, 60 (15) sec; 44°C, 1 min; 72°C, 2.5 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72°C, 5 min</td>
</tr>
</tbody>
</table>

Note. The program files in each reaction were linked automatically.<br><sup>a</sup>Denaturation time settings used with the Perkin-Elmer Cetus DNA Thermal Cycler (original model) are indicated first, followed by a number in parentheses indicating the time setting used with the Perkin-Elmer GeneAmp PCR System 9600 model.<br><sup>b</sup>These are nine-thermal-segment super cycles each consisting of two high-stringency and one reduced-stringency (see Figure 1).

Chapter 3

Results of dEAAT1 and GS2 Analysis

One focus of our work has been on the genetic analysis of glial function. The experiments performed within this research program attempted to characterize the function of the glial cells, specifically the roles of dEAAT1 and GS2 in glutamate regulation. A transgene was created to knockdown the expression of GS2 (Figure 3.1).

Figure 3-1. Scheme for GS2 RNAi Transgene

A hairpin RNA was created through placement of complementary GS2 cDNA sequences in an inverted pattern. The GS2 dyad was then shuttled into vector, pUAST.
The UAS-dEAAT1 RNAi transgene markedly reduced expression of the protein, which caused significant neurodegeneration and a startling phenotype (Figure 3.2).

Figure 3-2. UAS-dEAAT1 RNAi Knockdown Causes Severe Effects

(A) dEAAT1 knockdown in glia produced severe motor impairment and immobilization. (B) Transverse head sections of 1 day-old adult males stained with toluidine blue. a, c, The wild-type brain contains darkly staining cells at the cortex-neuropil border (box). b, In the dEAAT1 knockdown brain, abundant neurodegenerative lesions are observed (arrow heads). d, Enlarged view of the boxed region in b. (C) Confirmation of dEAAT1 knockdown by Western analysis of head homogenates. Glial-specific expression of the UAS-dEAAT1-IR transgene produced a marked reduction in dEAAT1 protein levels.

Flies were unable to stand and experienced intense seizures, culminating in premature death at 1-2 days old. This control was compared against mutant phenotypes to assess improvement in the reduction of glutamate excitotoxicity. The screen using dEAAT1 knockdown flies generated several promising mutant suppressors that were able to stand and walk noticeably better than their control counterparts. In particular, Suppressor 7-72 was able to walk and had an increased life span compared to that of control flies. This mutation was mapped to the second chromosome, but meiotic mapping is challenging with the presence of the UAS-dEAATI RNAi transgene also on the second chromosome. To facilitate mapping of the 7-72 mutant, the location of the UAS-dEAAT1
transgene was determined by molecular mapping. Degenerate PCR and subsequent sequencing of the purified product identified the location of the insertion to be 2L 35D2. Western blotting showed that the increase in health was not by increasing the levels of dEAAT1 (Data not shown), which suggests the mutation decreased glutamate excitotoxicity through another mechanism.

RNAi lines to knockdown the function of GS2 were created and western blotting showed a significant reduction in the expression of the protein (Figure 3.3). Expression of the UAS-GS2 RNAi transgene was exclusively driven in glial cells using a pan-glial driver, Repo-GAL4.

**Figure 3.3. Glial Specific RNAi Knockdown**

**Glial-Specific RNAi Knockdown (KD) of GS2 - Western**

- **A** Specific Detection of GS2 and EGFP-GS2
  - Blot: α-GS2
  - α-TUB
  - WT EGFP-Gs2
- **B** Gs2-RNAi KD (Repo-Gal4 UAS-Gs2-RNAi)
  - Blot: α-GFP
  - α-TUB
  - WT EGFP-Gs2

*Western blots demonstrating specificity of the GS2 antibody and confirmation of successful GS2 RNAi KD. UAS transgenic lines were generated for expression of the fluorescent fusion protein, EGFP-GS2, and a dsRNA “hairpin” for RNAi against GS2. Transgene expression was driven by the glial-specific GAL4 driver, repo-GAL4. (A) α-GS2 recognizes both endogenous GS2 and the larger EGFP-GS2 transgene product in head homogenates. (B) Glial-specific GS2 KD produces a marked reduction in GS2 protein.*
The specificity of the antibody was verified by western blot analysis of the EGFP tagged GS2, which was blotted with both α-GFP and α-GS2. This western showed both the endogenous GS2 and transgenic EGFP-GS2 at their respective molecular weights (Figure of GFP 3-3 A). In addition, immunocytochemistry using α-GS2 with repo-GAL4 showed the disappearance of GS2 from the glial cells (Figure 3-3B).

**Figure 3-4. Knockdown of GS2-Immunocytochemistry**

Confocal immunofluorescence images showing glial expression of GS2 at DLM neuromuscular synapses and a marked reduced in GS2 after knockdown. WT - wild-type. dEAAT1, GS2- pan-glial markers, HRP-neuronal membrane marker.

Together, these experiments support the effectiveness of the RNAi construct at reducing GS2 expression. Despite the marked reduction in GS2, the flies appeared behaviorally normal and their synapses had normal function with no evidence of excitotoxicity. The phenotype of the GS2 RNAi flies was markedly different from the very dysfunctional dEAAT1 knockdown flies, which were unable to stand and experienced seizures. Although the GS2 knockdown flies did not display a phenotype, the
GS2 RNAi transgenic lines we generated were of particular use in later work to differentiate perisynaptic glial cells from other glial cell types.
Chapter 4

Characterization of Perisynaptic Glia

Previous work has established the presence of tripartite synapses, within *Drosophila*, even within the peripheral nervous system (Danjo, Kawasaki, & Ordway, 2011), Figure 4.1. The genetic tools available within the *Drosophila* system make the further development of this tripartite synapse model important; it is the only invertebrate system to possess these glial-synaptic relationships. Previously, there was no information about the glial cells which send processes to form tripartite synapses. Our work has further defined the cellular basis of tripartite synapses in *Drosophila* and pursued further analysis of the participating glial cell type.

**Figure 4-1. Presence of Tripartite Synapses in Drosophila**

(A-C) Confocal immunofluorescence images of DLM neuromuscular synapses. (A) Anti-HRP labels the neuronal plasma membrane and anti-BRP labels presynaptic active zones. (B) Anti-dEAAT1 labels glial processes and (C) reveals their close association with axons and synapses. The postsynaptic muscle membrane is not labeled and appears dark. All images are maximum projections of three consecutive optical z-sections. (D) Ultrastructure of glia-synapse interactions at DLM neuromuscular synapses.
No specific markers have been identified for glial cells that form tripartite synapses in *Drosophila*. Antibodies against GS2 or dEAAT1 do not distinguish between ensheathing glia and the glial processes that extend into the synapses. GAL4 drivers can distinguish between different types of glial cells, but there is no driver that expresses exclusively in these tripartite glial extensions. Repo-GAL4 is a pan-glial driver expressed in all glia and dEAAT1-GAL4 drives expression in most glial cells. However, moody-GAL4 is a driver selective for ensheathing glia, but not for the glial cells extending within the synapses (Figure 4.2).

**Figure 4-2. Moody-GAL4 Drives Expression in Ensheathing Glia**

![moody-GAL4 UAS-mCD8-GFP](moody-GAL4: Schwabe, Bainton, Fetter, Heberlein and Gaul (2005) Cell)

Anti-HRP shows the neuronal membrane. Anti-dEAAT1 shows the presence of glial cells. The Moody-GAL4 driver shows expression of GFP in only the ensheathing glia.

Availability of the GS2 RNAi lines we had generated, as well as the Moody-GAL4 driver which does not express in glial processes at synapses, provided a possible strategy for specific labeling of glia participating in tripartite synapses. In combination, these tools were used to achieve specific knock down of the pan-glial marker GS2 within ensheathing glia. This resulted in the signal from the ensheathing glia becoming negligible, and revealed a GS2 signal within a newly defined glial cell type participating in tripartite synapses (perisynaptic glia) (Figure 4.3).
Figure 4-3. Expression of GS2 RNAi

Specific labeling of perisynaptic glia by GS2 KD in *moody*-positive glia

*WT*  *moody-GAL4 UAS-GS2-RNAi*

*Adult DLM immunocytochemistry preparation. Anti-GS2 specifically labels a perisynaptic glial cell following GS2 RNAi KD using the moody-GAL4 driver.*

Perisynaptic glial cells were associated with neuronal axons only at synapses (Figure 4-3H, arrow heads), avoiding the ensheathed processes and areas without active zones (Figure 4-3H, arrow). These findings demonstrated that the glial cells extending processes to synapses were distinct from those ensheathing other regions of the axon. Comparison of these observations of neuromuscular synapses in the adult flight motor with those of the larval body wall (a widely used experimental preparation) revealed striking differences. The UAS-GS2 RNAi transgene was also expressed at larval neuromuscular synapses using the moody-GAL4 driver (Figure 4.4).
Figure 4-4. Expression of GS2 RNAi in the Larval Synapse

Immunoctyochemistry with a larval synapse. Anti-GS2 is a pan glial marker, anti-SYT is against synaptotagmin, a synaptic vesicle protein associated with the active zone, and HRP is a neuronal membrane marker.

Notably, the pattern of GS2 expression within the larval synapses is remarkably different from the adult synapses. First, no glial processes are observed in proximity to synapses. Second, the knockdown eliminates GS2 in all the observed glial cells, indicating that no perisynaptic glia are present at this synapse. These findings reveal different mechanisms for glutamate regulation at larval neuromuscular synapses and our additional work (not shown) indicates that glutamate transporters at larval synapses are located in the muscle rather than glia. This marked difference between larval and adult synapses reinforces the importance of the tripartite synapse model in the adult fly as a genetic model for glia-synapse interactions.
Chapter 5

Conclusions and Ongoing Work

The work described in this thesis involved genetic analysis of two key glial proteins involved in glutamate regulation, the dEAAT1 glutamate transporter and GS2. Studies of dEAAT1 included a forward genetic screen, which recovered a suppressor of glutamate excitotoxicity. Suppressor 7-72 is located on the second chromosome and was shown to suppress the excitotoxic phenotype without increasing dEAAT1 levels. Analysis of GS2 involved the first knockdown of this enzyme in Drosophila. Knockdown of GS2 demonstrated no phenotypic or synaptic abnormalities, despite a marked reduction in the protein level. However, the RNAi lines proved vital for characterizing perisynaptic glial cells involved in tripartite synapses. Knockdown of the RNAi in only the sheath cells with the moody-GAL4 driver allowed for specific labeling of perisynaptic glia and represents an important experimental tool for their further analysis.

dEAAT1 Genetic Screen

The strong dEAAT1 knockdown phenotype clearly demonstrates the necessity of glial glutamate transport from the extracellular space in limiting excitotoxicity. New mutations that suppress this effect are of importance for many diseases. In particular, amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in humans. The neurodegeneration caused by this disease has been linked to abnormal expression of glutamate transporters that results in increased extracellular glutamate concentrations (Sheldon & Robinson, 2007). Finding suppressors of the phenotype
through novel mechanisms could prove important for devising therapies, as extensive
efforts to develop glutamate receptor antagonists based drugs have proved unsuccessful
(Sheldon & Robinson, 2007). The question of whether glutamate excitotoxicity is a cause
for disease or an effect of pathology remains to be established for many disorders.
Different studies have suggested that changes in glutamate transporter expression may be
either a cause or consequence of neurodegeneration. The role of glutamate transporters in
the appearance of pathology will remain an interesting topic of research until present
conflicting results are resolved. Interestingly, platelets of ALS patients had normal
 glutamate transporter levels but increased levels of GS. These results suggest a role for
GS2 in excitotoxicity despite the inconsistency of studies concerning the necessity of this
enzyme (Bos et al., 2006).

**GS2 Knockdown**

The lack of a noticeable phenotype due to knockdown of GS2 provides useful
information from the first study of this kind in an invertebrate genetic model system. It
may be that glutamate-glutamine cycling is not necessary for neural function. This would
support the finding that pharmacological inactivation of GS in rat hippocampal slices did
not interfere with synaptic transmission (Kam and Nicoll, 2007). However, these results
are in contrast to the finding that GS knockout within astrocytes causes premature death
due to starvation in mice (He et al., 2010). The latter work did not find any difference in
 glutamate levels required for synaptic transmission, but instead a decrease in glutamine.
Knockdown of GS2 does not seem to cause rising glutamate levels in the extracellular
space. The health of the GS2 knockdown flies might also result from a significantly
reduced amount of GS2 that can still function adequately to recycle the glutamate
required for synaptic transmission. Our studies of GS2 RNAi knockdown define the effect of GS2 reduction in the *Drosophila* system for the first time, but do not explicitly address the necessity of glutamine cycling for synaptic transmission.

Another possible contributor to glutamate-induced toxicity is gliotransmission. Astrocytes can release neurotransmitters such as glutamate in response to increased intracellular calcium. Overexcited astrocytes could release higher levels of glutamate which could also contribute to neurodegeneration. Epileptic tissue has been demonstrated to have enhanced calcium excitability, suggesting contribution from glutamate gliotransmission (Halassa, Fellin & Haydon 2007). Defining the relative contributions of glutamate transporter inhibition, glial overexcitability, and glutamate metabolism toward excitotoxicity is an important focus for future research that will be crucial for the development of therapies.

**Perisynaptic Glial Involvement in Tripartite Synapses**

The presence of tripartite synapses has only recently been established within *Drosophila* system (Danjo, Kawasaki, & Ordway, 2011). In order to better define and study glia forming tripartite synapses, a method of distinguishing them from other glia is required. Knockdown of the pan-glial marker, GS2, driven by a GAL4 driver specific for ensheathing glia, moody-GAL4, allowed for immunocytochemical analysis to reveal a new type of glial cell. These perisynaptic glia function to remove and metabolize the glutamate from the extracellular space around peripheral synapses. Previous work has demonstrated the presence of astrocyte-like glia within the CNS but not the PNS. The development of this tripartite model allows for genetic and molecular analysis of these cells and their functions in synaptic transmission. The ability to differentiate between
ensheathing and perisynaptic glial cells is an important step towards defining their different roles in the nervous system.

**Ongoing Work**

Our work on glial cell involvement in excitotoxicity has led to further studies that address the relationship of neurodegeneration caused by excitotoxicity with that resulting from environmental stress in the form of heat shock. The heat shock response is highly conserved and can occur in response to heat, oxidative stress, heavy metals, toxins, and bacterial infections (Akerfelt et al., 2010). In *Drosophila*, a series of 3 heat shocks inhibits flight ability and causes degeneration of the flight muscles and associated neurons. On this basis, a genetic screen was initiated and has thus far identified a new mutant (Hsm26) that retains its ability to fly following heat shocks and is resistant to stress-induced neurodegeneration. The molecular mapping of this mutation is ongoing and may lead to identification of novel gene products implicated in the heat shock response and its role in protection from neurodegenerative disease. In addition to a forward genetic approach, we are studying the involvement of molecular chaperones induced during the heat shock response to environmental stress. These broader studies of neurodegenerative mechanisms resulting from either glutamate excitotoxicity or environmental stress may reveal common mechanisms of importance to neurodegenerative disease and remain an important focus of our research.
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Professional Experience
• Undergraduate Research, Penn State Neuroscience Lab January 2010-present
  Conducting undergraduate thesis research in the laboratory of Dr. Richard Ordway. Planned and executed a genetic screen employing classical Drosophila genetics and RNAi to identify and map mutations which can suppress neural excitotoxicity and neurodegeneration. Planned and executed the generation of related molecular constructs and transgenic Drosophila lines using a range of molecular biology methods and tested the expression using western blotting.

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  Taught 24 student lab component of Honors Cell Biology Course. Helped troubleshoot and design experiments. Responsible for grading and designing lab lecture material.
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**Presentations**


**Undergraduate Involvement**

• Actor, Penn State’s No Refund Theatre, 2009-2013
• No Refund Theatre Secretary, 2012-2013
• Penn State Dance MaraTHON Rules and Regulations Committee, 2010-2012
• Schreyer Honors College Scholar Advancement Team, 2011-2012