# MOLECULAR REGULATION OF SYNAPTOGENESIS IN *DROSOPHILA*

by

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A DISSERTATION

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Title: Molecular Regulation of Synaptogenesis in *Drosophila*

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DISSERTATION ABSTRACT

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Dynamic regulation of the actin cytoskeleton is required for synapses to form and maintain their shape. The actin cytoskeleton is regulated by Rho GTPases in response to genetic and extracellular signals. Rho GTPases are regulated by guanine nucleotide exchange factors and GTPase activating proteins (GAPs). Syd-1 is a protein that has been identified as necessary for synapse formation in worms, with similar proteins in flies, and mice. Little is known about the molecular mechanism by which Syd-1 is acting. While genetic techniques are great tools for examining synapse development, they are limited by their inability to consider the molecular nature of the protein product. By studying the biochemical nature of synaptic proteins, we can begin to understand their function with a new level of clarity. Syd-1 has a predicted Rho GAP domain; however it is thought to be inactive. The activity of the fly protein, Dsyd-1, has never been examined although it has been speculated that it is inactive in all invertebrates. Recently the mouse version was reported to have Rho GAP activity. By performing GTPase activity assays on purified proteins, I found the GAP domain of Dsyd-1 increased the GTPase activity of Rac-1 and Cdc42 but not RhoA. Members of our lab found the activity of Dsyd-1 is necessary for proper synapse formation both at the *Drosophila* neuromuscular junction as well as in R7 neurons. In *Caenorhabditis elegans*, Syd-1 was found to interact with

presynaptic protein RSY-1. Since RSY-1 is evolutionarily conserved, I tested whether or not RSY-1 has a similar effect on R neurons in *Drosophila*. I also isolated mRNA from R neurons and evaluated the possibility of analyzing mutant neurons using comparative transcriptomics.

This dissertation includes previously unpublished coauthored material.

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# CHAPTER I

## INTRODUCTION - PREFACE

A majority of human diseases can ultimately be described as variations in behavior (Marteau and Hollands, 2012). In the study of neuroscience, the ultimate goal is to gain insight into the molecular, cellular, and systemic, mechanisms underlying behavioral phenomenon (Ryan and Grant, 2009). To understand an organism's behavior, we must examine its nervous system (Chiel and Beer, 1997). The human nervous system consists of some 100 billion neurons (van Spronsen and Hoogenraad, 2010). Connecting these neurons to muscles and each other are trillions of specialized cell junctions known as synapses (Wu, Xiong, and Mei, 2010; Scheiffele, 2003). Synapses typically connect presynaptic axons, when transmit information to post synaptic receptors via small molecule neurotransmitters (Rolls, 2007). Axons and dendrites both extend out from the cell body of the neuron but form morphologically distinct structures expressing different sets of proteins (Shen and Cowan, 2010). When neurons "fire", ion influx into the pre-synaptic cell triggers vesicles containing neurotransmitters to fuse with the membrane and release neurotransmitter into the extracellular space known as the synaptic cleft (Sollner and Rothman, 1994). Neurotransmitters bind to receptors on the postsynaptic cell which then initiate ionotropic and metabotropic responses that can stop, modify, or propagate the signal (Rosenmund and Rettig, 2003). In order for neural networks to function correctly, synapse formation must be precisely regulated such that individual circuits are isolated from one another and not short circuited, or cross wired (Cohen-Cory, 2002). Furthermore, each synapse must express the proper cell adhesion molecules

(Dalva, McClelland, and Kayser 2007) ion channels (Rosenmund and Rettig, 2003), cytoskeleton regulators (Dillon and Goda, 2005), and produce a pool of ready to release neurotransmitter containing vesicles (Ziv and Garner, 2004).

While ultimately variation in behavior is a result of variation in neural connectivity, the complex nature of these connections requires a more in depth examination of synaptic structures (van Spronsen and Hoogenraad, 2010). Before we delve into questions about connectivity of neural networks, we must first understand the genetic and biochemical processes underlying synapse formation.

***Axon Growth and Guidance***

After adopting the neuronal fate, many neurons extend out axons from the cell body with bulbous extensions known as growth cones or butons located at the tip of the extension (Sanchez-Soriano and Goncalves-Pimentel, 2009). Growth cones are dynamic structures whose motility allows axons to travel great distances (Shen and Cowan 2010). During neuronal development, guidance cues in the form of secreted molecules allow growth cones to navigate to stereotyped target regions with the goal of making and maintaining synaptic connections (Shen and Cowan 2010). Guidance cues are often ligand receptor interactions acting through signaling cascades which mediate morphological changes in growth cone structure underlying its motile nature (Ziv and Garner, 2004; Dillon and Goda, 2005). Growth cone motility is maintained by retracting and extending filopodia, thin finger-like protrusions in the membrane at the very tip of the axon periphery (Goldberg and Burmeister, 1986). The mechanical force required for axon outgrowth is provided by differential adhesion of filopodia (Letourneau, 1975). Guidance cues are received by the growth cone which is expressing membrane bound

receptors for which the guidance cues are ligands (Shen and Cowan, 2010). It is thought that filopdia are able to scan the extracellular environment for guidance cues and that this increases the likelihood of the axon contacting the proper dendrite with which to synapse (O'Donnell, Chance, and Bashaw, 2009) Upon detection of an attractive cue, filopodial extensions are stabilized, alternatively, filopodia coming into contact with a repulsive cue retract (Fan et al., 1993). Through dynamic regulation of the growth cone cytoskeleton, extracellular guidance cues are translated into motile behavior. (Dent and Gertler, 2003) In addition to affecting the cytoskeleton, guidance cues can affect the activity of transcription factors which can then alter gene expression (Colon-Ramos, 2009). Once the growth cone initiates contacts with its target, the process of synaptogenesis is only beginning. The contact must first stabilize, then presynaptic components must accumulate and assemble before neurotransmission can be accomplished (Cohen-Cory, 2002; Shen and Cowan, 2010).

***Synaptogenesis***

In order for synapse formation to proceed, the dynamic growth cone must transform into a stable synapse (Li and Sheng, 2003). During axon guidance the goal is to translate guidance cues into cytoskeletal dynamics; during synapse formation, it is to recruit and organize synaptic components. Synapses are highly specialized asymmetric cell-cell junctions (Sollner and Rothman, 1994; Scheiffele, 2003; Wu, Xiong, and Mei, 2010). Presynaptic boutons of axons are filled with synaptic vesicles filled with neurotransmitter while the postsynaptic membrane is densely clustered with neurotransmitter receptors, referred to as the post synaptic density (PSD) (Rosenmund,

Rettig, and Brose, 2003; Wu, Xiong, and Mei, 2010). Electrical signals from the presynaptic cell are relayed as chemical signals to the postsynaptic cell in the form of neurotransmitter release (Ziv and Garner, 2009). Specifically, the influx of ions into the presynapse triggers synaptic vesicles to fuse with the plasma membrane, releasing neurotransmitter (Rosenmund, Rettig, and Brose, 2003). Synaptic vesicle fusion to the presynaptic membrane is governed by a complex protein network called the Active Zone (AZ) (Rosenmund, Rettig, and Brose, 2003). Proteins residing in the active zone include calcium channels as well as scaffolding proteins BRP/ELKS, and vescicle fusion machinery such as SNARE proteins, as well as many others. There are two distinct steps during synaptogenesis: first, synapses must choose targets with which to connect and second, the pre and post synapse must accumulate the proper components and assemble them correctly. During synapse development, AZ proteins and synaptic vesicles accumulate in the presynaptic buton while simultaneously transmembrane bound receptors and ion channels gather at the PSD (Sieburth, Ch'ng, and Tavazoie, 2005). In order for efficient neurotransmission to occur, the proteins of the AZ and PSD must align precisely (Schoch and Gundelfinger, 2006). To accomplish this, the development of the PSD and the AZ must be coordinate in both space and time (Ziv and Garner 2009). This coordination is organized by trans synaptic signaling between pre and post synapse (Shen and Cowan, 2010; Dalva, McClelland, and Kayser, 2007).

The process of synapse assembly can occur remarkably quickly, initiating only an hour after initial axo-dendritic contact (Rosenmund, Rettig, and Brose, 2003). It is believed that the presynaptic AZ assembles in a stepwise process, where AZ proteins are recruited to the AZ by other AZ proteins (Scheiffele, 2003) Understanding the structure

of the AZ protein network is essential for synaptic function as fluctuations in the composition of the AZ protein complex can result in nonfunctional or dysfunctional synapses (Schoch and Gundelfinger, 2006).

### *The Role of the Cytoskeleton in Neural Development*

The transduction of extracellular signals into changes in cell morphology, motility, and protein expression essential for synaptogenesis is a miracle of molecular coordination. Perhaps unsurprisingly, actin dynamics are required for almost every step during neural development. The cytoskeleton is the ultimate target of most of the signaling induced by axon guidance receptors (Bashaw and Klein, 2010). During the migration of neurons, polymerizing actin at the leading edge of the growth cone cell and depolymerizing at the other side allows the cell to navigate with surprising precision (Mogilner and Keren, 2009). Similarly, rearrangements in the F-actin network are the underlying force behind growth cone dynamics (Dent and Gertler, 2003). Filopodia are dynamic actin allows the growth cone to scan the environment for attractive or repulsive cues as well as respond to them (Geraldo and Gordon-Weeks, 2009). Filopodia formation is the result of changes in the actin cytoskeleton (Faix, Stradal, and Rottner, 2009). The turning of a growth cone in response to a repulsive or attractive cue is a result of cytoskeletal remodeling (Tojima et al., 2007).When axons are extending, actin is polymerized in areas of the growth cone nearest the attractive guidance molecules, and this differential stabilization of actin is most likely what allows growth cones to navigate their environment (Dickson, 2002; Dent and Gertler, 2003; Tojima et al., 2007). While extensive research has been conducted on the involvement of regulation of the F-actin

network in various neural processes, relatively little is known about the role of F-actin in presynaptic assembly.

F-actin serves in part as a scaffold for protein protein interactions at the presynaptic terminal (Sankaranarayanan et al., 2003). Research demonstrates that an elaborate actin network organizes the presynaptic bouton, tethers synaptic vesicles, and modulates neurotransmitter release (Dillon and Goda, 2005). Experimental inhibition of actin polymerization led to the mis-localization of presynaptic proteins, suggesting that the F-actin network is responsible for their organization (Zhang and Benson, 2001). Accordingly, a number of presynaptic proteins possess F-actin binding domains (Dillon and Goda, 2005) including N-Cadherin (Dalva, McClelland, and Kayser, 2007). Transmembrane receptor activation leads to regulation of cytoskeletal dynamics which results in axon repulsion or attraction (Dickson, 2002; Dent and Gertler, 2003; Dalva, McClelland, and Kayser, 2007). During the formation of a synapse, the interaction of trans-synaptic adhesion proteins results in changes in the cytoskeleton which effect synaptic buton growth and also localization and organization of pre- and postsynaptic protein components (Shen and Cowan, 2010). Though understanding the regulation of the cytoskeleton is essential in understanding presynaptic assembly, little is known about how it is affected by extracellular guidance cues. As has been implied by many studies to date, it is likely that changes in the cytoskeleton are regulated by the activity of upstream Rho GTPases (Dent and Gertler 2003).

***Rho GTPase Family Proteins***

Connections between membrane bound receptors and Rho GTPases as well as between Rho GTPases and the actin cytoskeleton have been topics of recent discovery, and mutations in these signaling pathways have been implicated in human neurological diseases, highlighting their importance in neural development (Ramakers, 2002; Faucherre and Desbois, 2003) Rho GTPases are a family of structurally related proteins which bind GTP and hydrolyze it yielding GDP and phosphate. (Luo, 2000) Rho GTPases (Rho GTPases) regulate actin cytoskeleton organization and are essential for proper nervous system development and function (Garcia-Matas et al. 2006; Brouns et al., 2001; Govek, Newey, and Van Aelst, 2005; Wong et al., 2001; Rosso et al 2005; Ng and Luo, 2004). GTPases are often referred to as "molecular switches" that transduce signals from both inter and extracellular stimuli to the actin cytoskeleton and the nucleus (Ng and Luo, 2004; Ridley and Self, 2003). When GTP bound, Rho GTPases, acting through effector proteins, induce F-actin nucleation, tubulin polymerization and actin contractility (Kimura et al., 1996; Rohatgi et al., 1999). Upon hydrolysis of GTP, conformational changes regulate the GTPase's interaction with downstream effectors (Scheffzek and Ahmadian, 2005). Typically they turn on or off as a result of ligand receptor interactions at the plasma membrane (Schiller, 2006). Rho GTPases regulate cytoskeletal dynamics downstream of numerous axon guidance receptors and cell adhesion molecules, functioning to integrate signals from different axon guidance pathways (Govek et al., 2005). In addition, RhoGTPases regulate cytoskeletal dynamics downstream of a number of axon guidance receptors and also adhesion molecules. (Hall and Lalli 2010). Rho GTPases are responsible for mediating cytoskeletal rearrangements

during all stages of neuron development involving morphological changes (Govek et al., 2005). Interestingly, the addition of Rac1 is sufficient to induce membrane ruffling when injected into cells in culture.

Much attention has been given to three distinct Rho GTPases which have been well conserved throughout evolution: Rac1, RhoA, and Cdc42. N-WASP activates Arp 2/3 downstream of Rac1 and Cdc42, this leads to the nucleation of new actin filaments at the leading edge of the cell leading; this leads to the membrane ruffling and filopodial extensions that are characteristic of neurons seeking to form synapses (Aspenström, Pontus 1996; Rohatgi, 1999). Since the intrinsic rate of GTP hydrolysis is slow, GTPases are essentially stuck in on or off states. As a result, many protein effectors bind to them to accelerate their GTPase activity (Scheffzek and Ahmadian, 2005). The nature of this regulation is of great interest to cell biologists as well as those who benefit from their research.

***Regulation of Rho GTPase Activity***

Precise spatial and temporal regulation of Rho family GTPase activity is required for neurons to develop and function (Tolias and Duman, 2011). How Rho GTPases interact with axon guidance receptors is the subject of current research (Schiller, 2006) For the most part, the activity of Rho GTPases are modulated by their interactions with guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) which allow GTPases to cycle between active and inactive states (Gamblin and Smerdon, 1998).

GAPS bind to the GTP bound form of the GTPase and stabilize the transition state such that GTPase hydrolysis can occur (Scheffzek and Ahmadian, 2005). Since GTPase tend to have high affinity for GDP, the interaction with GEFs allows for GDP to be released and a news GTP molecule to be bound (Fig. 1; Tolias and Duman, 2011). An arginine residue has been identified in RhoGAPs which is thought critical for their ability to increase the rate of GTP hydrolysis (Graham, Eccleston, and Lowe, 1999). The presence of the conserved arginine in the Switch II region of the protein is not sufficient to confer GAP activity as this is the case with the p85 subunit of PIP3 Kinase which binds both Rac-1 and Cdc42 yet does not increase GTPase activity and instead acts to maintain the GTP bound state (Scheffzek and Ahmadian, 2005). In addition, an asparagine residue is thought to participate in the stabilization of the binding interaction (Rittinger et al., 1997). Interestingly, these residues are not required for the activity associated with RanGTPases (Scheffzek and Ahmadian, 2005). It is interesting to note that GEFs and GAPs can interact directly with both membrane bound receptors and GTPases, putting them in a prime position to relay such signals (Govek et al.,2004; Pinto et al., 2010). There's more to GEFs and GAPs than simply activating and inactivating the target GTPases. Multiple protein interaction domains significantly complicate the issue of what these molecules are doing.

Rho GEFs and GAPs are numerous and diverse. The human genome contains 61 Rho GEFs and 68 Rho GAPs yet only 22 Rho GTPases (Tcherkezian and Lamarche-Vane, 2007). The excess of GAPs relative to GTPases demonstrates the complex nature of RhoGTPases regulation. The large number of GAPs allows for Rho GTPase signaling to be linked to specific signals transduced by specific upstream receptors. As a result,

GAPs can be localized to specific transmembrane receptors which would lead to local regulation of GTPase activity in response an extracellular signal and thus location specific changes in motility and structure. Thus the proper molecular code must be present in both pre and post synaptic membrane for GTPase mediated modification of the cytoskeleton. In addition to regulation by GAPS and GEFs, molecules known as guanine nucleotide disassociation inhibitors (GDIs) can lock GTPases in a GDP bound state (Bernards and Settleman, 2004).

Much attention has been given to regulation of Rho GTPases by GEFs, less is known about the GAPs which particicpate in these regulatory signaling events. Most GAPs are known to have membrane binding domains, many specific to certain phosphoinostitides (Moskwa, Paclet, et al., 2005; Karimzadeh and Primeau, 2012; Bernards and Settleman, 2004). A number of GAPS have been reported to exist naturally in an auto-inhibited conformation, which in some cases can be relieved by membrane binding or prenylation (Moskwa, Paclet, et al., 2005).

***Rho GTPase Regulation in Neuronal Development***

RhoGTPases are regulatory hubs of cytoskeletal dynamics which coordinate cytoskeletal dynamics of most eukaryotic cells including neurons (Luo, 2002; Garcia-Mata, 2006; Tolias and Duman, 2011). Rho GTPases are known to play roles in axon growth and guidance, dendrite elaboration, synapse formation, and synaptic plasticity (Hall and Lalli, 2010). The precise regulation of GTPase activity in space and time is required for a cell to develop, mature, and maintain homeostasis (Bernards, 2004). Due to their central role in the regulation of cell morphology, mis-regulation of RhoGTPases and their regulators by mutation, infection, or environmental cues can result in a number

of different neurological diseases (Rathinam and Berrier,2011) (Miyake et al., 2008, Ramakers, 2002). Since many GTPases are used at multiple steps during development, examining their role in synaptogenesis is difficult because removing them too early results in axons and dendrites not making it close enough to begin forming synapses. Also, it is thought that many GTPases have overlapping functions such that in the event that one is removed, redundant GTPases allow for cellular processes to proceed uninterrupted.

Removing Ced-10, a Rac-1 homolog from *C. elegans* prevents proper clustering of pre-synaptic vesicles (Stavoe and 2012). Also, knock out of p190RhoGAP, a regulator of RhoA results in axon guidance defects (Brouns et al., 2001). In addition, the binding of Sema3A to its receptor Plexin-A/Npn-1 results in a repulsive cue regulated by activation of Rac1 (Jin and Strittmatter, 1997).

Investigating the role of GEFs and GAPs during neuronal development will allow us greater understanding of diseases caused by their malfunction. Gene expression profiling studies of metastatic brain tumor tissue have lead to the observation that misregulation of ARHGAPs could be an underlying cause of tumor formation, although more research is require to distinguish cause from symptom (Zohrabian and Nandu 2007). ORCL, which contains an inactive GAP domain, has been found to be mutated in Lowe's Syndrome, a rare form of mental retardation. (Faucherre and Desbois, 2003) In addition, LAR a membrane bound receptor which interacts with liprin-α, and signals via Rac GTPase has been found to be up regulated in breast cancers and carcinomas (Chagnon and Uetani, 2004). Mutations have been identified in a number of Rho GTPase

regulating proteins in the drosophila synapse which result in metal retardation (Raymakers, 2002) (Kasri and Van Aelst, 2008).

Further studies have implicated a Rho GEF dubbed Vav, which also plays a role in synapse formation (Kiraly and Eipper-Mains, 2010). In mammals, when ephrins bind their Eph receptors, Vav becomes transiently activated when phosphorylated and locally promotes Rac-dependent endocytosis of the ephrin/Eph complex, a key event in axonal repulsion (Kiraly and Eipper-Mains, 2010). Since removing all three Racs from the R neuron has a worse phenotype than vav knockout, more GEFS and GAPS must be regulating these GTPases. Both in axon guidance and synapse formation, GAPs and GEFs are essential players regulating changes in the cytoskeleton downstream of Eph- and BMP-receptors (Ball et al. 2003).

***Drosophila: A Model for the Study of Synaptogenesis***

*Drosophila* is an excellent model in which to further study molecular function at the synapse because of the ability to precisely control when and where proteins can be expressed. The availability of well studied genetic tools for drosophila researchers allows for ease of and predictability of manipulation. Many members of the presynaptic active zone protein complex are evolutionary conserved in the three well studied model organisms: worm, *fly,* and mouse. 120 conserved presynaptic proteins in vertebrates and insects (Ryan and Grant, 2009).

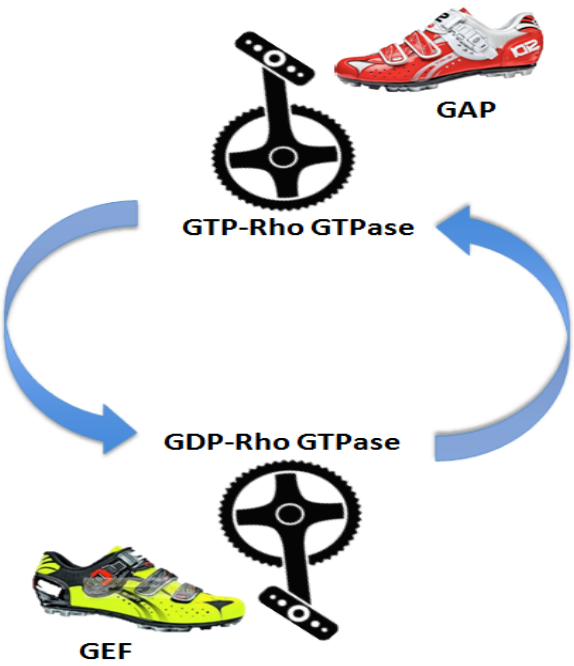


Figure 1: GTPases Cycle through GTP and GDP bound states with the help of GEFs and GAPs.

The relative simplicity of drosophila R neurons makes them excellent models for the study of synapse formation. The visual system is an especially attractive model because eye defects are not lethal (Sanchez-Soriano et al., 2009). The development of each of the ~750 photoreceptor R neurons results in a single terminal synapse which under normal conditions is organized into columns (Rolls et al., 2007). The reproducibility of this organization allows for a convenient way to score defects in synapse formation. Retinotopic map formation leads the neurons to form stereotypical synapse targets which are easily visualized microscopically with fluorescent antibodies or genetically encoded florescent proteins (Ting et al., 2007). The entire eye forms from sensory organ precursor cells, some of which then adopt neuronal fate decisions which lead to inter and intra cellular signaling mechanisms that allow the developing neurons to extend in the proper direction, and express the proper genes to form the proper synapses (Rolls et al.,2007). Unique to *Drosophila*, electron dense regions of active zones known as T bars are the sites of vesicle fusion and neurotransmitter release (Schoch and Gundelfinger, 2006). Easy to access, dissect, and visualize, the neurons of the NMJ are an excellent model in which to study synapse formation. The MARCM technique allows the late removal of genes from specific cell populations using UAS Gal4 and GAL80 along with FRT/FLP recombinase which ensures that within each animal are homozygous mutant and heterozygous wild type cells within the same population (Lee and Luo, 1999).

In *Drosophila* expressing constitutively active or dominant negative Rac1 or Cdc42 in neurons have distinct phenotypes in motor axon guidance, suggesting that Rac1

and Cdc42 are involved in mediating guidance cues (Luo et al., 1994). According to a 2010 review of GEFs in neuronal development: "It is clear that many signaling and additional regulatory components await discovery, and molecular and genetic approaches, including sensitized genetic screens in Drosophila and C. elegans, will continue to identify these missing components" (Kiraly and Eipper-Mains, 2010). The Drosophila genome contains relatively few rho GTPAses: drac1,drac2, mtl dcdc42,rhoA , however they are still far outnumbered by the 22 rhoGEFs and 24 rho GAPs which regulate their activity. Furthermore, at least nine of them are known to be expressed in the CNS (Johndrow and Magie, 2004).

The motor neurons of the drosophila neuro-muscular junction (NMJ) form large butons on skeletal muscle fibers that allow for in depth analysis of synaptic substructures (Wu and Xiong, 2010). Trio, a dual Rho/Rac GEF has recently been implicated in NMJ synapse formation, however the details of what Trio's role in synapse formation remains unclear (Debant, A., Serra-Pagès, 1996; Awasaki et al. 2000; White, Ball, et al., 2001). Over expressing Trio rescues the LAR loss phenotype (Hofmeyer K, Maurel-Zaffran, 2006). The role of Trio seems evolutionarily conserved as knock-out mice also demonstrate axon guidance defects (Briançon-Marjollet et al., 2008) Trio has also been found to signal through Netrins (Briançon-Marjollet et al., 2008). Both Trio and another GEF, DOCK180 are able to signal downstream of transmembrane receptor DCC however it is unknown whether they act redundantly or in parallel (DeGeer, Boudeau, and Schmidt). Trio is also known to signal downstream of liprin-α/LAR in drosophila neurons. While many potential regulators have been identified through bioinformatic efforts, these predictions must be verified using genetic and biochemical approaches.

### *Syd-1 Is Required for Synaptogenesis*

The protein synapse defective-1, Syd-1, has emerged recently as a key factor in synapse development (Hallam et al., 2002; Wentzel and Sommer, et al., 2013). First identified in a screen for synapse formation components in *C. elegans*, Syd-1 is the only AZ protein in *C. elegans* that is essential for the localization of synaptic vesicles *in vivo* (Dai et al., 2006; Patel et al., 2006, Hallam et al., 2002). Experiments in worm have led to a model where two presynaptic proteins, synapse defective-1 (Syd-1) and synapse defective-2 (Syd-2) are master regulators of synapse development directly downstream of trans-membrane adhesion proteins (Hallam et al., 2002; Patel et al., 2006; Patel and Shen, 2009).Similarly, The Syd-1 homologue in *Drosophila*, Dsyd-1, is essential for the assembly of the AZ at the fly NMJ (Owald et al., 2010). In addition, there exist two orthologs of Syd-1 in mouse, Msyd-1 a and b, which are also necessary for synaptogensis (Wentzel and Sommer, et al., 2013). For the rest of this dissertation, I will refer to the worm protein as Syd-1, the fly protein as Dsyd-1, and the mouse protein as Msyd-1.

Like the worm version, Dsyd-1 contains an N-terminal PDZ domain, followed by a C2 domain, and a proline rich SH3 domain, as well as a potential GAP domain (Hallam et al., 2002). PDZ and SH3 domain interactions are a common mode of protein-protein interaction between many proteins of the AZ. These domains could be involved in interactions with other AZ proteins. C2 domains bind to phospholipids via a calcium sensitive mechanism, which means that Syd-1 could potentially be localized to the membrane in response to calcium ions entering the cell. The presence of the GAP homology domain suggests that Syd-1 could be regulating synaptogenesis by modulating

RHO GTPase activity. It has been proposed that the invertebrate Syd-1 GAP domain is inactive due to lack of conservation of residues thought to be critical for the acceleration of GTP hydrolysis and for efficient binding of the GTPase (Hallam et al., 2002; Graham et al., 1999; Scheffzek and Ahmadian, 2005). Both in *C. elegans* and *Drosophila* Syd-1 directly interacts with the ELKS family protein Bruchpilot (BRP). (Owald et al., 2010; Patel and Shen, 2009). Syd-l co-localizes with Syd-2/ liprin-α at nascent synapses. (Hallam et al., 2002) The loss of *syd-1* mislocalizes synaptic vesicles and AZ proteins to non-synaptic regions (Hallam et al., 2002; Patel et al., 2006). It is thought that Syd-1 localizes presynaptic components by positively regulating Syd-2/ liprin-α (Hallam et al., 2002). Both Syd-l and Syd-2/ liprin-α function in HSN axons to localize cargo to presynaptic sites. (Hallam et al., 2002). Syd-2/ liprin-α is mis-localized in Syd-1 mutants Seemingly, the only role of Syd-1 in this system is to promote the activity of Syd-2/ liprin-α as the phenotype caused by *syd-1* loss can be rescued by gain of function mutant S*yd-2* (Dai et al., 2006); however the Syd-2 mutant does not rescue in an Elks-1 mutant background. Syd-1 directly enhances the ability of Syd-2/ liprin-α to bind the active zone protein ELKS-I. (Dai et al., 2006).

The Rho GAP domain of Syd-1 is atypical in that it is missing a conserved arginine in catalytic core, and demonstrates no obvious Rho family GTPase activity (Hallam et al., 2002) Additionally, a conserved asparagine residue thought to be important for GTPase binding is also mutated. In other GAPs the arginine residue was found to be essential for efficient GTP hydrolysis to GDP (Graham et al., 1999) and the aspargine is thought to be important for the stability of the RhoGTPase-GAP complex (Rittinger et al., 1997). The functional significance of these amino acid changes in Syd-1

have not been tested. Regardless, it has been hypothesized that Syd-1 might function as scaffold or platform for RhoGTPases and effector proteins (Hallam et al., 2002). Though the domain has been identified as having significant homology to RhoGAP domains, it could have evolved to recognize other divergent GTPases such as Ran or Rab, which do not require the conserved arginine for effective catalysis (Scheffzek and Ahmadian, 2005). It is unknown weather this domain is capable of promoting GTP hydrolysis, let alone which GTPases it acts on in vivo. While the GAP activity of *Syd*-1 has never been assayed, it was found that its presence is required for proper synapse formation to occur as worms expressing a mutant Syd-1 with the GAP domain deleted had mislocalized synaptic components. (Hallam et al. 2002)

While this research was being performed, the mouse version of Syd-1, msyd-1a was found to be necessary for synapse formation and to also have an active GAP domain (Wentzel and Sommer, et al., 2013). mSYD-1A was found to have significant GAP activity towards RhoA and this activity was autoinhibited by its N terminus (Wentzel and Sommer, et al., 2013). Additionally it was found that targeting mSYD-1A to the plasma membrane increases its GAP activity (Wentzel and Sommer, et al., 2013). Similar to worm Syd-1, mSYD-1A can interact with liprin-α (Wentzel and Sommer, et al., 2013). Liprin-α is known to interact with the receptor tyrosine phosphatase LAR, and this signaling is known to promote accumulation of presynaptic components (Woo et al., 2009). mSYD-1A may act downstream of LAR signaling to regulate RhoA and thus the cytoskeleton in a way that promotes synapse development.

The *Drosophila* version of SYD-1, Dsyd-1 also plays a major role in synapse development where it plays a role in both pre and post synaptic assembly (Holbrook et al.

2012). Dsyd-1 is required for proper terminal synapse formation both in R7 neurons as well as neurons of the Neuromuscular Junction (NMJ) (Owald et al., 2010; Holbrook et al. 2012). Presynaptic Dsyd-1 is required to properly localize liprin-α to AZ, however, liprin-α is not required for DSyd-1 targeting (Owald et al., 2010; Holbrook et al. 2012). Loss of function in *Dsyd-1* results in fewer synaptic release sites and ectopic AZs (Owald et al., 2010; Holbrook et al. 2012). At the NMJ, *Dsyd-1* mutants have smaller presynaptic terminals and less neurotransmitter release sites (Owald et al., 2010). Like Syd-1, Dsyd-1 has also been reported to biochemically interact with BRP. The AZs that do form in Dsyd-1 mutants were structurally abnormal and had abnormal accumulation of BRP (Owald et al., 2010). Its removal has two distinct phenotypes in R7 neurons (Holbrook et al. 2012). A percentage of axons in dsyd-1 -/- mutants fail to reach their target layer, stopping short by hundred of microns (Holbrook et al. 2012). Other axons make it to the target layer but then continue to grow, occasionally branching or forming ectopic synapses with neighboring neurons (Holbrook et al. 2012). In vivo imaging has confirmed that Dsyd-1 arrives early at nascent synapses, before liprin-α and BRP (Owald et al., 2010). This indicates an assembly process where Dsyd-1 is necessary for liprin-α and BRP to localize to the AZ. Presynaptic Dsyd-1 is required to properly localize liprin-α to AZ, however, liprin-α is not required for Dsyd-1 targeting (Owald et al., 2010; Holbrook et al. 2012).

The failure to contact M6 layer phenotype, but not the overextension phenotype caused by loss of Dsyd-1 in R7 neurons can be can be rescued by over expressing liprin-α. Interestingly, overexpression of the Rho/Rac GEF Trio partially rescues both defects, suggesting that both Dsyd-1 and Trio are acting in the same pathway, perhaps to promote

Rac-1 cycling. Alternatively, the GAP activity of Dsyd-1 could be relatively slow compared to the GEF activity of Dsyd-1 such that they have a similar effect; to promote GTP bound Rac-1. It is important to remember however, that there are a number of Rho GTPases and many more regulators which could also be contributing to these phenotypes.

Although Dsyd-1 does posses the arginine residue thought to be essential for GAP activity, it does not have the asparagine residue thought to stabilize the interaction with the GTPase (Fig. 2; Gamblin, Smerdon, 1998). The results of previous biochemical assays come into question with the recent observation that GAPs can be auto inhibited by their N terminus (Wentzel and Sommer, 2013). Sequence variation in worm and fly Syd-1 proteins led to speculation that their GAP domains might be nonfunctional. Mutations introduced into the msyd-1a GAP domain (R436 and N552) did reduce GAP activity of a minimal GAP domain construct, but did not eliminate it entirely (Wentzel and Sommer, et al., 2013). Thus although invertebrate Syd-1 proteins may have significantly less GAP activity than Msyd-1a, this experiment suggests that they are functional GAPs. When considering the similarities in presynaptic assembly between *C. elegans*, *Drosophila* and Mouse, and the critical role that SYD-1 plays in presynaptic assembly we decided to characterize the GAP activity of Dsyd-1. Additionally, since many GAPs are known to affect multiple GTPases, we further decided to characterize which GTPases it is acting on specifically.

### *Scope of This Thesis*

With this research, we aim to demonstrate that Dsyd-1 possesses the ability to catalyze GTP hydrolysis on Rho family GTPases and that this GTPase regulation is affecting synapse formation. Additionally, further research was performed to characterize the specificity of GTPase catalysis. In the research descried in this dissertation, we identified and characterized molecules involved in regulating synaptogenesis as well as investigated a new technique to accomplish neuron specific transcriptional analysis. In Chapter II I will present a previously unpublished manuscript of our findings of the biochemical activity of Dsyd-1, as well as how it relates to synapse development in *Drosophila* R neurons and NMJ. The manuscript in Chapter II was coauthored by my Advisor, Dr. Tory Herman, Mike Spinner, a fellow graduate student in the lab, and myself. In Chapter III I present my investigation on the role of RSY-1 in synaptogenesis, as well as the relevance of its genetic interaction with DSyd-1. Chapter IV summarizes my efforts to isolate mRNA exclusively from R7 neurons for the purpose of transcriptome analysis.

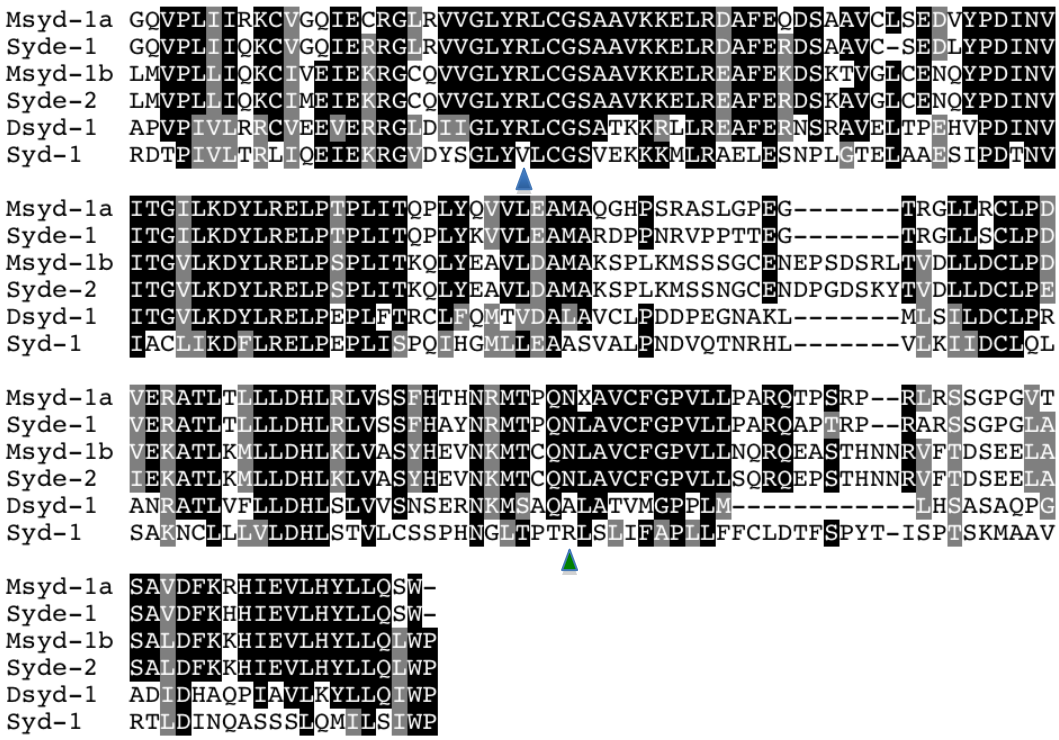


**Figure 2**: Alignment of Syd-1 GAP domain to homologous sequences from fly, mouse, and human. The blue carrot marks the essential arginine residue, the green carrot marks the conserved asperigine.

# CHAPTER II

## *DROSOPHILA* SYD-1 HAS RHOGAP ACTIVITY THAT IS REQUIRED FOR PRESYNAPTIC DEVELOPMENT

This chapter contains material from an article that will be published with coauthors: Tory Herman, Mike Spinner, and David Walla. Dr. Herman contributed by advising and and providing lab space and equipment as well as performing the experiments summarized by the results for Figure 5, Mike Spinner contributed the experiments summarized by the data presented in Figures 2-4, and I contributed by designing the experiments, performing the biochemistry summarized in Figure 1, and in interpreting the data in the discussion.

### Introduction

Neurons are organized into circuits by asymmetric cell-cell junctions known as synapses (Sollner and Rothman, 1994). Each presynaptic cell contains specialized regions of membrane - active zones (AZs) - at which synaptic vesicles can fuse in response to a change in voltage, thereby releasing their neurotransmitter contents. Directly apposed to AZs are neurotransmitter receptors in the postsynaptic cell which translate the chemical signal back into an electrical signal. Synapse development requires cytoskeleton remodeling, adhesion between pre- and post-synaptic cells, and the recruitment of scaffolding proteins which recruit additional synaptic components (Sieburth, Ch'ng, and Tavazoie, 2005). Recent evidence suggests that among the earliest events at developing presynaptic sites is the accumulation of cytoskeletal actin.

Rho GTPases comprise three families of proteins - Rac, Rho, and Cdc42 - that coordinate cytoskeletal dynamics of most eukaryotic cells including neurons (Luo, 2002; Garcia-Mata, 2006; Tolias and Duman, 2011). Rho GTPases regulate axon growth and guidance, and dendrite elaboration, but have more recently been implicated in regulating synapse development (Hall and Lalli, 2010). Like other small GTPases, Rho GTPase are modulated by interactions with guanine nucleotide exchange factors (GEFs) and GTPase

activating proteins (GAPs) which allow them to cycle between active and inactive states (Gamblin and Smerdon, 1998). Disrupting Rho GTPases, their regulators, or their downstream effectors can result in human neurological disease, highlighting their importance in neural development (Ramakers, 2002; Faucherre and Desbois, 2003)

The RhoGAP-like protein Syd-1 has emerged as a key factor in synapse development (Hallam et al., 2002; Owald, 2010; Wentzel et al., 2013). Both in *C. elegans* and *Drosophila*, Syd-1 is required for presynaptic assembly and directly interacts with the AZ ELKS family protein Bruchpilot (BRP; Owald et al., 2010; Patel and Shen, 2009). Loss of *syd-1* from either organism causes mislocalization of AZ proteins and synaptic vesicles to non-synaptic regions (Hallam et al., 2002; Patel et al., 2006). Two orthologs of Syd-1 have recently been described in mouse, and the one tested - mSyd1a - is also required for normal synapse development (Wentzel et al., 2013). While invertebrate and vertebrate Syd-1 proteins contain RhoGAP domains, it has been proposed that only vertebrate Syd-1 has GAP activity (Hallam et al., 2002; Wentzel et al., 2013). In particular, the RhoGAP domain of worm Syd-1 is missing the conserved arginine thought to be essential for catalysis (Graham et al., 1999; Scheffzek and Ahmadian, 2005) and neither binds nor has activity toward multiple Rho GTPases tested (Hallam et al., 2002). And both worm and fly Syd-1 proteins lack a conserved asparagine residue thought to be important for GTPase binding (Rittinger et al., 1997). By contrast, mouse mSyd-1a was recently shown to have GAP activity toward RhoA in vivo (Wentzel et al., 2013). Because mutating mSyd1A to more closely resemble fly Syd-1 eliminated GAP activity toward RhoA, it was proposed that fly Syd-1 itself likely lacks GAP activity (Wentzel et al., 2013). While mouse mSyd1A's Rho GAP activity is not required for its gain-of-function effect on

synapse morphology, its involvement in normal synapse development has not yet been tested (Wentzel et al., 2013).

We set out to test directly whether fly Syd-1 might have RhoGAP activity and, if so, whether that activity is required for synapse development. Here we present our evidence that Syd-1 has GAP activity toward Rac-1 and Cdc42 but not RhoA and that this activity is required for some though not all aspects of Syd-1's role in presynaptic assembly at both neuron-neuron and neuron-muscle synapses.

## Materials and Methods

***Transgenes:*** The predicted RhoGAP domain (amino acids 643 to 1164) of fly Syd-1 isoform C was tagged with a C-terminal HA epitope and fused in-frame to GST in the PGEX 4T1 vector. An identical construct containing the R979A mutation was ordered from Stratagene. Full-length fly Syd-1 (identical to that described in Holbrook et al., 2010) was tagged with three C-terminal FLAG epitopes, placed under control of the UAS promoter, and used to transform *yw* mutant flies by standard methods. An identical construct containing the R979A mutation was also used to transform *yw* mutant flies.

***Protein expression and purification in bacteria:*** PGEX GTPase expression plasmids (from DGRC) and the PGEX constructs containing wild-type and R979A mutant Syd-1 GAP domains were transformed into Rosetta (DE3) pLysS (Novagen) competent *E. coli* cells. Protein expression was induced and the resulting GST fusions purified by standard methods (Amersham Pharmacia Biotech; Garcia-Mata et al., 2006). Protein concentration was determined by the Bradford assay and protein purity by Coomassie Blue-staining of SDS-polyacrylamide gels.

***GTPase activity assay:*** GAP activity was measured using the EnzChek Phosphate Assay Kit, E-6646 as described by the product information provided (Molecular Probes; Scheffzek and Ahmadian, 2005). Briefly, 1 mM GST (control), Rho, Rac, and Cdc42, and p50GAP (Cytoskeleton Inc.) were each added to a 1X HBS solution containing 1 mg/ml BSA, 0.1 mm dithiothreitol, 20 mM MESG, PNP (1 unit), supplied 1X reaction buffer. Solutions were incubated at room temperature for 5 minutes at which time 1 mM GTP was added along with either the buffer alone, or 1mM of the wild-type or R979A mutant Syd-1 GAP or 1mM of the p50 RhoGAP positive control. A no GTP control was included as well. Somples were loaded into a 96 well plate with flat bottom clear wells and read at 360 nm on a Tecan Safire 2 spectrophotometer.

***Co-immunoprecipitation:*** Adult flies of genotype Actin (Act)-GAL4 (obtained from the Bloomington *Drosophila* stock center), UAS-FLAG-tagged wild-type or R979A mutant Syd-1 were homogenized in cold lysis buffer and centrifuged using a Beckman-Coulter Microfuge 18 to remove insoluble cellular components as described by Emery (2007). A western blot was performed to determine the approximate yield of FLAG-tagged protein per fly. 500 ul lysate was added to an eppendorf tube containing either 50ul of GST sepharose or 50 ul of GST-Rho GTPase and incubated on ice for 1 hr. Samples were washed with cold lysis buffer 3x and then cold HBS 3x. Western blots were performed using anti-FLAG antibody on PVDF membranes using 5% methanol in standard transfer buffer. Membranes were blocked with 2% low fat powdered dry milk.

***Genetics:*** Genotypes used for analyzing NMJ were (a) *BG380-Gal4*, (b) *BG380-Gal4/+;;Syd$^{w46}$/Syd$^{CD}$*, (c) *BG380-Gal4/ UAS-wild-type-Syd-1;; syd-1$^{w46}$/syd-1$^{CD}$*, and (d)

*BG380-Gal4/+; UAS-R979A-syd-1/+; syd-1$^{w46}$/syd-1$^{CD}$*. The *syd-1$^{w46}$* and *syd-1$^{CD}$* alleles were generated as previously described (Holbrook et al., 2012). Only third-instar females were dissected. Animals were raised at 25°C under standard laboratory conditions. Individual homozygous R7s were generated and labeled using the *GMR–FLP*/MARCM technique (Lee and Luo, 1999; Lee et al., 2001); homozygous cells expressed UAS–Synaptotagmin (Syt)–GFP under the control of Act–Gal4 (flies containing the UAS constructs and Act–Gal4 were obtained from the Bloomington Drosophila Stock Center). In each rescue experiment, the UAS-Syd-1 construct to be tested was also present and therefore specifically expressed in homozygous GFP-labeled cells. Only adult females were dissected. Animals were raised at 25°C under standard laboratory conditions.

***Image acquisition:*** Confocal images were collected on a Leica SP2 microscope with a z-stack of either 0.5µm or 1µm with a 63x 1.4 NA oil immersion objective, and analyzed with Leica or Fiji software (http://fiji.sc/Fiji; Schindelin et al., 2012). A complete z stack was acquired for all NMJ and rendered as a maximum projection. All quantifications of both NMJ and R7s were performed blind.

***Immunostaining of larval NMJs and adult R7s:*** NMJ dissections were performed in Schneiders insect media (Sigma. Dissections were either fixed with 4% paraformaldehyde in PBS for 25 min or in Bouins fixative for 15min. After fixation, Samples were washed with PBS with 0.1% Triton-X 100 and blocked for 30 min in 5% normal goat serum. For immunostaining, the larvae were incubated with primary antibodies at 4°C overnight and washed with PBT. Larvae were then incubated overnight with secondary antibodies at 4°C and washed. Larval pelts were mounted in Vectashield

(Vector Laboratories). Antibody dilutions were: 1:250 M- α-DLG and 1:100 M-α-Nc82 (Developmental Studies Hybridoma Bank); Rb-α-GluIIC (Marrus et al., 2002); 1:500 GP-α-dNRX (Li et al., 2007). Fluoresence conjugated anti-HRP (Jackson Immuno Labs) were used at 1:250. All secondary antibodies were diluted 1:500. Staining for dNRX was conducted as previously described (Li et al., 2007)

***Quantifications of NMJ size and AZ/PSD number and size:*** Quantification of Bouton number was conducted at muscle 6/7 of segment A3. Total boutons were visualized by staining of HRP and DLG. BRPNc82 size quantification was performed as previously described (Odwald et al., 2010) All images for synapse quantification from samples were stained in the same vial. Images were acquired using the same microscope gain settings.

## Results

### *Drosophila Syd-1 has GAP activity toward Rac1 and Cdc42 in vitro and interacts with all six RhoGTPases in vivo*

The RhoGAP-like domain within invertebrate Syd-1 proteins has been hypothesized to be catalytically inactive (Hallam et al., 2002; Wentzel and Sommer, 2013). While *C. elegans* Syd-1 has previously been assayed for GAP activity, *Drosophila* Syd-1 has not. To do so, we expressed specifically the RhoGAP domain of fly Syd-1 in *E. coli* and assayed its ability to increase the GTPase activity of the three major Rho family members: Rac1, RhoA, and Cdc42, from fly. We found that fly Syd-1's RhoGAP domain significantly increases the GTPase activity of Rac1 and Cdc42 but not that of RhoA (Fig. 1A-C). The degree of increase is similar to that caused by the control p50RhoGAP. This increase is abolished by a mutation (R979A) that disrupts the

predicted arginine finger, essential for RhoGAP activity in previously characterized RhoGAPs. We conclude that, contrary to previous hypotheses, the RhoGAP-like domain of fly Syd-1 does have RhoGAP activity.

We next wanted to test whether fly Syd-1 interacts with Rho GTPases in vivo. To do so, we tested the ability of fly Rho GTPases to co-immunoprecipitate full-length Syd-1 from adult. The GTP-bound form of Rho GTPases to which RhoGAPs bind can be transitory. To maximize the chance of binding, we instead used the constitutively-active mutant form of each fly Rho GTPase, which stably mimics the GTP-bound form. We found that each of the six fly Rho GTPases co-immunoprecipitates with full-length fly Syd-1 expressed ubiquitously in adult (Fig. 1D). We conclude that full-length fly Syd-1 can interact with Rho GTPases in vivo. This interaction is not disrupted by the R979A mutation: full-length Syd-1 containing the latter still co-immunoprecipitates with all six Rho GTPases (Fig. 1D). The latter result suggests either that the R979A mutation specifically disrupts the catalytic activity of the Syd-1 GAP without affecting its ability to bind Rho GTPases or that the interaction between Syd-1 and Rho GTPases is not mediated by Syd-1's GAP domain. To distinguish between these possibilities, we assayed the ability of Syd-1's GAP domain to interact with the representative Rho GTPase Rac1. We found Syd-1's GAP domain co-immunoprecipitates with endogenous Rac1 (Fig. 1E), indicating that the GAP domain itself interacts with Rho GTPases and that the R979A mutation disrupts GAP activity but not binding.

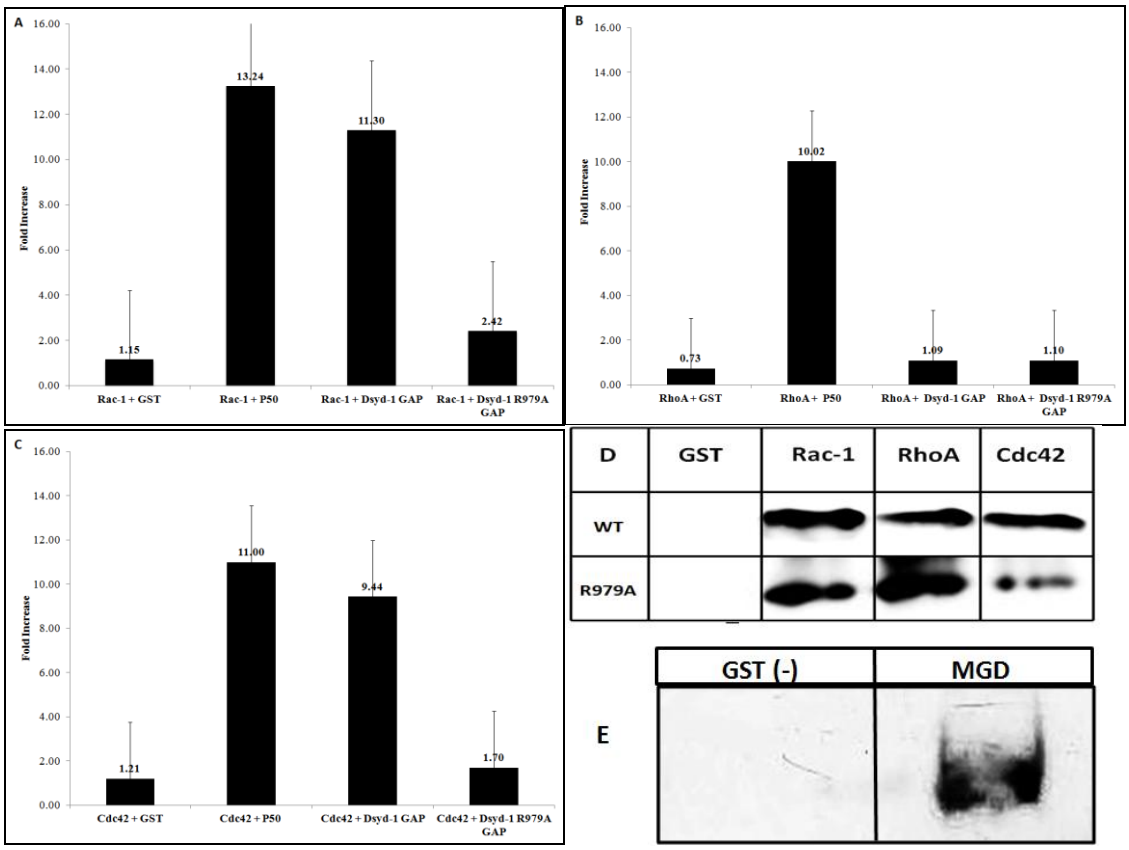


**Figure 1**: *Drosophila* Syd-1 binds Rac1, RhoA, and Cdc42 and enhances the GTPase activity of Rac1 and Cdc42 in vitro.

***A-C***. endpoint GTPase activity assays of wild-type Rac1, RhoA and Cdc42 alone or in the presence of the control p50RhoGAP, the predicted GAP domain of fly Syd-1, or the GAP domain of fly Syd-1 in which the conserved arginine of the arginine finger has been replaced by alanine.

***D***. Both FLAG-tagged full-length wild-type fly Syd-1 and FLAG-tagged full-length R979A mutant fly Syd-1 expressed ubiquitously in adult was pulled down with GST-tagged constitutively active mutant forms of Rac1, RhoA, or Cdc42.

***E***. Endogenous Rac1 was pulled down from wild-type adult flies with the GST-tagged GAP domain of Syd-1.

***Syd-1 overexpression in wild-type animals causes an increase in NMJ size independent of its GAP activity***

A mouse Syd-1 ortholog, mSyd1A, was recently also shown to have RhoGAP activity (Wentzel and Sommer, 2013). Whether this activity is required for mSyd1A to promote normal synapse development has not yet been tested. Presynaptic overexpression of mSyd1A can increase presynaptic terminal number even when its RhoGAP domain has been disrupted, suggesting that this protein's GAP activity may not be important for its synaptogenic function (Wentzel and Sommer, 2013). To test whether the same is true of fly Syd-1 we examined whether forced expression of Syd-1 in wild-type animals causes an analogous gain-of-function effect.

Syd-1 is among the first presynaptic proteins to accumulate at presynaptic sites, to which it recruits the active zone protein Bruchpilot (Brp) (Hallam et al., 2002; Dai et al., 2006; Patel et al., 2006; Owald et al., 2010). We first tested whether our FLAG-tagged wildtype Syd-1 would localize properly to AZs and, if so, whether disrupting Syd-1's RhoGAP activity might prevent this localization. We expressed wild-type and R979A mutant fly Syd-1 in the motorneurons of wild-type third instar larvae and found that both versions of the protein localized to NMJ synaptic boutons in a similar pattern to that observed for endogenous Syd-1, forming clusters that co-localized with the AZ marker Brp (Fig. 2A,B). We found that R979A mutant Syd-1 expressed in motorneurons of *syd-1* mutant animals also co-localized with Brp, indicating that this localization does not depend on the presence of endogenous Syd-1 (Fig. 2C). We conclude that Syd-1's GAP activity is not required for its localization to AZs.

We next quantified NMJ size in these animals. We found that expressing wild-type Syd-1 in the motorneurons of wild-type animals caused an increase in NMJ bouton number (Fig. 2D-F). A similar increase was caused by R979A mutant Syd-1 (Fig. 2D-F). We conclude that, like mouse mSyd1A, fly Syd-1 overexpression can have a gain-of-function effect on synapse development independent of its RhoGAP activity.

***Syd-1's GAP activity is required for normal NMJ size***

We next wanted to test whether Syd-1's RhoGAP activity is normally required for its ability to promote presynaptic assembly. To do so, we assayed the abilities of wild-type and R979A mutant fly Syd-1 to rescue the synaptic defects of *syd-1* mutant animals. Loss of *syd-1* has previously been shown to cause a decrease in NMJ size that can be fully rescued by presynaptic expression of a wild-type Syd-1 transgene (Owald et al, 2010). We therefore first compared the abilities of wild-type and R979A mutant Syd-1 to restore NMJ to normal size when expressed in the motorneurons of *syd-1* mutants. We found that, consistent with previous reports, our wild-type Syd-1 fully rescued the NMJ size defect (Fig. 3). By contrast, we found that *syd-1* mutant NMJs expressing R979A mutant Syd-1 were indistinguishable from *syd-1* mutant NMJs alone (Fig. 3). Given that the wild-type and mutant Syd-1 proteins are expressed and localized to AZs at similar levels, we conclude that the difference in their abilities to rescue the NMJ size defect reflects the difference in their RhoGAP domains. We conclude that Syd-1's RhoGAP activity is required presynaptically for normal NMJ development. We note that, by contrast, identical overexpression of either wild-type or R979A mutant fly Syd-1 in wild-

type animals increased NMJ size beyond that of wild type. These results suggest that endogenous Syd-1 is required for this gain-of-function effect.

***Syd-1's GAP activity is required for localization of the active zone component Brp***

Syd-1 has been shown to bind and localize the AZ component Brp: loss of Syd-1 results in enlarged Brp clusters at presynaptic sites (Owald et al, 2010). We compared the abilities of wild-type and R979A mutant Syd-1 to restore the pattern of Brp localization when expressed in the motorneurons of *syd-1* mutants. We found that wild-type Syd-1 fully reduces the size of Brp clusters in *syd-1* mutants but that the R979A mutant Syd-1 is unable to do so (Fig. 5). We conclude that Syd-1's RhoGAP activity is required for normal Brp localization. Because Brp is required for normal NMJ size, its mislocalization in the absence of Syd-1's RhoGAP activity may at least partly explain the inability of the Syd-1 R979A mutant to restore *syd-1* mutant NMJs to normal size.

***Syd-1's GAP activity is not required for R7 photoreceptor axon terminals to contact their M6 synaptic target layer but is required for a later phase of R7 presynaptic development***

R7 photoreceptor neurons provide a system in which to investigate the development of neuron-neuron synapses. Previous work has identified several differences between R7s and NMJ in the molecular mechanisms that underlie this process (Hofmeyer and Treisman, 2009; Astigarraga et al., 2010). We have found that *syd-1* is required in R7s for two distinct phases of presynaptic development (Holbrook et al., 2010). Each R7 axon terminal normally contacts the M6 layer of the medulla and, by the mid-pupal stage develops into a bouton that contains multiple active zones.

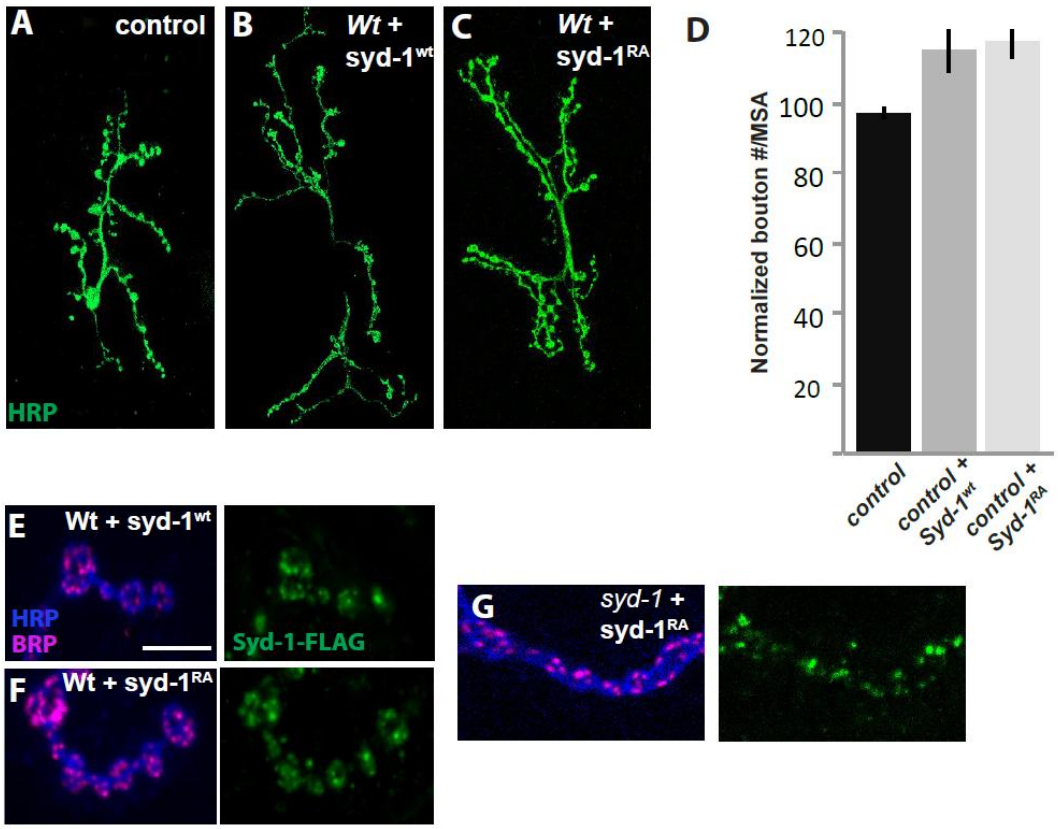


**Figure 2:** Overexpression of Syd-1 causes increased synaptic growth /Syd-1R979A Localizes Normally.

A. WildType

B. Syd-1 Mutant

C. WT

D. R979A

E. quantification.

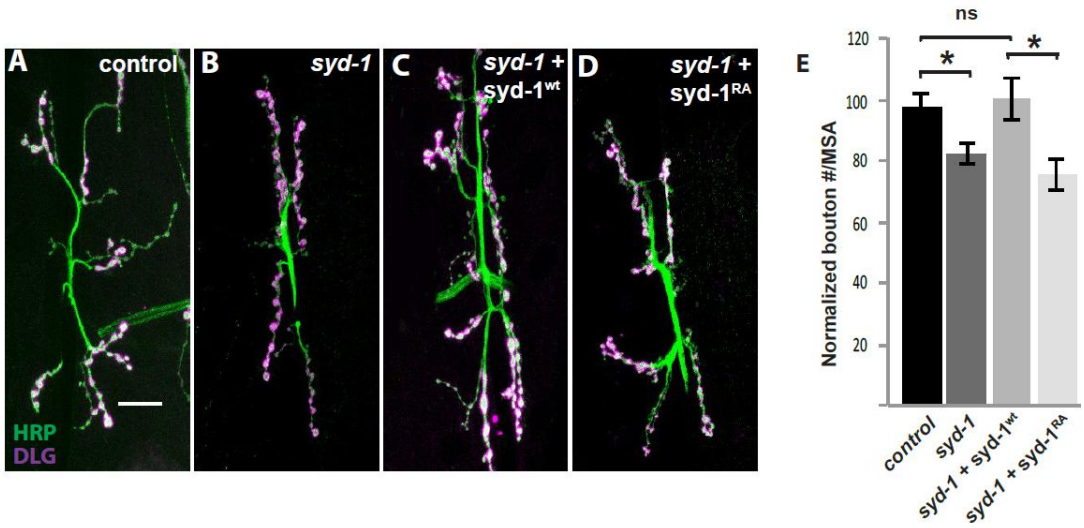


**Figure 3:** The GAP activity of Syd-1 is required for normal NMJ size.

***A-D.*** NMJs stained with anti-HRP and anti-Dlg

***A***. wild type

***B***. *syd-1*$^{CD}$*/syd-1*$^{w46}$ mutant

***C***. *syd-1*$^{CD}$*/syd-1*$^{w46}$ mutant in which motorneurons express wild-type Syd-1

***D***. *syd-1*$^{CD}$*/syd-1*$^{w46}$ mutant in which motorneurons express R979A mutant Syd-1

***E***. The average NMJ size in animals of genotypes ***A-D***.

Loss of *syd-1* initially prevents R7 axon terminals from maintaining contact with their M6 target layer (Holbrook et al., 2010). Later, those *syd-1* mutant R7 axon terminals that remain in contact with M6 project thin extensions beyond M6 (Holbrook et al., 2010). We next tested whether Syd-1's RhoGAP activity might be required to prevent either of these defects. We used the *GMR-FLP*/MARCM technique to express either wild-type or R979A mutant Syd-1 in individual *syd-1* mutant R7s. We found that both constructs fully rescued the failure of *syd-1* mutant R7 terminals to contact M6 (Fig. 5). By contrast, only the wild-type version of Syd-1 was able to prevent *syd-1* mutant R7s from later projecting thin extensions (Fig. 5). We conclude that Syd-1's RhoGAP domain is only required for the second stage of R7 presynaptic development.

Finally, we were curious as to whether Syd-1 overexpression in wild-type R7s, as in wild-type motorneurons, might have a gain-of-function effect on presynaptic development. Indeed, we found that using the *GMR-FLP*/MARCM technique to express wild-type Syd-1 in wild-type R7s resulted in an increase in R7 boutons with thin extensions beyond M6 (Fig. 5). As at NMJ, this gain-of-function effect is dependent on the presence of endogenous Syd-1, since we did not observe it when we identically expressed Syd-1 in *syd-1* mutant R7s (Fig. 5). And, as at NMJ, the effect is independent of Syd-1's RhoGAP domain, since using *GMR-FLP*/MARCM to express R979A mutant Syd-1 in wild-type R7s caused a similar increase in extensions beyond M6 (Fig. 5). However, because the phenotype caused by Syd-1 overexpression is identical to that caused by *syd-1* loss, we cannot distinguish whether the gain-of-function effect of R979A mutant Syd-1 also depends on endogenous Syd-1.

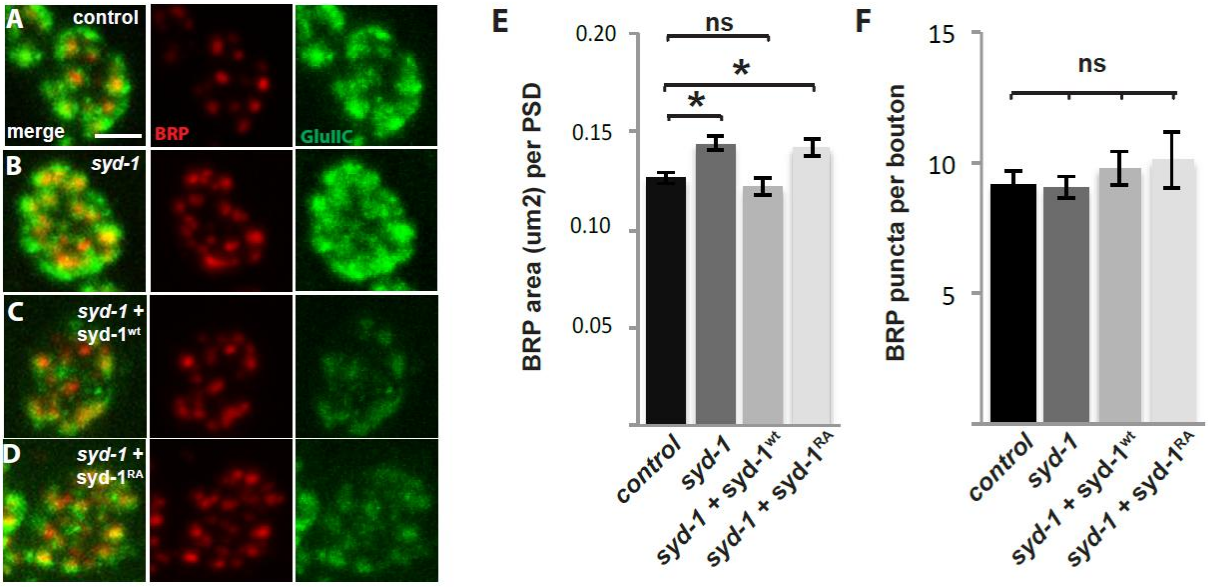


**Figure 4:** Syd-1 Gap activity is required for BRP localization
***A-D***, third instar NMJs stained with anti-HRP and anti-Nrx
***A***, wild type
***B***, *syd-1$^{CD}$/syd-1$^{w46}$* mutant
***C***, *syd-1$^{CD}$/syd-1$^{w46}$* mutant in which motorneurons express wild-type Syd-1
***D***, *syd-1$^{CD}$/syd-1$^{w46}$* mutant in which motorneurons express R979A mutant Syd-1
***E***, The average intensity of BRP staining per puncta
***F,*** The average intensity of BRP staining per buton

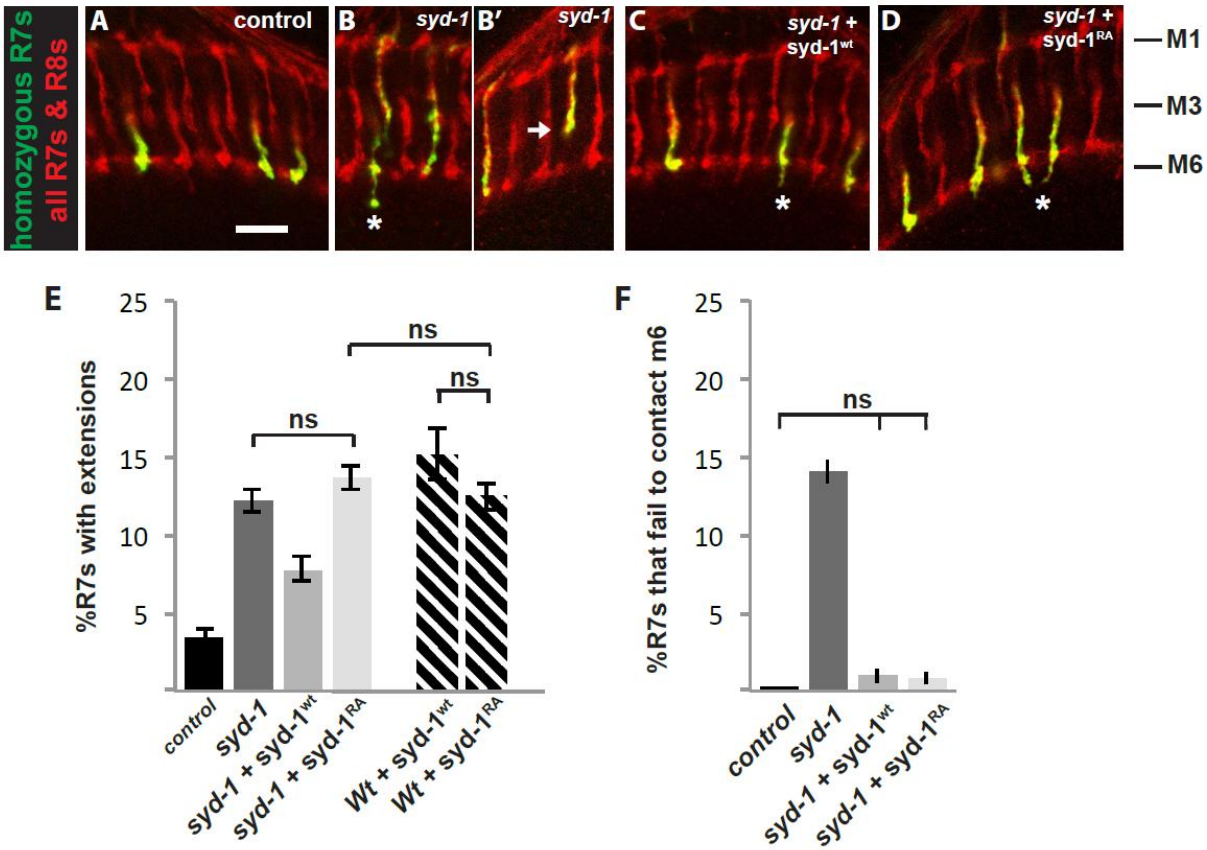


**Figure 5: Syd-1 Gap activity is partially required for R7s**.

a) Wild type

b) Syd-1 Mutant

c) +wt

d) +R979A

e) quantification

## Discussion

### *Fly Syd-1 has RhoGAP activity*

We found that the predicted RhoGAP domain of Syd-1 has GAP activity toward Rac1 and Cdc42 but not RhoA. Many other GAPs have previously been shown to exhibit this specificity, suggesting that it may be a conserved mechanism for regulating cytoskeletal dynamics (Bernards, 2003; Scheffzek and Ahmadian, 2005). One piece of evidence leading to the incorrect hypothesis that fly Syd-1 is not an active GAP was the finding that altering mouse mSyd1A to more closely resemble fly Syd-1 eliminated its activity toward RhoA (Wentzel et al., 2013). This result is consistent with our findings. Whether mouse mSyd1A might, like fly Syd-1, have GAP activity toward Rac or Cdc42 was not reported (Wentzel et al., 2013). One possibility is that the two vertebrate Syd-1s specialized and that the second mouse Syd-1 homolog, mSyd1B, may instead be a Rac or Cdc42 GAP.

While fly Syd-1's RhoGAP domain does not act on RhoA in vitro, we found that full-length fly Syd-1 co-precipitates with all six fly Rho GTPases in vivo. These results are not contradictory, since disrupting Syd-1's GAP activity by disrupting its arginine finger did not disrupt its ability to co-immunoprecipitate with Rho GTPases. They therefore suggest that Syd-1 may normally interact with RhoA without affecting its GTPase activity. However, Syd-1 may instead have GAP activity toward RhoA that is dependent either on the presence of the full length protein or on some regulatory modification. For example, MgcRacGAP was found to have Rac- and Cdc42-specific GAP activity unless phosphorylated on Ser387, which confers RhoA GAP activity (Benards and Settleman, 2004). This serine is present in fly Syd-1.

***Fly Syd-1's RhoGAP activity is not required for the ability of overexpressed Syd-1 to cause gain-of-function effects on presynaptic development, but the presence of at least some wild-type Syd-1 protein is***

The GAP domain of mSyd1A is not required for mSyd1A's gain-of-function effect on presynaptic development, including an increase in synaptic terminal number and synaptic vesicle clustering (Wentzel et al., 2013). Consistent with this result, we found that overexpressing fly Syd-1 in wild-type animals also causes gain-of-function effects on presynaptic development that are independent of its RhoGAP activity. At NMJ, overexpression of wild-type or R979A mutant fly Syd-1 caused indistinguishable increases in bouton number. And in R7s, overexpression of either protein caused late projection of ectopic extensions from the R7 terminal bouton. We have unpublished evidence that the latter represent sites of ectopic synaptogenesis, and the formation of excessive NMJ boutons is consistent with this interpretation.

GAP-inactive mouse mSyd1A is sufficient to increase synaptic terminal number and synaptic vesicle clustering even in cultured neurons lacking mSyd1A. By contrast, we have here shown that disrupting the fly Syd-1 RhoGAP domain eliminated its ability to rescue decreased synaptogenesis at NMJ and in R7s. How to reconcile these results? The presynaptic defects caused by loss of mSyd1A are far milder than those of the invertebrate *syd-1* mutants, suggesting that other proteins, perhaps among them the second mouse Syd-1 homolog mSyd1B, have taken over some functions that depend on Syd-1 in invertebrates. We found that RhoGAP-inactive Syd-1 can promote increased synaptogenesis in the presence of wild-type endogenous Syd-1. Perhaps mouse mSyd1A lacking the RhoGAP domain can promote increased synaptogenesis even in mSyd1A

knockout cells because wild-type mSyd1B is present. It will be interesting to examine the effects of deleting both mouse Syd-1 proteins and to test the functionality of mutant versions of those proteins in that double mutant background.

What might be a mechanism by which GAP-inactive Syd-1 promotes synaptogenesis only in the presence of wild-type Syd-1? Syd-1 and the trans-synaptic adhesion molecule Neurexin (Nrx) have been shown to depend on one another for localization to AZs. One possibility, though purely speculative, is that GAP-inactive Syd-1 may still be able to use its intact PDZ domain to bind and cluster Nrx. Increased Nrx clustering may in turn recruit additional Syd-1 molecules which can promote increased presynaptic growth that depends on the RhoGAP domain and that therefore can only occur if endogenous wild-type Syd-1 is present.

***By contrast, fly Syd-1's RhoGAP activity is required for some but not all aspects of its role in promoting presynaptic development***

Whether mouse mSyd1A's RhoGAP activity is normally required for synapse development has not yet been tested. We tested the requirement for Syd-1's RhoGAP activity in two different cell types: motorneurons, which synapse onto bodywall muscle and R7 photoreceptor neurons, which synapse onto neurons in the optic lobe of the brain. In each case we found that GAP-inactive Syd-1 can rescue some but not all aspects of the presynaptic defects in *syd-1* mutants. Because the R979A substitution that we used to disrupt Syd-1's RhoGAP domain significantly reduced GAP activity but did not appear to affect the ability of Syd-1 to co-immunoprecipitate with Rho GTPases, we conclude that it is Syd-1's GAP activity that is important, rather than simply the ability to bind Rho

GTPases. We found that this activity is required for normal localization of Syd-1's direct binding partner Brp to AZs. Syd-1 also regulates Brp localization by clustering the scaffold protein Liprin-alpha. In the future it will be of interest to test whether GAP-inactive Syd-1 is no longer able to localize Liprin-alpha as well as to test whether GAP-inactive Syd-1 can, as we predict, still bind and cluster Nrx.

**Bridge to Chapter III**

One of my first projects after joining the Herman Lab was investigating the genetic interaction between Dsyd-1 and Regulator of Synaptogenesis 1 (Rsy-1). This interaction has been characterized previously in worms however the role of Rsy-1 or its interaction with Dsyd-1 has not yet been investigated in higher organisms. Scott Holbrook, a previous student in the lab had bequeathed to me a number of different fly stocks including a dsyd-1 custom deletion allele as well as a deletion allele removing Rsy-1 as well as UAS-Rsy-1.RNAi.

# CHAPTER III

# EVALUATION OF THE GENETIC INTERACTION BETWEEN DSYD-1 AND RSY-1

## Introduction

The development of a nervous system is a truly miraculous feat. It is no trivial matter for an axon to extend out from the cell body, seek out its target, recognize that target, and form a synapse with it (Ziv and Gardener, 2004). Even more remarkable is the fact that although thousands of neurons are navigating this process simultaneously, they are able to maintain an organization to their connectivity, rarely cross-wiring or mis-targeting. (Astigarraga and Hofmeyer, 2010.) One can imagine that since there exist molecular signaling pathways capable of extending axons and forming synapses, there must also exist molecular mechanisms for preventing axons from targeting or forming synapses inappropriately. It is of great interest to modern medicine to identify molecules involved in the regulation of this process. While much effort has been devoted to the identification and study of positive regulators driving axons extension and synapse formation, much less is known about negative regulators of synaptogenesis. In order to better understand how and why a synapse forms, we need to study not only the molecules which promote this process, but also the ones which prevent it from happening at the wrong time or place. (Hallam 2002) In *C. elegans*, Syd-1 and Syd-2/ liprin-α are both required for normal motor neuron synapse formation. (Hallam et al. 2002) In the absence of Syd-1, synapses do not accumulate necessary pre-synaptic components and do not form functional active zones. It was found, however, that loss of Syd-1 can be rescued by over expression of Syd-2/ liprin-α, indicating that Syd-1 acts as a positive regulator of

synaptogenesis. (Taru and Jin, 2011) A suppressor screen was performed to identify negative regulators of this process. A mutation in the gene Regulator of Synaptogenesis 1 (Rsy-1) was able to suppress the phenotype caused by loss of Syd-1 (Patar and Shen, 2009). Rsy-1 accumulates early during presynaptic assembly where it localizes to developing active zone sites and binds to Syd-1 (Patar and Shen, 2009). Binding between Syd-1 and Rsy-1 is thought to negatively regulate the binding interaction between Syd-1 and ELKS (Patar and Shen, 2009). There are two different isoforms of Rsy-1; isoform A is a 589 kD protein with coiled coil, proline rich, and serine/arginine rich domains (Patar and Shen, 2009). The serine/arginine rich domain contains predicted nuclear localization sequences (NLS) ((Patar and Shen, 2009). Isoform B is missing this serine/arginine rich region, and instead has a unique NLS (Patar and Shen, 2009). Rsy-1 specifically localizes to the active zone of the developing synapse where it binds to Syd-1 (Patar and Shen, 2009).

While Rsy-1 has been identified as a negative regulator of presynaptic assembly in *C. Elegans*, it has also been observed that it is evolutionarily conserved in *Drosophila*, as well as vertebrates (Patar and Shen, 2009). Little is known about its function in vertebrates except the fact that it interacts with pinin, a protein involved in cell adhesion (Patar and Shen, 2009). Before this research, the role of Rsy-1 in synaptic assembly in *Drosophila* neurons had not yet been investigated, however its conservation throughout evolution would suggest its involvement.

Removal of Dsyd-1 is known to cause two distinct phenotypes during R7 neuron development in *Drosophila*. (Herman et al., 2012) While some R neurons extend past their target layer, forming ectopic synapses in inappropriate places, others fail to stabilize

a connection with the target M6 layer and instead stop short, forming synapses in the M3 layer. (Herman et al., 2012) In worms, removal of Rsy-1 is sufficient to rescue defects caused by Syd-1 loss. (Patar and Shen, 2009) I analyzed the requirement of Rsy-1 for proper R neuron development by removing it using mosaic analysis with a repressible cell marker (MARCM), as well as RNAi. I evaluated the epistasis relationship between Rsy-1 and Dsyd-1 by comparing the single and double mutants to determine if Rsy-1 is acting as a negative regulator of Dsyd-1 activity in *Drosophila* similar to its function in the worm HSN synapse.

**Results**

I assessed the role of Rsy-1 in presynaptic development by removing it genetically from the R neurons of the *Drosophila* brain and examining them for synapse formation defects. A deletion allele removing Rsy-1, as well as other genes was analyzed for synapse formation defects in R7 neurons using the mosaic analysis of a repressible cell marker (MARCM) technique. MARCM creates mutant R7 clones at a low frequency in the developing brain. Mutants R7s are label in green marking synaptotagmin, a protein associated with synaptic vesicles, while wild type neurons are label red marking chaoptin in all R neurons. While the role of Dsyd-1 in R neuron presynaptic assembly has been described previously, I wanted to make sure that in my hands the loss of dsyd-1demostrated the phenotype described by others and this control is necessary for me to be able to assess whether or not Rsy-1 is interacting with Dsyd-1 genetically.

***Gross Axon Morphology Intact in Deletion Removing Rsy-1***

The deletion removing Rsy-1 seemed to have little to no effect on R7 axon development. For the most part, axon tiling was normal, however I did observe a small

frequency of invasion of neighboring R7 cells known as tiling defects. R7 axons demonstrated normal morphology as indicated by synaptotagmin GFP staining. These mutant R7 axons correctly targeted the M6 layer of the medulla. They also had well formed butons, and properly localized synaptotagmin GFP suggesting proper localization of synaptic vesicles at active zones.

***Rsy-1 RNAi does not disrupt axon morphology, tiling, or targeting in R7 neurons***

Since the deletion allele removes more than just Rsy-1, I next wanted to remove only Rsy-1 to be sure than it's phenotype is not masked by the deletion of other genes. There are no known null alleles of Rsy-1 so I used a UAS-Rsy-1 RNAi construct to knock down gene expression. Similar to the deletion phenotype, I observed a small frequency of defects in R7 axon tiling however overall axon targeting and morphology was similar to that of wild type flies.

***Rsy-1 RNAi Rescues the Ectopic Synapse Formation Caused by Dsyd-1 Loss***

Removal of Dsyd-1 from R7 axons resulted in numerous occurrences of axons sprouting extensions that either invade neighboring neurons synapses or grow past the M6 target layer, forming ectopic synapses. (Fig 1C) Additionally, on occasion Dsyd-1 mutant axons would fail to extend down to the M6 target layer, residing instead in the M3 layer. (Fig 1B) Surprisingly, the axon extension phenotype observed in the dsyd-1 mutants was significantly reduced (Fig 1F, G) (p value=.001) It appears that Dsyd-1 mutants cannot form ectopic synapses in the absence of Rsy-1

In experiments with both Dsyd-1 and Rsy-1 removed, R7 axons stop short of their target layer at a higher frequency than that of Dsyd-1 single mutants however the increase was not found to be statistically significant. (p value =.008) (Fig 1H)

**Discussion**

In C. Elegans, Rsy-1 is required at an early step of synapse development where it was found to interact with and negatively regulate the activity of Syd-1/Syd-2/l liprin-α (Patar and Shen, 2009). The conservation of Rsy-1 is not just in sequence homology, but also in its ability to function at the synapse. Surprisingly, unlike in worms, I found that in flies, Rsy-1 acts as a positive regulator of synapse formation.

Although Rsy-1 deletion did not have an explicit phenotype caused by its removal, there is precedent for the removal of essential synaptic components not having an effect on axon morphology. The reason for the lack of phenotype from Rsy-1 loss is unclear. Our evaluation of the Rsy-1 mutant phenotype was limited to low resolution, gross axonal morphology. It is possible that there is a more subtle phenotype associated with Rsy-1 loss however our analysis was not sophisticated enough to detect it. Future efforts could employ high- resolution two-photon microscopy to examine proper localization of synaptic components within the active zone. Additionally, we could examine the post-synaptic density for signs of trans-synaptic signaling affecting synapse structure. It could be that Rsy-1 is acting redundantly in concert with other genes, or that it is required late in synapse formation, after the majority of the synapse has already formed. Likewise it could be that it is require exclusively for activity or maintenance of the synapse. The RNAi phenotype similar to that of the deletion was almost nonexistent except for to a few occurrences of defective tiling.

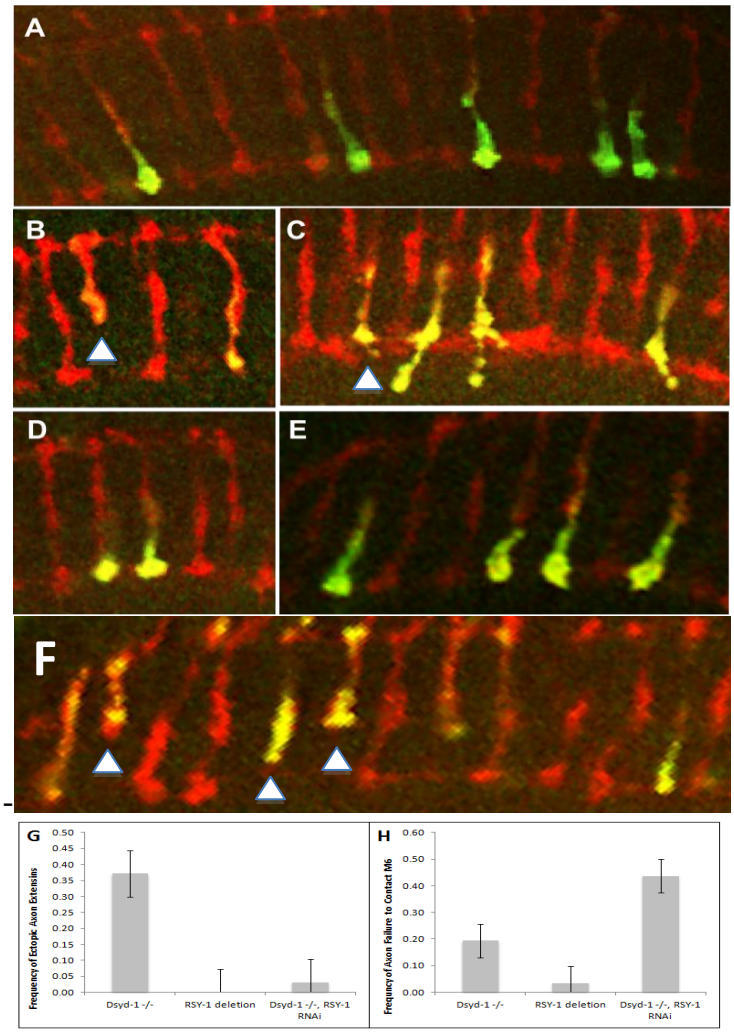


**Figure 1**: Epistasis Analysis of Rsy-1, Dsyd-1

A-F. MARCM labeling of mutant R7 neurons in green (syt-GFP, synaptic vesicles), red: mab24B10 (wild type R neurons).

A. Wild Type (Oregon R)
B. Dsyd-1 -/- axon extension failure
C. Dsyd-1 -/- ectopic extension defect.
D. Rsy-1 deletion .
E. Rsy-1 RNAi.
F. Dsyd-1 -/-, Rsy-1 RNAi.
G. Quantification of axon extension phenotype frequency. Error bars represent standard deviation. Statistical significance was determined using a t-test. (P=.001)
H. Quantification of axon short stop phenotype frequency. Error bars represent standard deviation. Statistical significance was determined using a t-test. (P=.08)

In flies, it has been reported that endogenous dicer expression in low (Bellés, 2010). Since dicer is require for RNAi processing, many RNAi constructs require the expression of UAS-Dicer (DCR) which aids in knocking down expression (Bellés, 2010). It could be that the Rsy-1 RNAi is not completely preventing Rsy-1 expression, and the phenotype is dosage sensitive to the amount of protein present. It could also be that Rsy-1 is required as a negative regulator for specific subset of neurons other than photoreceptors, such as motor neurons.

Rescue of the ectopic synapse formation phenotype caused by Dsyd-1 loss in the absence of Rsy-1 suggests that Rsy-1 acts downstream of Dsyd-1 as well as in a parallel pathway to promote synaptogenesis. This is an interesting result that suggests that Rsy-1 is required for ectopic synapse formation. We could also say that Rsy-1 is necessary for proper bouton structure. A number of different mutations in different presynaptic genes all give rise to an ectopic synapse formation phenotype similar to that observed with the loss of Dsyd-1. It would be interesting to test the general requirement of Rsy-1 for ectopic synapse formation in R7s. We could test this by determining if removal of Rsy-1 is sufficient to rescue the ectopic synapse formation phenotype caused by the loss of genes other than Dsyd-1.

Perhaps Rsy-1 can affect the GTPase activity of Dsyd-1 or is required for BRP localization. Since the binding interaction between Rsy-1 and Syd-1 prevents ELKs recruitment by Syd-1 in *C. elegans*, it would be very interesting to determine if a binding interaction exists between Dsyd-1 and Rsy-1 in *Drosophila*. We found the GAP activity of Dsyd-1 to be necessary for its ability to recruit BRP to the synapse. The homolog of ELKs protein in flies is BRP, thus it would be worthwhile to investigate if the interaction

between Rsy-1 and Dsyd-1 has an effect on Dsyd-1's GAP activity. Since the role of Rsy-1 seems to be opposite of its role in worms, perhaps a binding interaction between Dsyd-1 and Rsy-1 could be necessary for BRP localization.

## Materials and Methods

### *Genetics*

The deficiency line Df (3R) Exel 6162 (or 6163?), from which Rsy-1 is absent, was used to characterize Rsy-1 loss. The MARCM system was used to create GFP labeled homozygous mutant R7 cells in an otherwise heterozygous wild-type brain. Expression of FLP recombinase in R7s was under control of the GMR promoter and targeted FRT 82 sites on chromosomes in trans containing either the repressor Gal80 or the dsyd- w28 mutation. Homozygous mutant R7s were labeled with UAS-Synaptotagmin GFP driven by Actin Gal4. Heterozygous wild type neurons are labeled in red with mab24B10 which recognizes Chaoptin in R neurons. UAS-Rsy-1 RNAi was driven by GMR Gal4. The dsyd-1 CD allele was created by Scott Holbrook, a previous member of the lab.

### *Microscopy*

The brains of *Drosophila* pupae were dissected at P 48 and fixed with 1% paraformaldehyde PLP for five minutes then washed with 1X PBS three times before blocking with FGS and incubating in primary antibodies overnight. Primary antibodies were removed and brains were again washed 3X with 1X PBS before incubation overnight in secondary antibodies from the appropriate species. The secondary

antibodies were removed and brains were again washed before mounting on slides and imaging using a Leica SP1 confocal scanning laser microscope.

**Bridge to Chapter IV**

An ongoing challenge in our lab has been to understand how different R neurons are able to target different regions in the brain as targets with which to form synapses. Since each one of eight R neurons in contained within a single ommnatidium, it is difficult to physically separate them for analysis of expressed transcripts or proteins. Mike Miller, a former student of Dr. Chris Doe had suggested to me that I could adapt a current technique that would allow for the sequencing of individual cell types. Obviously intrigued, I developed a modified protocol for use in *Drosophila* pupae with the purpose of obtaining an R7 specific transcriptome.

# CHAPTER IV

# TECHNIQUE DEVELOPMENT: TU-TAGGING R NEURON MRNA

## Introduction

A precise spatio-temporal control of gene expression is essential for a functional synapse to be produced (Goda and Davis, 2003). In order for a functional circuit to be wired, neurons must grow axonal processes capable of spanning significant distances and forming synapses with other neurons or muscle (Bashaw et al., 2009). As developing axons stretch their way to a compatible target, they undergo dramatic morphological changes that allow synapse formation to proceed (Bashaw et al., 2009). When a synapse forms, axons slow their growth, find and adhere to the proper target regions, and accumulate neurotransmitter containing synaptic vesicles (Colon-Ramos, 2009). Underlying these morphological changes are changes of their molecular nature via modification of gene expression and protein-protein interactions (Polleux, Ince-Dunn, 2007). To understand these transcriptional changes is to understand the genetic basis of synaptogenesis. Although great progress has been made in identifying proteins present in the synapse, little is known about which transcripts are regulated during synapse development.

REST is a major vertebrate transcription factor known to regulate the transcription of a host of essential neuronal genes. (Ballas et al., 2005) Early experiments with REST demonstrated that it serves to silence expression of neuronal genes in non-neuronal cells through binding upstream regulatory elements of neuronal genes and promoting heterochromatin formation. (Schoenherr and Anderson, 1995) Later experiments demonstrated that REST expression doesn‘t actually prevent a cell from

becoming a neuron; it controls the temporal progression of the neuronal differentiation program. (Ballas et al., 2005) Tramtrack69, the REST ortholog in *Drosophila*, is required in post-mitotic glia to maintain their differentiated state. (Badenhorst 2001) Also, over expression of ttk69 has been shown to repress a subset of neuroblast markers and subsequent neuronal differentiation. (Badenhorst 2001) Consistent with its role as a repressor of neuronal fate, over expression of ttk stalls R7 axon outgrowth. (Kniss, Holbrook 2013) It has been observed, however, that ttk69 is indeed expressed in some neurons later in development, after the neuronal cell fate has been adopted. (Lai, Ying, 1999) It has been previously found that Ttk69 is expressed naturally in R7 neurons, and that this expression is important for R7 neurons to be able to form proper synapses. (Kniss, Holbrook 2013) Since Ttk69 is a DNA binding Zinc finger containing transcription factor, it is of interest to examine how its interaction with DNA alters gene expression. (Zollman and Godt, 1994) By determining the gene targets of Ttk69, we can answer important questions about what genes are necessary for neuronal specification. By understanding how the transriptome changes in R7 cells during final target selection/synapse stabilization, we can gain new insights into the molecular mechanisms regulating when a where a synapse can form.

We know from previous experiments that the activin pathway is regulated by Ttk69 and R7 cells and partially responsible for its ability to properly form synapses, however there must be more genes participating in this activity. (Kniss, Holbrook 2013) Our lab is interested in identifying genes that are affected by ttk and thus activin signaling to be evaluated for their roles in synapse formation. Since the advent of high

throughput Illumina sequencing, a number of new techniques have been developed which allow us to examine cells is ways never before thought possible.

RNASEQ is a method by which one can obtain the sequences of all the RNA in a sample, also known as the transcriptome. (Wang and Gerstein, 2009) This is accomplished by isolating RNA and converting it to cDNA, then using massively parallel Illumina sequencing. (Wang and Gerstein, 2009) The mRNA transcripts can be isolated from the total population of RNA using a number of techniques including taking advantage of its interaction with magnetic poly T beads. Obviously the information obtained with RNASEQ will vary with the tissue sampled. Even when dissecting out specific tissues they often contain a number of different cell types. The presence of multiple cell types could confound potentially important transcriptional differences, for example the transcriptional differences between neurons and neighboring epithelial cells. In order to obtain the transcriptome from a single cell type, one must be able to isolate the mRNA of that single cell type. This can propose quite a technical challenge to the researcher, with many cell types being difficult if not impossible to dissect or otherwise purify. This is true especially if one is interested in studying neurons which are often deeply entangled within a matrix of other cell types.

Since R7 neurons are only 1 of 20 cells in the fly retina, we cannot simply collect RNA from whole retinas (Hsiung and Moses, 2002). It is not possible to dissect out and isolate R7 neurons without severing axons. A new technique has been developed to address this issue which uses the Gal4 expression system to express an enzyme uracil phosphoribotransferase (UPRT) that incorporates 4 thio uracil (4TU) into the RNA of targeted cells. (Cleary and Meriring, 2005) The 4TU labeled mRNA can then be

biotinylated and then purified from the total retinal mRNA on streptavidin beads in order to perform RNASEQ on labeled RNA only (Miller and Robinson, 2009). The aim of this research is to specifically label the mRNA of R7 neurons with 4TU such that it can be purified and sequenced. By comparing the transcriptome of R7 neurons before and after Tramtrack signaling, we can identify transcripts associated with synapse formation. Alternatively we can compare the transcripts of wild type R7s with ttk-/- mutant R7s to obtain similar data.

R neurons extend and form synapses with their target layer during the pupal stage of development, between 24 and 48 hours APF. (Hadjieconomou, D., Timofeev, 2011) To study the transcripts involved in the development of R neurons, one must dissect the brains of *Drosophila* pupae, purify out the tagged transcripts, and sequence them. 4TU must be provided to the cells within a few hours to be incorporated into mRNA and is typically administered orally to *Drosophila* larve or adult flies. (Miller and Robinson, 2009) Due to the fact that pupae do not feed, feeding them 4TU is not possible. I developed a novel protocol for administering 4TU to *Drosophila* pupae.

In order to perform the sequencing experiments, I will need to use an R neuron specific Gal driver. While these experiments were being performed, there were no known Gal4 drivers that are expressed in R7s cells only. I performed a Gal 4 driver expression test using UAS LacZ and B galactosidase immunostaining to examine the expression patterns of a number of Gal4 drivers demonstrating expression in the developing retina. Many researchers have used GMR Gal4 to express proteins specifically in the eye, however a recent study pointed out that it is actually expressed in a number of tissues throughout development including the wing. (Li and Li 2012) Similarly Elav Gal4 was

reported to be expressed specifically in post-mitotic neurons, but later found to be expressed in embryonic glia, as well as proliferating longitudinal glioblasts. (Berger and Renner 2007) Chaoptin-Gal4 was gifted to our lab and reported to be expressed specifically in photoreceptor neurons (Larry Zipursky, unpublished communication 2009). We identified Zwilch Gal4 (ZWGAL4) as being located very close to the chaoptin gene and thus potentially have a similar expression pattern although it had not been previously reported to be R neuron specific. PM 181 Gal4 has been used to drive expression specifically in R7 neurons, but has not been extensively characterized. (Lee and Herman 2001) These Gal4 lines were examined for their expression patterns and intensity in *Drosophila* central nervous system with a focus on the retina and R neurons. After selecting an appropriate Gal 4 driver I wanted to determine if it was capable of simultaneously expressing UAS-UPRT and UAS-Ttk69 RNAi as we hypothesize that expressing multiple UAS constructs using the same Gal4 could reduce their expression level due to Gal4 sequestration.

## Results

### *Characterization of Gal4 driver lines*

Gal4 drivers are often expressed in many different types of cells and demonstrates temporal variation in expression levels. If Gal4 is to be used to label RNA from specific cell types, it is important to know which cells are expressing Gal protein. It is also useful to know how much Gal protein is being produced as well as when and for how long it is expressed and stable. I determined which cells in the *Drosophila* brain express candidate Gal4 driver by using it to overexpress UAS-LacZ. LacZ was chosen because it typically

demonstrates robust expression, is not endogenous to *Drosophila*, and can be easily detected using a GFP conjugated anti beta galactosidease antibody.

Chaoptin Gal4 was a gift to our lab from the Zipursky lab and was described as R neuron specific. I found that it demonstrated high levels of expression in R7 neurons specifically. There was also a significant amount of staining in R1-R6, although the intensity was much lower. Its expression pattern was similar to that of Elav however staining intensity would suggest it is expressed at lower levels. Notably, I did not observe expression of ChpGal4 in any other parts of the brain, nor any other cells in the retina.

Zwilch Gal4 has not been extensively characterized, and this is the first characterization of its retinal expression pattern. Interestingly, Zwilch Gal4 had a remarkably similar expression pattern as Chaoptin Gal4, which it is located next to it on the *Drosophila* chromosome. Significant staining was noted in R7 neurons expressing LacZ under ZWGal4. Less intense staining was noted in R1-R6. Occasionally an ommnitidia would contain a single R neuron lacking any detectable stain. Although similar in intensity and expression pattern to Chp Gal4, there was considerably more variation in both metrics. Zwilch was not exclusive to the retina as staining was seen in other parts of the brain however expression in the optic lobe is restricted to R neurons. (data not shown)

The Gal4 driver Elav was chosen for its renown as being neuron specific. (Berger and Renner, 2007) I found that the chaoptin Gal4 driver to be R neuron specific in its retinal expression pattern however it was expressed in many other parts of the fly brain.

It demonstrated consistent expression in R7 cells and was expressed at lower levels in all R neurons in the retina, although GFP intensity varied slightly.

181 Gal4 demonstrated strong expression specifically in R7 neurons as described previously. R1-R6 were not even visible due to lack of LacZ expression. (Fig. 1d) LacZ expression was restricted to the retina, however by P24 it was found to be expressed in each of the four surrounding cone cells, and by P48 was expressed in non-neuronal bristle cells as well.

*GMR*-GAL4 was reported to drive the expression of target genes in all cells posterior to the morphogenetic furrow in the developing eye (Song et al., 2000). Since then, many overexpression studies have been carried out in *Drosophila*, using the *GMR*-GAL4 driver line with attention being focused on the developing eyes. (Li and Li 2012) Additionally, many variations of this driver have been constructed using the split Gal4 technique. (Luan and Peabody, 2006) I found that the classic "R neuron specific driver", Long GMR, is actually, not R neuron specific, as it is expressed in glia, as well as other places in the developing brain. This finding has since been replicated and described in detail. (Li and Li, 2012) Since there was significant expression in so many different parts of the brain, I did not further characterize this driver.

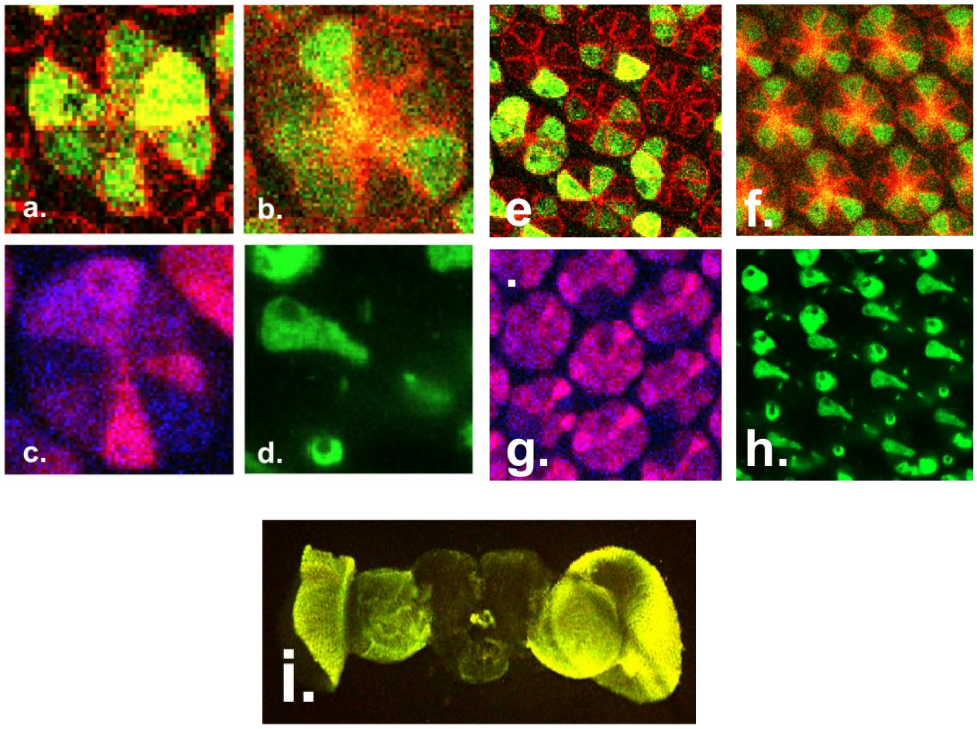


**Figure 1**: Gal4 promoters expressing UAS-LacZ

A. Single ommnitidium, Elav GAL4.

B. Single ommnitidium, ChpGAL4.

C. Single ommnitidium, ZWGAL4

D. Single ommnitidium, 181GAL4

E. Elav GAL4. Same image as a but lower resolution.

F. ChpGAL4. Same image as b but lower resolution.

G. ZWGAL4

H. 181GAL4

I. Whole brain, longGMR Gal4

### *4-Thio-Urcail is Incorporated into Retinal mRNA*

Since 4 thio-uracil is not naturally synthesized in *Drosophila*, it must be injected in order for UPRT to incorporate into a nucleotide. (Cleary and Meriring, 2005) Once 4TU is phosphoribosylated, it can be incorporated into RNA transcripts. In order for the mRNA of a *Drosophila* R neuron to incorporate 4TU, it must simultaneously express Gal4, UPRT, and 4TU must be present. (Miller and Robinson, 2009) Since I am injecting the 4TU solution into the thorax, it must travel via the haemolymph and into the brain, across the optic lobe and into the retina.

To ensure that 4TU was being incorporated into the mRNA of flies expressing UAS-UPRT under control of the Chaoptin Gal4 driver, I dissected retinas and extracted total retinal RNA from which I purified the total mRNA population using poly T beads. Next I used a EZ-Link HPDP-Biotin Kit (Pierce) containing pyridyldithiol-activated, sulfhydryl-reactive biotinylation reagent to create disulfide bonds between the 4TU mRNA and biotin molecules. The biotylated RNA was then purified using magnetic streptavidin beads, eluted, and run on an agarose gel. Ethidium bromide staining indicated that some mRNA had been purified using this technique. (fig 2a) I performed a northern blot on this RNA with streptavidin conjugated to HRP and developed with ECL detection reagent. Bright staining bands indicate that 4TU labeled mRNA was present. (fig 2b) Although 8 hours was determined to be a sufficient incubation period post injection for 4TU incorporation into retinal mRNA, low yields could indicate that additional optimization of this step is required. The approximate yield of mRNA per retina was 10 ng. With long exposure times, it seems like all three Gal4 drivers tested

expressed similar amounts of 4TU mRNA however, upon a shorter exposure time it, there was significantly less staining in from the no Gal4 control (fig2c).

***Driving TTK69 RNAi, with CHPGal4 knocks down TTK protein expression from R neurons***

After determining chaoptin Gal 4 to be an appropriate R neuron Gal4 driver, I created flies containing chaoptin Gal4 as well as UAS-Tramtrack69 RNAi to verify that Ttk69 protein expression has been reduced. I used anti-Ttk69 antibody to indicate the location and abundance of Ttk protein and anti-Chaoptin, also known as mab24b10 to mark R neurons. Wild type flies begin expressing Ttk69 36 hours APF and by 48 hours, demonstrate robust expression in all R neurons. (fig. 3a) Flies expressing UAS-UPRT as well as UAS-ttk69 RNAi demonstrated significant loss of Ttk69 staining at P48, indicating that the RNAi construct has successfully knocked down Ttk69 protein expression. (fig 3b) Gross morphology of the ommnatidia was intact and normal.

***Loss of TTK caused by RNAi is sufficient to cause axon tiling defects***

Usually R neurons form stereotyped, bulb-like butons. (fig 4a) In order to preserve spatial information, they are well organized into columns (fig 4f) however a number of mutations are known to upset this order. In the absence of Ttk69 for example, R7 axons often extend past their target layer, mis-localize pre-synaptic components, and invade adjacent neurons, forming ectopic synapses. To verify that the Ttk protein was sufficiently removed, the R7 neurons were examined for signs of synapse tiling defects, a phenotype that has been reported previously.

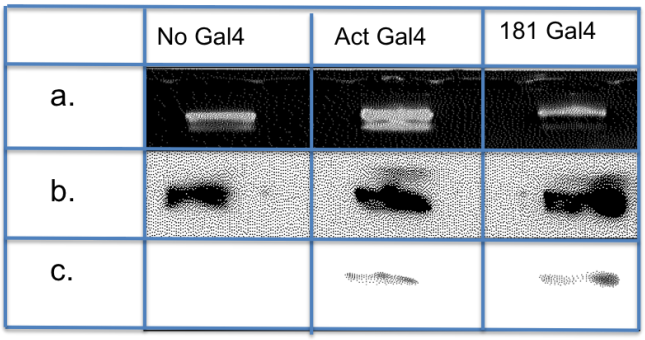


**Figure 2**: 4TU incorporation into retinal mRNA.

a. Agarose gel of total mRNA stained with ethidium bromide

b. Northern Blot anti Streptavidin-HRP

c. Same blot as b. with reduced exposure time.

Since RNAi often knocks down a genes expression without being able to stop it entirely, I wanted to ensure that a sufficient amount of Ttk69 protein had been removed to recapitulate the phenotype observed from its complete loss. I found that even with the presence of an UAS-UPRT transcript, sufficient ttk69 RNA was affected to cause significant axon targeting defects including extensions (fig4b,c,d,e) and invasions (fig 4g).

**Discussion**

The possibility of obtaining cell type specific expression data has the potential to exponentially increase the rate of discovery of genetic contributions to biological processes. One of the many attractive features of using *Drosophila* as a model to study developmental biology is the development of a collection of Gal4 driver lines which vary which cells they are expressed in and when and for how long they are expressed. With the power of the Gal4 system combined with the power of 4TU tagging, an untold number of new experiments are possible.

Long GMR Gal4 had been previously characterized as having its expression pattern restricted to R neurons but I found this to be inaccurate as using long GMR Gal4 to express LacZ resulted in distinct staining of the entire optic lobe as well as regions of the deep midbrain. The expression in deep midbrain possibly explains why early expression using longGMR Gal4 often results in embryonic or larval lethality.

The expression pattern of neuron specific driver Elav Gal4 did indeed drive lacZ expression exclusively in neurons. In the retina, a majority of R neurons were GFP

positive, however the staining demonstrated a stochastic expression pattern where R neurons from different ommnatidia vary considerably in their GFP expression.

In addition to being expressed in neurons throughout the entire animal, Elav Gal4 expresses earlier than other drivers, such that over-expressing RNAi or UPRT may cause cell fate issues. The expression of LacZ using 181 Gal4 was robust, however it was not specific to R neurons in the retina as it also was found to be expressed in non neuronal cone cells and bristle cells. Even though we cannot specifically express UAS-UPRT in R7 neurons, we can express it in all R neurons, and compare that data to data obtained from flies which have only had their R7 neurons removed. By comparing the data, we should be able to identify transcripts regulated specifically in R7 cells.

The chaoptin Gal4 driver demonstrates promise as an R neuron specific Gal4 driver. It is consistently expressed exclusively in R neurons. The expression intensity seemed strongest in R7 neurons which will be useful for future experiments. At times, the expression intensity seemed to vary amongst different R neurons however that could simply be bleaching of antibody fluorophores. Similar to chaoptin, the expression pattern of zwilch Gal4 was R neuron specific. Indeed, their expression patterns are almost identical (Fig. 1 F and G) Little is known about zwilch except for the fact that it is involved in kinetochore function (Williams et al., 2003). Given its expression pattern, it seems likely that the zwilch gene product is expressed in R neurons, and perhaps plays a role in their development or function. It would be worthwhile for future studies to investigate the role of zwilch in R neuron development. After this research was concluded, an article was published describing an R7 specific Gal4 driver line called R20C11-Gal4.

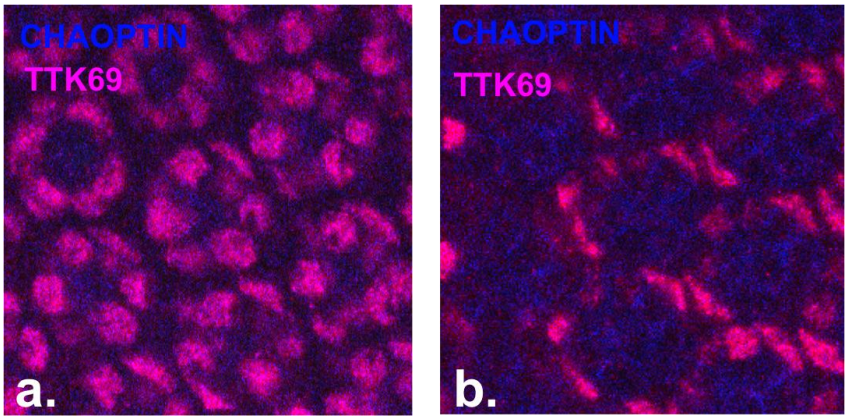


**Figure 3**: Ttk69 is absent from R cells expressing ttk69 RNAi

a. Control. Wild Type ORR P48 retina.

b. ChpGal4→UAS-ttk69 RNAi P48 retina.

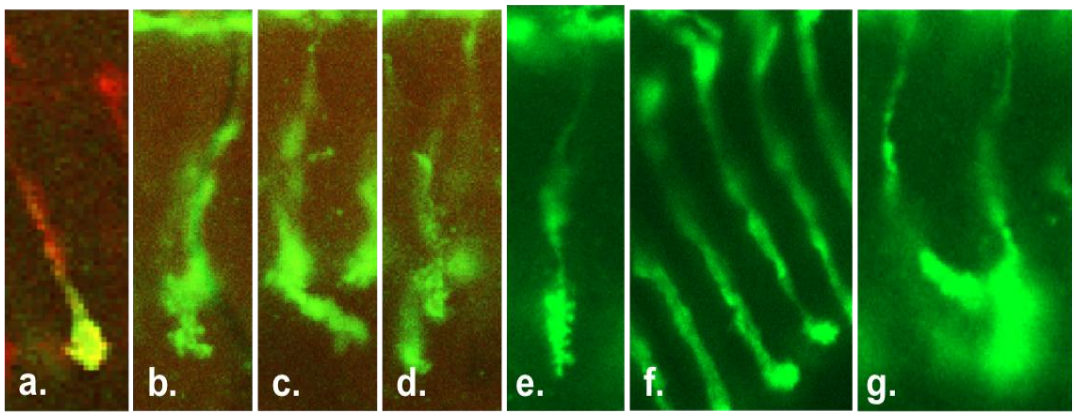


**Figure 4**: Chaoptin Gal4 x UAS-Ttk RNAi recapitulates R7 axon tiling defect.

a. Wild type R7 axon.

b.-e. CHPGal4 → UAS-Ttk RNAi.

f. Normal axon tiling in wild type R7 neurons.

g. Axon tiling defect characteristic of Ttk69 mutants.

This driver would be ideal for expressing UAS-UPRT exclusively in R7 neurons.

I developed a novel protocol for administering 4TU to *Drosophila* pupae. This technique allows for incorporation of 4TU into R neurons during critical stages of synapse formation. It is likely that this injection technique will allow for 4TU incorporation anywhere in the developing pupae. Since there are approximately only 750 R7 neurons per retina, many brains are required to obtain a sufficient quantity of RNA for downstream purification, labeling, and sequencing. Loss during these steps can lead to little RNA being recovered and poor sequence quality. An average eye disc yields only 10 ng of mRNA, and the R7 neuron is an estimated .1% of this quantity. I estimate that it would require over 5000 flies dissected to obtain a quantity of RNA to provide reasonably clear sequence data.

RNASEQ is a promising new technique, but is not without its technical challenges. The result of 4TU tagged RNA isolation from UAS-UPRT control flies indicates that a small number or UPRT molecules must be expressed in these flies in the absence of a Gal4 driver. UAS-UPRT seems to have a leaky promoter is of cause for concern as this could cause incorporation of 4TU into mRNA from undesired cell types. Although an amplification step is standard during the RNASEQ procedure, this has the potential to introduce considerable bias depending on which sequences are amplified and how frequently. It has been reported that many smaller RNAs are washed out during the 75% ETOH wash thus larger transcripts are more likely to be purified and amplified than smaller ones.

As an alternative method of obtaining large quantities of R7 mRNA, we could induce the formation of ectopic R7 cells genetically using the sevenup mutation; although it is unclear whether extra R7 cells would have the same transcriptome as native R7s.

In the years since this research was performed, new advances in the sample prep for RNASEQ have facilitated successful sequencing from as little as 50ng of RNA. It would definitely be worth revisiting this technique in the future as it would require the dissection of a more practical number of retinas.

**Materials and Methods**

Gal4 expression was performed using a Leica SP2 CSLM to analyze the retinas of flies expressing UAS-LacZ stained with anti-beta galactosidease. UAS-Ttk69 RNAi flies from Vienna were crossed to ChpGal4, UPRT flies and the retina of the progeny were dissected and analyzed for defects in photoreceptor synapse formation. 4TU labeling was accomplished by injecting embryos with 1 microliter of 50mM 4-thio Uracil in the ventral thoracic cavity of pupae 24 hours after puparian formation. Biotylation of RNA was accomplished using a kit EZLink Biotin HPDP. RNA purification was performed using MPG streptavidin beads (Pierce). In the northern blot, 1 ug total RNA was run in an agarose gel and stained with EtBr. The RNA was then transferred to a Nylon membrane where it was stained with streptavidin-HRP and detected using luminol, p-coumaric acid, and H202.

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