THE ROLE OF THE TUMOR SUPPRESSOR LETHAL GIANT LARVAE DURING NEURAL DEVELOPMENT

By

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Abstract

The tumor suppressor *lethal giant larvae* (*lgl*) is essential for development and regulates cell polarity in epithelia and neuroblasts by interacting with two tumor suppressors, *discs large* (*dlg*) and *scribble* (*scrib*). Due to its earlier requirement in neuroblasts, the role of *lgl* in postmitotic neurons has remained understudied. Molecular and genetic epistasis experiments suggest that Lgl functions upstream or in parallel with the Fragile X syndrome gene (dFmr1) during neural development. These data has led us to hypothesize that Lgl functions independently of dFmr1 in neuronal development and to test this, I began to investigate the *lgl* loss of function phenotypes at the larval neuromuscular junction (NMJ). Studies of two different allelic combinations, lgl^{1}/lgl^{U334} and lgl^{1}/lgl^{DV275} indicate that although loss of *lgl* does not significantly affect the overall size of the NMJ, individual synaptic boutons appear enlarged and malformed. Immunohistochemistry experiments, using antibodies against the Bruchpilot protein indicate that loss of *lgl* leads to an increased number of active zones on the presynaptic side. This is the first evidence that *lgl* functions in neurons, a role previously confounded by its requirement in neural stem cell division. Future studies on Lgl's impact on glutamate receptor content will be important to determine whether Lgl also functions postsynaptically.

Introduction

Characterization of the lgl locus

When the *lethal giant larvae (lgl)* allele was first isolated in the 1930s by Bridges and Brehme, the gene's ability to serve as a tumor suppressor was unknown (Bridges and Brehme 1944; Bilder 2004). However, in the 1960s Elizabeth Gateff and Howard Schneiderman studied the null allele lgl^4 and found that the loss of function *lgl* phenotype causes malignant tumor growth and acts in a recessive manner (Gateff and Schneiderman 1967).

Embryogenesis is carried out relatively normal in homozygous *lgl* mutants because the Lgl protein is maternally provided into the egg. Once the maternal supply of Lgl runs out during the larval stage the mutant offspring cannot produce its own Lgl. Zygotic transcription is required for proper development and with the inability to transcribe Lgl, the offspring cannot survive. As a result, mutant larvae live until third instar due to the presence of the maternally loaded Lgl protein (Manfruelli et al., 1996; Bilder and Hariharan 2006). These mutant offspring become "giant larvae" and neoplastic tumors metastasize in the imaginal and neural tissues (Wodarz 2000). The metastasis seen in *lgl* mutants has been linked to a loss of polarity within the mutant cells and to a loss of cell proliferation control (Humbert et al., 2008; Grzeschik et al., 2007). In homozygous *lgl* deficient mutants, the tumorous growth can be first seen during the third instar larval stage in the form of bloating of the larval body, underdevelopment of the ring gland and atrophy of the fat bodies (Merz et al., 1990). The overgrowth of the larvae and the resulting death during the larval stage is what accounts for the origin of the name of this gene.

Roles of Lgl in the cell and its functional partners

Lgl has been found to carry out a versatile array of roles in addition to maintaining epithelial polarity and cell proliferation, such as asymmetric cell division, cell migration and invasion (Wirtz-Peitz and Knoblich 2006; Humbert et al., 2008). In order to carry out its role in all of these cellular processes, Lgl receives assistance by its two functional partners: Scribble (Scrib) and Discs Large (Dlg) (Vasioukhin 2006; Wirtz Peitz and Knoblich 2006; Humbert et al., 2008). The mutant phenotypes of any one of the three tumor suppressors results in overgrowth of the brain lobes and imaginal discs, as well as loss of apicobasal polarity of the epithelial cells. Therefore, it is assumed that Lgl, Dlg and Scrib all function within the same pathway (Bilder and Hariharan 2006). Tumor suppressors that affect larval imaginal discs and brain tissue such as these three are categorized as either hyperplastic (when overgrowth occurs without loss of epithelial structure) or as neoplastic (when overgrowth is accompanied by loss of apical basal polarity causing an abnormal epithelial cell shape). Of the 50 known *Drosophila* tumor suppressors*, Dlg, Scrib, and Lgl are the only tumor suppressors in which mutations lead to neoplastic formation of tumors in the brain lobes and the imaginal discs (*Watson et al., 1994; Humbert et al., 2003).

Role of Lgl in Epithelial Cell Polarity

Lgl, Scrib, and Dlg are all considered to be polarity genes in addition to being tumor suppressor genes on account of their important role in establishing and maintaining polarity in epithelial tissue (Assemat et al., 2008). Apicobasal polarity is required for the development of the epithelial barrier function. Apicobasal polarity is essential for the

correct localization of membrane proteins and receptors to distinct cellular domains so that the proper interaction of cells with other cells and the extracellular matrix can occur (Tepass et al., 2001).

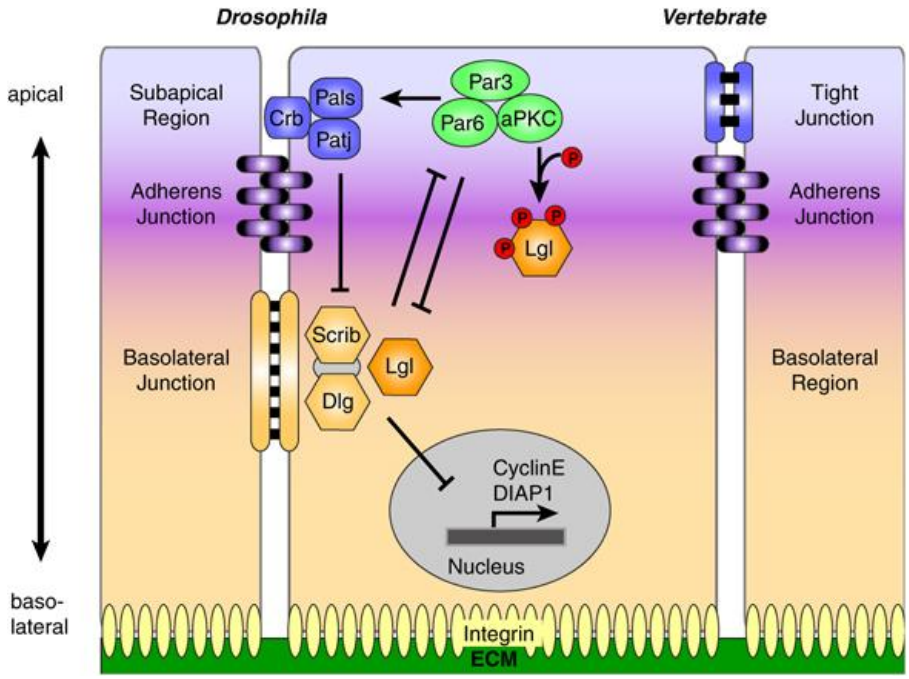

Figure 1. Regulation of apicobasal cell polarity in epithelial cells by means of the Scribble and Par Complex. Scribble and Dlg are localized to the cortex basal to the adherens junction at the septate junctions. Lgl is predominantly localized basolaterally in epithelial cells, however its localization to the plasma membrane is dependent upon its phosphorylation state by the Par complex. aPKC phosphorylates Lgl which inhibits Lgl from localizing to the cortex and renders it inactive. Lgl when bound to the basolateral membrane along with its functional partners Dlg and Scrib is not phosphorylated so is able to target proteins selectively to the basal cortex and inhibit aPKC activity. Figure from Humbert et al., 2008)

There are three polarity modules that cause the establishment and maintenance of apicobasal polarity in epithelial cells. These modules are: Scribble, Par and Crumbs polarity modules. The Scribble polarity module is comprised of the three tumor suppressors: Scrib, Dlg, and Lgl. Dlg and Scrib are constitutively localized to the basolateral region of the plasma membrane whereas Lgl localization to the basolateral region is dependent upon phosphorylation of Lgl by the Par polarity module as seen in

Figure 1. The Par polarity module is comprised of the proteins Par3 (Bazooka in *Drosophila*), Par6, and aPKC (Yamanaka et al., 2003).

When the PAR complex is localized to the apical membrane, aPKC phosphorylates Lgl to inactivate it and thus exclude Lgl from the apical membrane (Yamanaka et al., 2003). Lgl is restricted to the basolateral membrane and acts to inhibit aPKC activity in the basolateral domain and confine specific determinant proteins to the apical membrane. The correct localization of Lgl to the basolateral membrane is demonstrated by examining *lgl* mutants. *Lgl* mutants cannot be phosphorylated by aPKC and therefore Lgl is no longer restricted to the basolateral membrane (Betschinger et al., 2003). The determinants of the apical membrane mislocalize to the basolateral domain. This mislocalization is then accompanied by the disorganization of adherens junctions, which are normally positioned at the interface between the two domains (Bilder 2000; Hutterer et al., 2004).

Role of Lgl in Asymmetric Cell Division

Dlg, Lgl and Scrib are known to be required in neuroblasts to induce asymmetrical segregation of fate determinants like Prospero (Pros) and Brain-tumor (Brat) (Albertson and Doe 2003; Betschinger et al., 2003). In asymmetrically dividing cells, fate determinants are proteins that segregate unequally into one daughter cell but not the other. The unequal inheritance of cell fate determinants either promotes or represses a certain cell fate (Betschinger and Knoblich 2004). The neuroblast divides into one daughter cell that will retain the neuroblast identity and another daughter cell that will become a ganglion mother cell (GMC). The GMC inherits the fate determinants

and then divides into either neurons or glial cells after an additional cell division (Goodman and Doe 1993). During interphase fate determinants are equally distributed, but during mitosis the fate determinants move from the cytoplasm into the cortical crescent that is positioned in the center of the basal pole of the dividing cell. If the mitotic spindle is oriented correctly then only one daughter cell (the GMC) inherits the fate determinants (Ohshiro et al., 2000; Peng et al., 2000). In *lgl* mutant embryos, asymmetric cell division is disrupted and fate determinants cannot migrate to the basal cortex. Therefore, both daughter cells of the neuroblast receive equal amounts of fate determinant and instead of dividing into a neuroblast and a GMC, the neuroblast symmetrically divides into two neuroblasts.

Role of Lgl in Cell Migration, Metastasis, and Invasion

Cell migration is a fundamental process required for normal development and is destabilized by different pathological situations including tumor cell invasion and metastasis. The Scribble polarity module has been found to regulate cell migration in embryonic dorsal closure, postmitotic ovarian follicular epithelia, and clonal patches of eye imaginal discs (Brumby and Richardson 2005).

While the Scribble polarity module regulates cell migration in different cell types, the role of each of its components: Lgl, Dlg, and Scrib in either promoting or restricting cell migration is dependent upon cellular context (Humbert et al., 2006; Dow and Humbert 2007). For example, in the migration of ovarian follicular boarder cells, *scrib* mutants act to reduce migration whereas *lgl* and *dlg* mutants act to increase boarder cell migration. Therefore, in this particular instance, the roles of Lgl, Dlg, and Scrib differ in

that Lgl and Dlg normally act to suppress migration and Scrib normally acts to promote migration (Zhao et al., 2008).

Defects in cell migration properties that develop in *scrib*, *dlg*, and *lgl* mutants also lead to overproliferation and metastasis of tumors. The effects of Scrib are not so clearly defined in metastasis as compared to Dlg and Lgl. Even though *scrib* mutants show a loss of polarity, they show less invasive behavior than *lgl* or *dlg* mutants (Zhao et al., 2008). The increase in boarder cell migration due to the loss of Dlg or Lgl leads to follicle cells overproliferating, changing in cell polarity and invading between germ cells (Goode et al., 2005; Zhao et al., 2008). The overproliferation that results from irregular cell migration can be further demonstrated by means of transplantation of *lgl* or *dlg* mutant imaginal disc epithelial or brain tissue into a wildtype adult host. The transplantation of this mutant tissue into normal adult tissue results in tumor proliferation and metastasis. This metastasis occurs by means of degradation of the basement membranes and penetration of the muscle layers to infiltrate the host ovary (Woodhouse et al., 1998; Beaucher et al., 2007). However the mechanism that drives this metastatic behavior still remains not fully understood. It is possible that the different roles of these tumor suppressors are due to the differential stability of the cell-cell junctional complexes in different cell types and that this differential stability could be a crucial determinant for the effects of the loss of the Scribble Complex (Humbert et al., 2006).

Human Lgl

The *Drosophila* Lgl gene codes for a cortical cytoskeleton protein that binds myosin II and is involved in establishing and maintaining cell polarity. There are two

human homologues of Lgl called Hugl-1 and Hugl-2 that are also a part of a multiprotein cytoskeletal network. Hugl-1 contains many conserved functional domains found in Lgl including homo-oligomerization domains, a cluster of phosphorylation sites, a myosin II binding domain, and at least two WD-40 repeats (Kalmes et al., 1996; Strand et al., 1994; Grifoni et al., 2004). Hugl-2 has phosphorylation sites that are modulated by p32*, and is positively regulated by the transcription factor Sp-1 and is down regulated by EGF (*Bialucha et al., 2007; Zimmermann et al., 2007). The function of Lgl during development is conserved between Drosophila and humans by rescue of a *Drosophila lgl* mutant with its human homologue Hugl-1 and complementation of yeast mutations by human and Drosophila Lgl proteins (Grifoni et al., 2004). Furthermore, transplantation of *lgl* mutant tissue into a wildtype host behaves just like mammalian metastatic tumors (Agrawal et al., 1995; Woodhouse et al., 1998). Therefore, study of the *Drosophila* tumor suppressor Lgl provides insight into understanding tumor formation and progression, which is applicable to cancer development in humans.

Both Hugl-1 and Hugl-2 take part in the inhibition of many human cancers. The loss of *Hugl-1* has shown to result in colorectal carcinoma formation and an increase in cell adhesion and decrease in cell migration (Schimanski et al., 2005). This data indicates that Hugl-1 plays a role in the suppression of colorectal cancer. *Hugl-1* loss of function has been detected in advanced stages of malignant melanoma and indicates that Hugl-1 also has a suppression role in the development and progression of malignant melanoma. (Kuphal et al., 2006). Hugl1 also has a role in suppressing many other forms of cancer such as solid tumor formation in the prostate, breast, and lung, as well as ovarian cancer (Grifoni et al., 2004). Hugl-2 also proves to have a suppression role of

tumor formation and progression. Hugl-2 is the primary negative target of the zinc finger transcription factor Zeb1. Levels of Zeb1 and Hugl-2 are inversely related in human colon cancer and breast cancer with Hugl-2 expressed in high levels in the tumor and Zeb1 having little expression (Aigner et al., 2007). Furthermore, Zimmermann and colleagues have shown that treatment of primary hepatocytes with epidermal growth factor (EGF) suppresses Hugl-2 thus suggesting regulation of Hugl-2 by the EGF signaling pathway. This study supports the notion that Hugl-2 plays a role in tumor suppression by means of down regulation by EGF (Zimmermann et al., 2007).

The human homologue of Lgl functions in suppressing many forms of human cancer. The fact that Hugl and Lgl are conserved not only physically but also functionally demonstrates how useful the study of the *Drosophila* Lgl is in understanding cell polarity, asymmetric cell division, and metastasis of tumors. It is interesting to note that while Lgl has been shown to have so many roles in the cell its role in the nervous system remains unknown.

Lgl's relationship with Fmr1 during Neural Development

Fragile X syndrome (FraX) is the most commonly inherited form of mental retardation. This disease occurs as a result of a loss of function for the *FMR1* gene on the X chromosome (O'Donnell and Warren, 2002). There is a *Drosophila* homologue of FMR1 known as dFmr1. The *dFmr1* mutants are viable and display defects in synaptic morphology and function, neuronal arborization and circadian rhythm (Lee et al., 2003; Zhang et al., 2001). In addition to having a neuronal function, dFmr1 has several mRNA targets, such as the Drosophila MAP1B (Futsch) which suppresses a *dFmr1* loss of

function phenotype in its mutant form. Zarnescu and colleagues found in 2005 that Lgl is a functional partner of dFmr1 by performing a genetic screen for dominant modifiers of retinal overexpression of *dFmr1* in *Drosophila* (Zarnescu et al., 2005).

They found in their study that dFmr1 and Lgl form a macromolecular complex that includes specific mRNAs as cargo molecules during synaptic development and/or function. They were also able to show that Lgl functions upstream of dFmr1 by studying the effects of *dlgl* overexpression in *dFmr1* mutants at the neuromuscular junction. They found that in *dFmr1* mutants, there is a significant decrease in total number of synaptic boutons at the neuromuscular junction. When the *dFmr1* mutant phenotype is rescued by overexpression of Lgl pre or post synaptically they found no significant change in the total number of synaptic boutons. This data indicates that Lgl functions upstream of dFmr1 during neural development (Zarnescu et al., 2005).

While it is interesting to see that Lgl interacts with dFmr1 during neural development in order to regulate the transport of specific mRNAs what is unknown is whether Lgl has any function independently of dFmr1 during neural development. Based on the interaction with dFmr1 and considering the versatility in roles that Lgl has in the cell, it is very possible and likely that Lgl also functions in the nervous system. Therefore, this paper proposes to asses the role of Lgl in the nervous system through study of the neuromuscular junction.

Model System

If Lgl functions upstream or in parallel with dFmr1 then this leads us to infer that mutant *lgl* phenotypes will be similar to *dFmr1* mutant phenotypes. However, because

Lgl has never been characterized in neural development it is possible that Lgl functions along other pathways to activate other target proteins involved in neural functions independent of dFmr1 (**Fig 1**). In order to characterize Lgl in neural development we have chosen to examine the phenotypic consequences of the loss of *lgl* function at the larval neuromuscular junction (NMJ) as our model system. The NMJ is a great model system for studying neural development for two reasons: i) it is a metabotropic type of synapse, similar to most synapses in the mammalian nervous system and ii) it is an established model for synaptogenesis.

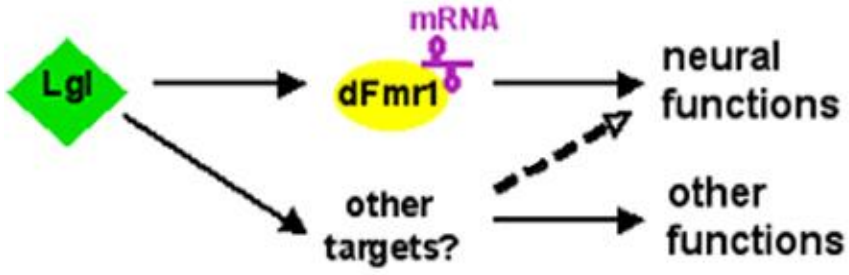

Figure 2. Lgl interacts with dFmr1 and several mRNA targets to produce specific neural functions. This model predicts that *lgl* loss of function in neurons will reveal its specific neuronal functions, which are primarily mediated by *dFmr1.*

The neuromuscular junction is a relatively simple pattern of muscles that are innervated by a few motor neuron axonal branches. The axonal branches of the motor neuron contain synaptic varicosities known as boutons and are arranged in characteristic numbers and patterns in individual muscles (Jia et al., 1993; Prokop et al., 1996). Ultrastructurally, boutons are located along the presynaptic side of the synapse and are covered by basement membranes on one side and closely linked to the muscle on the other side (Prokop et al., 1996). Boutons are categorized by both size and the branch they are located on. Bouton branches that are comprised of boutons ranging in diameter of 1-3 μm are considered small boutons (type Is) and bouton branches that contain

boutons ranging in diameter of 2-5 μm are classified as big boutons (type Ib) (Atwood et al., 1993; Jia et al., 1993). Sites of neuromuscular contact can be labeled with the monoclonal antibody mouse anti-CSP. Mouse anti-CSP binds to the synaptic vesicle associated protein cysteine string protein (CSP) at the NMJ (Bronk et al., 2005). In addition, the NMJ synaptic terminal is fluorescently labeled with an anti-HRP antibody that specifically labels a carbohydrate epitope in the neuronal membrane of NMJ boutons (Katz et al., 1988; Wang et al., 1994).

Inner bouton content is comprised of vesicles that store neurotransmitter and regions known as active zones that form at the neuron-muscle interface (Prokop et al., 1996). Ultrastructurally, active zones are specialized morphological regions consisting of electron dense projections of various shapes including: plaques, pyramids, T-shaped bars and ribbons that extend from the active zone into the presynaptic cytoplasm (Wagh et al., 2006). At the *Drosophila* larval neuromuscular junction the electron dense projection specifically found are in the shape of T bars. Many components that comprise the active zone region have been characterized. The vertebrate ELKS/CAST/ERC proteins are active zone components that take part in the molecular organization of the presynaptic active zone and regulate the release of neurotransmitter (Ko et al., 2003; Takao-Rikitsu et al., 2004). The *Drosophila* homologue to this vertebrate protein is Bruchpilot (BRP). BRP contains an N terminal and a significant amount of sequence homology to vertebrate ELKS/CAST/ERC (Wagh et al., 2006).

These active zones are areas where vesicles of neurotransmitter (at the larval NMJ glutamate is the primary neurotransmitter) get anchored to the inner wall of the bouton and then fuse with the bouton membrane in order to release the neurotransmitter content

into the synaptic cleft by means of exocytosis. The release of neurotransmitter from the presynaptic vesicles into the synaptic cleft is activated by an increase in the Ca^{2+} concentration in the presynaptic terminal. This increase in Ca^{2+} concentration can be attributed by the clusters of voltage gated Ca^{2+} channels that are located close to the vesicle docking sites of active zones (Kittel et al., 2006). The MAB NC82 antibody can be used to label against the BRP protein component of the presynaptic active zone in order to detect active zones at the presynaptic boutons (Hofbauer 1991; Wagh et al., 2006).

The *Drosophila* NMJ contains ionotropic glutamate receptors homologous to mammalian AMPA, NMDA, δ and kainate receptors (Sprengel et al., 2001). These receptors assemble as heteromeric tetramers by choosing from five subunits, GluRIIA, GluRIIB, GluRIIC (also known as GluRIII), GluRIID, and GluRIIE. All glutamate receptors contain the subunits GluRIIC, GluRIID, and GluRIIE which are essential for receptor formation and function (Qin et al., 2005). Each receptor includes a fourth subunit that divides the glutamate receptors into two different glutamate receptor subtypes: A class receptors and B class receptors (Chen and Featherstone 2005; Featherstone et al., 2005). A-class receptors contain the GluRIIA subunit and B-class receptors contain the GluRIIB subunit. A- and B- class receptors are thought to be molecularly identical to one another with the exception of the difference in the one subunit (either GluRIIA or GluRIIB). However, they differ functionally (DiAntonio et al., 1999).

In the studies of Pan and Broadie it was found that in *dfmr1* null mutants A-class GluRs accumulate and that B-class GluRs are reduced, while the total GluR content

remains the same. There is a pre and postsynaptic requirement of dFMRP and DmGluRA for regulating the presynaptic release of glutamate and also postsynaptic class specific regulation of glutamate receptors. These findings indicate that DmGluRA signaling and dFMRP function converge in order to regulate the expression of the two GluR classes synaptically, however independent pathways of DmGluRA signaling and dFMRP function also exist (Pan and Broadie 2007).

Regulating glutamate receptor class composition postsynaptically is crucial for controlling the neurotransmission strength and synaptic plasticity properties. Each class of GluRs has distinct functional properties. The A-class is negatively regulated by protein kinase A phosphorylation and is modulated by atypical protein kinase C. In addition, coracle and actin are required for proper clustering and stabilization of A-class GluRs but not B-class GluRs (Chen et al., 2005). The A-class GluRs are important in retrograde signaling and mediating larger, slower decaying transmission events with a smaller single channel conductance (Davis et al., 1998; DiAntonio et al., 1999; Chen et al., 2005). The mechanism by which B-class GluRs are anchored is due to Dlg. These findings come from studies showing that GluRIIB abundance correlates to Dlg levels, however GluRIIA levels remain unaffected by Dlg (Chen et al., 2005). Therefore, separate mechanisms for regulating A- and B- class GluRs exists.

Materials and Methods

Drosophila stocks and Genetics

All *Drosophila* stocks were maintained at 25°C on standard cornmeal/molasses/agar standard food under standard conditions. To serve as a genetic

background control of the hypomorph *lgl* lines we used a w^{1118} strain that was derived from the genetic background of the *w;* lgl^{1} *FRT40A/Kr:GFP CyO* crossed with a w^{1118} stock. To address any concerns regarding the genetic background effects of the w^{1118} stock, we also used an Lgl genomic rescue line that expresses a wild type Lgl as *P(neo lgl+)* in the presence of lgl^{U334} *cn bw* as a wild type. The Lgl stocks include: *w;* lgl^{1} *FRT 40A/Kr: GFP CyO*, *w;* lgl^{U334}*/Kr: GFP CyO*, and *w;* lgl^{DV275}*/Kr: GFP CyO*. Two different *lgl* allelic combinations were obtained from crossing the null allele lgl^{1} with lgl^{U334} and also with lgl^{DV275}. The genomic rescue line of Lgl was crossed with the *w;* lgl^{1} *FRT40A/Kr: GFP CyO* stock to use as a wild type. All crosses that were set up were comprised of 6 virgin females and 6 to7 males, and were flipped every two days to prevent overcrowding of larvae and potential issues with properly sized third instar larvae for the NMJ dissections.

Antibodies

For our morphology experiments, the larval preps were labeled with the monoclonal mouse α CSP-2 (6D6, a presynaptic marker) (Developmental Studies Hybridoma Bank) at a 1:100 dilution to detect boutons (Bronk et al 2005). The NMJ was co-labeled with the goat polyclonal HRP-FITC (Jackson ImmunoResearch Laboratories Inc.) at a 1:50 dilution. Muscles were stained with phalloidin 647 (Molecular Probes), a high affinity probe for F-actin, at 1:250. To detect the active zones of the NMJ, the monoclonal NC82 (the bruchpilot protein binding antibody) (Developmental Studies Hybridoma Bank) was used at a 1:50 dilution (Kittel et al 2006; Wagh et al 2006). All

the primary monoclonal antibodies were visualized using the same fluorescent dye-conjugated secondary antibody: mouse Alexa 568 (Molecular Probes) at 1:500.

Immunohistochemistry

For both the morphology and active zone experiments, NMJ dissections were conducted on wandering 3rd instar larvae in Ca^{2+} free standard saline solution containing 2MM L-glutamate. These dissections were then followed by fixation in 4% formaldehyde (Ted Pella Inc.) for 30 minutes. After fixation, the larval preps were then washed in 1xPBS twice for 10 minutes each. These washes were then followed by 2 washes in 1xPBHT twice for 10 minutes. These washes were then followed by blocking in PBHT supplemented with 10% normal goat serum. Next, the larval NMJs were then incubated with primary antibodies at 4°C overnight.

For the second day of washes and staining, the primary antibody was washed off the larval preps with two washes of 1xPBHT for 10 minutes each. Following these washes, the larvae were then blocked in PBHT supplemented with 10% normal goat serum twice for 10 minutes each. After this, the larval NMJ preps were incubated with secondary antibody at 37°C for one hour. The secondary antibody was followed by 2 1xPBHT washes for 10 minutes each and then 1 wash of 1xPBS for 10 minutes. These larval preps were mounted in the anti-fade mounting solution Vectashield, which was obtained from the Vector Labs, Orton Southgate, England.

Image Acquisition

Images of the 3rd instar larval NMJ muscle 6/7 segment A3 were acquired on a Zeiss LSM META confocal system (Carl Zeiss Inc., Thornwood NY). Different components of the NMJ were imaged using different objectives. In order to measure muscle area, muscle 6/7 was imaged at a 20X objective. The images of the muscle were taken as a single image. Boutons of the NMJ were imaged with a 40X objective and the active zones of each NMJ were imaged in 3 to 4 segments (NC82 staining) with a 63X objective. Images of the boutons and the active zones were taken as a Z-series which were then later projected into a single image for quantitative analyses.

Quantitative Analysis of the NMJ

In order to determine the size of the NMJ as well as count individual active zones we used MetaMorph Version 6.2r6 (Molecular Devices). The total area of muscle 6/7 for each NMJ was configured by measuring the amount of the microns in the muscle region of each image. To determine the number of boutons/arbor we manually counted each individual bouton by using a manually counting tool in the program that marks each individual bouton. We used the total number of boutons as an indirect method to measure the total size of the NMJ and normalized the size by dividing the total number of boutons by the muscle area. Bouton images were taken in a Z-series and therefore quantification was done on projections of this series.

Upon inspection of the NMJ images it was found that type Ib boutons in the *lgl* mutant appeared abnormally larger and malformed in shape in comparison to the wild type. We recorded the size of type Ib boutons on the Metamorph system by measuring

the total area of all boutons on a type Ib bouton branch. We then divided the different sized boutons for both wild type and *lgl* mutants into bins in increments of 5 μm^2. Bouton sizes were measured for the genotypes: *lgl1/lglU334* (n = 5 larvae and 10 NMJs), *lgl1/lglDV275* (n = 9 larvae and 16 NMJs) and for *w1118* (n = 9 larvae and 18 NMJs)

In order to determine the number of active zones/NMJ we used an automated count system in the program that counted each individual fluorescent dot (equivalent to an NC82 staining for an active zone). We then totaled the number of active zones per NMJ and normalized this data by dividing it by the total muscle area of muscle 6/7. These images were also taken as a Z-series and the quantification analysis was performed on projected images.

Results

Lgl regulates morphology of synaptic boutons at the Neuromuscular Junction

Based on previous studies mentioned Lgl regulates the transport of dFmr1 and specific mRNA types during neural development and Lgl functions either in parallel or upstream of dFmr1 (Zarnescu et al., 2005). Studies have shown that dFmr1 has a role in neural development at the NMJ morphologically as well as in neurotransmission. Loss of dFMRP causes NMJ over elaboration including overgrowth, overbranching and excess synaptic boutons (Zhang 2001). In addition, previous studies have also shown that *dfmr1* overexpression mutants have significantly larger synaptic boutons than controls (Pan and Broadie 2007). Based on our proposed model we set out to determine whether *lgl* mutants express a similar morphological phenotype to the *dfmr1* mutants at the third instar larval NMJ. We performed analyses on w^{1118} wild type controls as well as two

different hypomorphic allelic combinations of *lgl* homozygote mutants: lgl^{1}/lgl^{U334} and lgl^{1}/lgl^{DV275} in order to determine whether there was a change in the overall size of the NMJ. To address any concerns regarding genetic background effects, we also used a genomic rescue line that expresses a wild type copy of Lgl in an lgl^{1}/lgl^{U334} mutant background: $P(lgl+);lgl^{1}/lgl^{U334}$.

The size of the NMJ can be indicated by the number of synaptic boutons at each NMJ. Therefore, type Ib and type Is boutons per terminal were counted manually at muscle 6/7 segment A3 of each NMJ. The monoclonal MαCSP antibody was used to label synaptic boutons at the NMJ and HRP-FITC was used to label the neuronal membranes. To measure the area of muscle 6/7, the F-actin label Phalloidin 647 was used (Liu and Kao 2009).

Morphologically, we found that there was no significant difference in the number of boutons, and therefore, the total size of the NMJ between either one of our wild type controls and our homozygote *lgl* mutants as seen in **Figure 2G**. The average bouton number/muscle area for w^{1118} was .149 +/- .0052, and our N= 24 larvae and 42 NMJs. For our Lgl genomic rescue line our average bouton number/muscle area was .148 +/- .0062 and our N = 4 larvae and 7 NMJs. From this data we were able to verify that the rescue is a good control to use, for its average value was nearly identical to that of our w^{1118} control (**Figure 2G**). For lgl^{1}/lgl^{U334}, our average bouton number/muscle area came to .129 +/-5.14 x 10^-3 and our N = 22 larvae and 40 NMJs. For lgl^{1}/lgl^{DV275}, the average was .131 +/- 6.62 x 10^-2 and our N = 11 larvae and 18 NMJs. Our P values between lgl^{1}/lgl^{U334} and our w^{1118} control was 8.46 x 10^-3 and between w^{1118} and lgl^{1}/lgl^{DV275} our P value = 6.16 x 10^-2. We configured the P value by the Student's T-test. While, there

was no statistically significant difference in the overall size of the NMJ between our mutants and controls we did find upon closer examination that for each individual NMJ the *lgl* mutant boutons appeared abnormally large and malformed in comparison to the round and normal sized control boutons, as seen in **Figure 2 A-F**.

Upon inspection of each individual *lgl* NMJ, we only saw the morphological defect in bouton structure occurring in type Ib boutons. Therefore, we measured the area of each type Ib bouton in our w^{1118} control and both of our *lgl* mutants. We then binned the data into 5 μm^2 intervals with bin 1 corresponding to 0-4.99 μm^2, bin 2: 5-9.99 μm^2, etc. We found that overall; type Ib boutons of both lgl^1/lgl^{U334} and lgl^1/lgl^{DV275} mutants were distributed into significantly larger sized bins than w^{1118} as seen in **Figure 2 H**. The w^{1118} type Ib boutons on average, assumed the normal size of type Ib boutons (3-5 μm). In addition, upon inspection, the *lgl* mutant boutons appeared to also be abnormally shaped. This malformation in shape and size could be accounted for by one of three possibilities: i) the type Ib boutons are each abnormally large boutons that are malformed in shape, ii) the type Ib boutons are of normal size and each are composed of or surrounded by much smaller boutons which give them the appearance of being abnormally large and malformed in shape, or iii) the malformed type Ib bouton is actually a cluster of many small boutons that give the appearance of a significantly large and malformed type 1b bouton. We are currently investigating these results further in order to determine the exact effect of *lgl* on the bouton morphology at the NMJ.

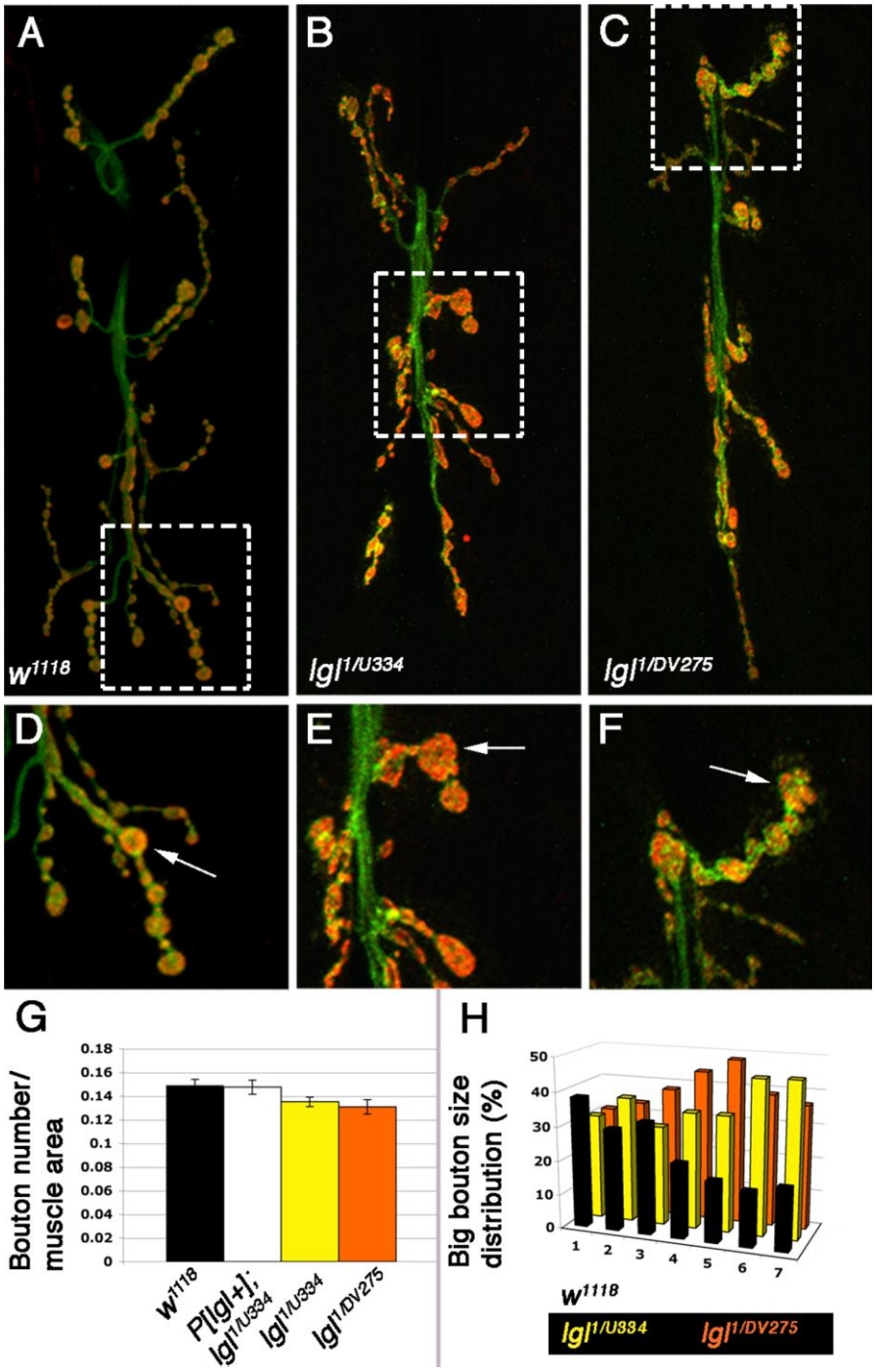

Figure 3. Comparative analysis of *lgl* mutants versus controls at NMJs. Muscle 6/7 segment A3 NMJs stained with CSP and HRP as seen in images (A-C). (D-F) are high magnification views of dashed areas in (A-C). B-C show abnormally large and malformed type Ib boutons (see arrows). (G) The number of boutons normalized to muscle size shows no significant difference between *lgl* and control. (H) *lgl* mutants show larger type 1b boutons compared to control. This data shows the normalized percentage of type Ib boutons per bin.

From what our morphological data has shown us we can infer that Lgl does not play a role in the overall size of the NMJ, however may have a role in regulating the proper size and shape of the synaptic Ib boutons at the NMJ. Our findings support previous findings on the phenotypes of *dfmr1* at the larval NMJ in terms of the increased number of active zones. In the *dfmr1* overexpression mutants, there are significantly larger boutons than found in the controls. This is an interesting observation because these results show to be different from the morphological phenotype that we found in our loss of function *lgl* mutants. Such results indicate that dFmr1 and Lgl do not share the same morphological role at the NMJ. Despite these differences in phenotype, the question of what Lgl's specific role is in regulating the size and shape of the boutons still remains. Does size and shape of these boutons have an effect on proper transmission of neurotransmitter from the presynaptic side of the synapse to the post synaptic side? In order to further explore the potential role of Lgl in neurotransmission we studied its effect on active zones.

Lgl regulates the total number of presynaptic active zones at the NMJ

Previous studies have shown that dFMRP functions not only in regulating morphology at the NMJ but also in other presynaptic functions. It has been found that *dfmr1* null mutants display significantly more active zones per bouton than controls (Pan and Broadie 2007). Despite our findings on Lgl's role in the morphology of synaptic boutons being different from dFmr1's role in the morphology of synaptic boutons, because Lgl functions upstream of dFmr1, it is still likely to expect that we will find

significantly more active zones in our *lgl* mutant as what was seen in the *dfmr1* null mutant.

In order to determine the total number of active zones found at each larval NMJ we stained the active zones with NC82, which labels Bruchpilot, an ELKS/CAST protein at the active zone (Kittel et al., 2006; Wagh et al., 2006). Each individual NC82 puncta at the NMJ was counted as one active zone within each synaptic bouton area. Each synaptic bouton area was defined with presynaptic membrane HRP staining. To determine, on average the number of active zones per NMJ we divided the total number of active zones by the total number of boutons divided by the total muscle area of muscle 6/7.

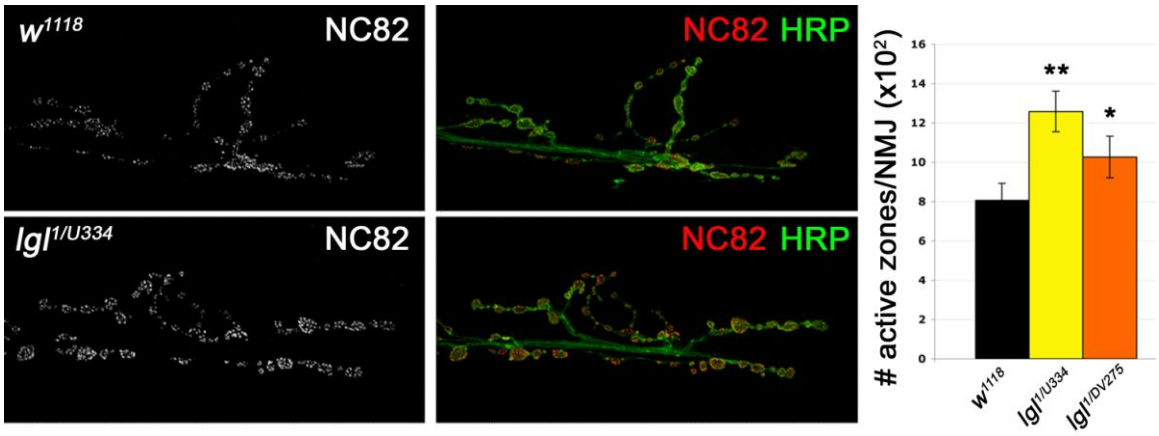

Figure 4. lgl NMJs contain more NC82 positive puncta. Wild type and *lgl* larvae were co-stained with nc82 and HRP as shown. The Student's T-tests were used to calculate the statistical significance of total number of active zones per NMJ (* - P = .05 and ** - P = .01). Analyses were performed at muscle 6/7 of the larval NMJ, segment A3.

We performed this study on the hypomorphic mutants: lgl^{1}/lgl^{U334} and lgl^{1}/lgl^{DV275} and we used w^{1118} as our control. We found from these studies that there were significantly more active zones per synapse in both *lgl* mutants (lgl^{1}/lgl^{U334}: .0126 +/- 1.03 x 10^-3, n = 9 larvae and 16 NMJs, p = 1.59 x 10^-4; lgl^{1}/lgl^{DV275}: .0103 +/- 1.06 x 10^-3, n = 11 larvae and 18 NMJs, p = 5.94 x 10^-2) than in the wild type control as seen

in **Figure 3** (WT: 8.09 x 10^-3 +/- 8.36 x 10^-4, n = 7 larvae and 13 NMJs). There was even a slight difference in the total number of active zones per NMJ between lgl^{1}/lgl^{U334} and lgl^{1}/lgl^{DV275}. This is likely due to the fact that different alleles may have a different effect on the NMJ. The null allele lgl^{1} in combination with another null *lgl* allele (lgl^{4}) is expected based on our findings, to also contain significantly more active zones at the larval NMJ just as found in the *dfmr1* null mutant and will be the next allelic combination of Lgl that we plan to study.

This data displays a similar phenotype to the *dfmr1* null mutant synapses and therefore supports our hypothesis of Lgl playing a role not only in the morphology of the synaptic boutons at the NMJ but also presynaptically by regulating the total number of active zones that form at the synaptic bouton membrane. A question that arises from this data is, while in *lgl* mutants there is significantly more active zones at the NMJ, do all these active zones function properly? A possibility that may occur in *lgl* mutants is that if there is a defect in synaptic transmission of the neurotransmitter glutamate (the NMJ neurotransmitter) than the way the cell compensates for this transmission defect is by producing more active zones even though they may not all necessarily be functional. Another possibility as to why there may be an increased number of active zones is to compensate for a defect on the postsynaptic side of the NMJ. It is possible that if glutamate receptors at the postsynaptic muscle are nonfunctional than in order to make up for this defect, more active zones are produced in order to increase the chances of glutamate uptake by the glutamate receptors that are still functional at the postsynaptic muscle. In order to determine exactly what the cause of this increase in active zones is in *lgl* mutants further analysis is currently being conducted on the expression of glutamate

receptors postsynaptically at the NMJ. This information will be able to help us determine whether or not Lgl shows only a presynaptic function or if it functions on both sides of the synapse.

Discussion

Here we report that Lgl plays a role in neuronal development through studies of the NMJ of *Drosophila melanogaster* 3rd instar larvae. We have examined the morphology of the *lgl* NMJ and have reported findings on Lgl having a role in the size and shape of individual synaptic boutons at the NMJ in addition to having a role in the number of active zones present.

It has been found that Lgl forms a large macromolecular complex with the Fragile X protein FMRP which is developmentally regulated and alters the morphology of the NMJ in *Drosophila melanogaster* (Zarnescu et al., 2005). The FMRP/Lgl complex contains a subset of mRNAs and interacts both physically and genetically with the PAR complex, an essential component for regulating cell polarity. Studies by Zarnescu and colleagues showed that Lgl functions upstream of FMRP to regulate the subset of mRNAs during synaptic development and/or function (Zarnescu et al., 2005). This data suggests that if Lgl functions upstream of FMRP then the two should share common mutant phenotypes. The basis of some our findings in characterizing *lgl* in neuronal development parallel some of the findings in *dfmr1* mutants. Therefore, our results suggest that Lgl has a role in regulating and maintaining the overall morphology of individual type Ib boutons as well as has a role in regulating the amount of active zones that develop presynaptically at the NMJ. Future studies will report on postsynaptic

function of Lgl as well as its role in neurotransmission of glutamate through physiological analysis of *lgl* mutants.

Lgl regulates the morphology of synaptic boutons at the NMJ

In our studies it was found that Lgl does not have a role in controlling the overall size of the NMJ. However, upon inspection of individual NMJ's it was found that the synaptic type Ib boutons appeared abnormally large and malformed in shape. The malformed shape of the synaptic boutons could be accounted for by one of three possibilities, i) each individual bouton is abnormally large and malformed in shape, or ii) each bouton is of normal size and several smaller boutons cluster around the central bouton in order to give it the appearance of being abnormally large and misshaped, or iii) the abnormal shape and size is accounted for by many small boutons clustering together to form what appears as one large malformed bouton. Any one of these possibilities indicate that Lgl is important in regulating the size of the type Ib boutons as well as the shape. This phenotype could be explained by defects in membrane trafficking, microtubule organization, and/or synaptic vesicle accumulation. The defects in shape and size of the big boutons could potentially have an impact on proper neurotransmission of glutamate from the presynaptic terminal of the bouton. These abnormalities could also indicate a role for Lgl in the proper internal structure of the bouton. Without Lgl, it is possible that active zones do not form properly and therefore neurotransmission cannot take place effectively.

Lgl regulates the total number of presynaptic active zones

The *dfmr1* null mutants show significantly more active zones per bouton than the control (Pan and Broadie 2007). Because Lgl has been found to function upstream of FMRP, we expected to find a similar phenotype for Lgl. In our studies we did find a significant increase in the total number of active zones that formed in both of our *lgl* mutants: lgl^{1}/lgl^{U334} and lgl^{1}/lgl^{DV275}. While it would be easy to assume that with a higher number of active zones, these *lgl* mutants have more release of glutamate from their presynaptic boutons, we cannot immediately make this assumption.

As previously discussed, the type Ib boutons of *lgl* mutants appear malformed in shape and abnormally large in size. This malformation in shape and size could account for the increased number of active zones formed in *lgl* mutants. Our results could also be indicative of these *lgl* mutants compensating for malformation of the size and shape of the boutons by having a higher number of active zones. While there are significantly more active zones in an *lgl* mutant, this does not necessarily mean that every active zone is functional. It is possible that because of the malformation of the boutons, active zones do not form properly and therefore many more active zones are made in order to compensate for the production of nonfunctional active zones. It is also likely that an increased number of active zones is produced in order to compensate for a postsynaptic defect. If glutamate receptors at the postsynaptic muscle are rendered nonfunctional than the increased number of active zones may be produced so that more glutamate can be released into the synaptic cleft and thus increase the chances of glutamate activating how ever many functional glutamate receptors are present. In order to determine whether there is a purpose for the increased production of active zones it will be necessary to

characterize the physiological properties of *lgl* NMJs in addition to performing rescue experiments to determine whether the increased number of active zones is in fact compensating for a postsynaptic defect.

Future Directions

So far we have been able to characterize the role of Lgl morphologically as well as show that Lgl may potentially have a role presynaptically and/or postsynaptically at the NMJ. In order to characterize the role of Lgl postsynaptically we plan to analyze the effect that the loss of function *lgl* phenotype has on GluRC, GluRA and GluRB content. We will use immunohistochemistry to study the effect of *lgl* on the total glutamate receptor content by means of staining for GluRIIC which is found in all glutamate receptors at the NMJ. We will then use immunohistochemistry to also stain for the individual classes of glutamate receptors: A-class and B-class by staining for their characterizing subunit either GluRIIA or GluRIIB. Studying the GluR class content of *lgl* mutants should provide insight into how A-class GluRs and B-class GluRs is distributed postsynaptically at the NMJ. Because A-class and B-class GluRs are regulated by different mechanisms and vary in postsynaptic function, this experiment will provide insight into how Lgl impacts the NMJ postsynaptically in neuronal development.

The glutamate receptor experiments also may be able to elucidate more of how Lgl affects neurotransmission of glutamate. If there is a decrease in the total GluRIIC subunit content this could indicate that glutamate may not be able to be taken up effectively by the postsynaptic membrane of the muscle and therefore may not be able to achieve the neuronal response from the presynaptic bouton to the postsynaptic muscle. In

addition a decrease in GluRC content could also indicate a variation in the GluRA/GluRB ratio which could also lead to other defects postsynaptically. If there is an increase in the total GluRC content this could indicate, again, a shift in the GluRA/GluRB ratio and could mean that either there are functionally more active glutamate receptors present to pick up glutamate from the morphologically defected boutons or that the postsynaptic membrane is compensating for nonfunctional glutamate receptors by producing more than the wildtype. Performing these experiments will provide great insight into how Lgl regulates neuronal development both pre and postsynaptically at the NMJ.

To determine whether or not the increased number of presynaptic active zones or the potential increase in GluR content is compensating for the morphological abnormalities at the presynaptic boutons or for nonfunctional active zones or glutamate receptors produced, it will be important to characterize Lgl physiologically. Recordings of the spontaneous release (mEJPs), evoked junctional potentials and the quantal content should help assist us in determining how Lgl affects neurotransmission at the NMJ. We can expect that if we find abnormal transmitter release that we will be able to answer the questions of whether abnormal transmitter release is partially compensated for by the loss of postsynaptic densities or if it is abnormal GluR trafficking and localization and a subsequent homeostatic effect that upregulates active zones.

While it is likely that Lgl functions both pre and postsynaptically, it is also possible that Lgl functions on one side of the synapse more than the other. In order to pinpoint the location of Lgl's function during neuronal development we will rescue the *lgl* mutant phenotype on either the pre or postsynaptic side of the synapse. This will be done by using the Gal4-UAS system. The GAL4 drivers that we will use for rescuing the

lgl mutant are c155 Gal4 (for pre-synaptic expression) and BG487 (for post-synaptic expression). Determining the location at which Lgl functions will also allow us to determine whether the increased number of active zones found in *lgl* mutants is due to a postsynaptic defect. If the *lgl* phenotype can be rescued at the postsynaptic muscle and result in a normal number of active zones than we can infer that the increased number of active zones does in fact compensate for nonfunctional glutamate receptors in an *lgl* mutant.

All of these experiments proposed should assist us in elucidating the role of Lgl during neural development at the NMJ and assist us in determining whether Lgl is required pre- and/or postsynaptically.

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