DROSOPHILA NEUROLIGIN 2 COORIDNATES PRE- AND POST-SYNAPTIC DEVELOPMENT, DIFFERENTIATION AND NEUROTRANSMISSION

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A dissertation submitted to the faculty of the Curriculum in Genetics and Molecular Biology at the University of North Carolina at Chapel Hill in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Chapel Hill
2012

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ABSTRACT

YU-CHI CHEN: *Drosophila* Neuroligin 2 Coordinates Pre- and Post-synaptic Development, Differentiation and Neurotransmission
(Under the direction of Manzoor A. Bhat)

Many cognitive functions including emotion, attention, language, social behavior, learning and memory depend on proper synaptic connectivity in the brain. Synapses are specialized asymmetric cellular junctions responsible for communication between neurons. Synaptic adhesion molecules, neuroligins and their binding partners, neurexins, have been suggested to play an important role in bridging the pre- and post-synaptic machineries across the synaptic cleft. However, detailed molecular mechanisms of how neuroligins function at the synapse *in vivo* still remain unclear. Recently, neuroligins and neurexins have drawn increasing attention due to the link between mutations in human *NEUROLIGINS* and familial autism spectrum disorders (ASDs) (Jamain et al., 2003). Therefore, understanding the role of neuroligins at the synapse may not only improve our knowledge of how synapses are organized but provide insights into the molecular basis of the pathology and etiology of ASDs.

Here we report the generation and phenotypic characterization of *Drosophila neuroligin 2* (*dnlg2*) mutants. Loss of Dnlg2 results in reduced synaptic development at neuromuscular junctions (NMJs) and decreased neurotransmission. *dnlg2* mutant synapses display defects in postsynaptic ultrasctural differentiation. Using UAS/Gal4 system, we demonstrat that both presynaptic and postsynaptic Dnlg2 are required for proper bouton

growth and synaptic transmission. We also show that postsynaptic overexpression of Dnlg2 leads to reduced bouton number and that both pre- and post-synaptic Dnlg2 overexpression leads to synaptic overgrowth at NMJs. Furthermore, we show that *dnlg2* and *dnrx* double mutants display phenotypes that resemble *dnlg2* and *dnrx* single mutants. Our results are in disagreement at multiple levels with those of Sun et al. (2011) which reported increased neurotransmission in *dnlg2* mutants and rescue of synaptic defects by postsynaptic expression of Dnlg2. Our results demonstrate that Dnlg2 functions both pre- and post-synaptically to coordinate synapse development and function at NMJs, thereby raising an interesting possibility that vertebrate Neuroligins may also be required pre- and post-synaptically for proper synapse development and function.

To my families and friends who have been supporting me through this journey.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Manzoor Bhat, who has guided and supported me. I would like to thank the committee members, Eva Anton, Jay Brenman, Steve Crews and Ben Philpot for their support and suggestions. I would like to thank all the past and present labmates, Swati Banerjee, Kevin Blauth, Elizabeth Buttermore, Liz Fisher, James Green, Afshan Ismat, Rosa Mino, Jingjun Li, Raehum Paik, Anil Pillai, and Courtney Thaxton. I am grateful for the help from our collaborator, Hugo Belen and the Bellen lab members, Yong Qi Lin, Koen Venken, Lita Duraine, Kuchuan Chen. I would also like to thank the directors in the core facilities. I thank the Curriculum in Genetics and Molecular Biology, Bob Duronio, Sausyty Hermreck and Cara Marlow, and the Department of Cell and Molecular Physiology, Alan Fanning and Michael Chua, as well as the Neuroscience Center, Vladimir Ghukasyan and JrGang Cheng.

PREFACE

The second chapter of this dissertation has been recently published. I appreciate all the co-authors for their contribution to the manuscript.

Chen Y-C, Lin YQ, Banerjee S, Venken K, Li J, Ismat A, Chen K, Duraine L, Bellen HJ, Bhat MA (2012) Drosophila Neuroligin 2 is Required Presynaptically and Postsynaptically for Proper Synaptic Differentiation and Synaptic Transmission. J Neurosci 32:16018–16030.

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CHAPTER 1

Introduction

1.1 Synapse structure

Our abilities to think, to process feelings and to memorize experiences all depend on the neural networks in our brain. These neural networks are composed of numerous neurons that interconnect with each other through highly specialized junctions called synapses (Li and Sheng, 2003). To insure that the signals are transduced from one neuron to the next in a proper direction, the structure of a synaptic junction is asymmetric. The presynaptic terminals contain synaptic vesicles that are filled with neurotransmitters. When an action potential arrives and induces the opening of Ca^{2+} channels, synaptic vesicles fuse with the plasma membrane and release neurotransmitters into the synaptic cleft. The receptors on the postsynaptic membranes can bind to neurotransmitters, then transduce the signal and lead to series of events at the postsynaptic terminal (Sanes and Lichtman, 2001).

In order to perform this complex process, well-organized presynaptic and postsynaptic machineries are required. Here, I will describe the synaptic apparatuses and the developmental process using the glutamatergic synapse at the vertebrate central nervous system (CNS) as an example (Fig. 1.1).

One of the most obvious characters of presynaptic terminals is a cluster of small vesicles (~40 nm in diameter) that are filled with neurotransmitters (Südhof, 2004). The membranes of synaptic vesicles contain neurotransmitter transporters, such as VGlut1in

glutamatergic synapses, and proton pumps that are required for maintaining the electrochemical gradient that drives neurotransmitter uptake. In addition, synaptic vesicles also contain proteins that are responsible for vesicle trafficking and fusion: Synaptotagmins serve as Ca^{2+} sensors, which translate the signal of Ca^{2+} influx into transmitter release. Rab3 aids the cycling and docking of the vesicles. Synapsin is involved in regulating the synaptic vesicle pool and vesicle recycling (Evergren et al., 2007). Synaptobrevin is part of the SNARE complex, a protein complex that directs the fusion of synaptic vesicle membranes to presynaptic plasma membranes. Vesicle fusion occurs when synaptobrevin binds to syntaxin and SNAP-25, two SNARE complex components on the plasma membrane.

The synaptic vesicle release site, named active zone, is a specialized region with protein complexes clustered at the presynaptic membranes. Ultrastructurally, the active zone is characterized by the electron-dense material on the presynaptic membrane directly adjacent to the synaptic cleft. The synaptic active zone in vertebrate CNS is usually a disk of diameter 0.2–0.5 um (Südhof, 2012). The major components of active zones include RIM, Munc13, RIM-BP, α-liprin, ELKS, piccolo and bassoon. These proteins together form a large platform that clusters Ca^{2+} channels and enables docking, priming and recycling of synaptic vesicles and, therefore, play an important role in mediating synaptic plasticity (Owald and Sigrist, 2009; Südhof, 2012). α-liprin can interact with scaffolding protein complex, CASK/Mint1/Veli, which further connect the active zone matrix to synaptic adhesion molecules and actin cytoskeleton (Butz et al., 1998).

The postsynaptic terminal is equipped with machineries to receive the neurotransmitter released from the presynaptic neuron and to convert the cues into electrical and biochemical signals in the postsynaptic neurons. Protein complexes cluster at the

postsynaptic terminal and form an electron-dense thickening at the postsynaptic membrane called the postsynaptic density (PSD), which is located directly opposed to the active zone (Sheng and Hoogenraad, 2007; Chen et al., 2008). The PSD of the glutamatergic synapses are composed of a large amount of proteins essential for propagating the signals: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are responsible for the fast synaptic transmission. NMDA (N-methyl-D-aspartate) receptors mediate synaptic plasticity. Metabolic glutamate receptors modulate synaptic activity and plasticity via signaling cascades that involve G-proteins (Niciu et al., 2012). In addition, the PSD also contain signaling molecules, such as CaMKII, small GTPase and their regulators, which help transduce the signals from the receptors to the downstream effectors to regulate synaptic activity and morphology. The major scaffolding proteins at the PSD is PSD-95 family proteins which interact with synaptic adhesion molecules, signaling proteins and receptors as well as other scaffolding proteins which further link this whole complex to actin cytoskeleton (Sheng and Kim, 2011).

1.2 Synaptic development and maturation

Development of such delicate apparatuses at the pre- and postsynaptic terminals involves a series of steps and each step is well coordinated temporally and spatially. The first is contact recognition between synaptic partners, which is regulated by the balance of a number of attractive and repulsive cues, including semaphorins, Wnt families, and polysialic acid as well as extracellular glutamate concentration (Scheiffele, 2003). The next step involves trans-synaptic signaling mediated by synaptic adhesion molecules, which then transduce the signals into the cells to recruit pre- and post-synaptic molecules to the nascent

synapse (McAllister, 2007). Studies showed that presynaptic proteins are pre-assembled as multi-molecule complexes in the cytoplasm and transported to the nascent synapse in a saltatory fashion (Zhai et al., 2001; Shapira et al., 2003). Two types of these complexes were observed at the axonal growth cones. The complex that arrives first at the nascent synapse contains active zone proteins such as Piccolo, Bassoon, RIM, Synatxin, SNAP-25, N-cadherin, Munc13, Munc18. Then, the next complex arriving at the synapse contains synaptic vesicle proteins, VAMP, voltage-gated Ca^{2+} channel, synapsin (Ahmari et al., 2000). Both of these precursor complexes reach at the nascent synapse prior to postsynaptic assembly. Synaptic scaffolding proteins, PSD-95, GKAP and Shank, appear to be the first postsynaptic protein complex assembled at the nascent synapse, followed by NMDA receptors, and then AMPA receptors (Washbourne et al., 2002, 2004; Li and Sheng, 2003; Gerrow et al., 2006; McAllister, 2007).

After the assembly of the key pre- and postsynaptic molecules, the newly-formed synapse gradually grows in size and changes its morphology. At the same time, the synapse strength is also increased mostly through the recruitment of more AMPA receptors (Li and Sheng, 2003). During maturation, these synapses then undergo activity-dependent modification that refines the neural networks in response to external stimuli and activity levels. Therefore, active synapses are stabilized or strengthened, while others become silenced or eliminated (Scheiffele, 2003). Recent studies suggest that the activity-dependent synaptic plasticity is mediated by multiple mechanisms (Malenka and Bear, 2004). One of the most extensively studied mechanisms is the NMDA receptor-dependent synaptic potentiation in CA1 region of the hippocampus. This process depends on the distinct feature of NMDA receptors: they respond to glutamate only when the postsynaptic membrane is

depolarized, which removes the Mg2+ block of NMDA receptors. Therefore, NMDA receptors can function as indicators for the timing of sequential synaptic activities. Upon the opening of NMDA receptors, Ca^{2+} influx activates CaMKII as well as many other downstream signaling cascades, which then result in a series of events at the postsynaptic terminal, including phosphorylation and recruitment of AMPA receptors as well as remodeling of actin cytoskeletons and adhesion molecules, thus increasing the synaptic strength and altering the size and morphology of the synapse (Lamprecht and LeDoux, 2004; Malenka and Bear, 2004; Sheng and Hoogenraad, 2007). In addition, studies suggested that the glutamate release machinery at the presynaptic terminal can also be potentiated through the retrograde signaling mediated by trans-synaptic adhesion molecules (Choi et al., 2000; Zakharenko et al., 2002; Malenka and Bear, 2004). Synaptic plasticity allows adjustments of synaptic strength in response to different activity patterns, and thus, the neural networks in the brain can store information acquired from previous experiences. Therefore, synaptic plasticity has been a popular candidate mechanism mediating experience-dependent development, learning and memory (Sheng and Hoogenraad, 2007; Citri and Malenka, 2008).

1.3 Synaptic adhesion molecules

During the processes of synaptic development and maturation, the trans-synaptic adhesion molecules have been proposed to play essential roles. Synaptic adhesion molecules are transmembrane proteins that interact with each other across the synaptic cleft through homophilic or heterophilic adhesions (Yamagata et al., 2003; Washbourne et al., 2004; Giagtzoglou et al., 2009). Intracellularly, the synaptic adhesion molecules interact with pre- and post-synaptic machineries and cytoskeletons directly or indirectly via scaffolding

proteins. Thus, synaptic adhesion molecules not only can serve as molecular "glue" of the presynaptic and postsynaptic terminals (e.g. cadherins and syndecan, nectins, integrins), they have also been implicated in regulating the initial steps of synapse formation (e.g. ephrinB, EphB2, SynCAM, SYGs, sidekicks, Dscam and neurofascin), specifying synapse connectivity, maintaining and aligning mature synapses (e.g. SynCAM, neuroligins, neurexins, LRRTMs and pentraxins) and modulation synaptic plasticity (e.g. N-cadherin, neuroligins, neurexins and NCAMs) (Yamagata et al., 2003; Waites et al., 2005; Craig et al., 2006; Sudhof, 2008; Tallafuss et al., 2010; Missler et al., 2012). Numerous studies tried to identify the roles of these molecules in specific steps of synapse development; however, it appears that each synaptic adhesion molecule involves in multiple processes throughout synapse development and maturation. Multiple redundant pathways could cooperate to organize synapse formation (McAllister, 2007). Among these pathways, neuroligins and their binding partners, neurexins, have received the most attention in this decade. Especially since mutations in human *NEUROLIGINS* were found in autism patients (Jamain et al., 2003), many attempts have been made to study their roles in synapse development and the underlying signaling events (Craig and Kang, 2007; Sudhof, 2008).

1.4 Neuroligin

Neuroligins are a family of single-pass transmembrane proteins localized in the postsynaptic membranes in the mammalian central nervous system (CNS). Neuroligin 1 was first discovered as a ligand of neurexin (Ichtchenko, 1995; Song et al., 1999). Most mammalian genomes contain four neuroligin genes, while human *NEUROLIGIN 4* on the X chromosome has a nonrecombining counterpart on the Y chromosome that is named

NEUROLIGIN 4Y or *NEUROLIGIN 5* (Bolliger et al., 2001). Neuroligin-1 and -2 are exclusively localized to the excitatory and inhibitory synapses respectively, while neuroligin-3 expresses in both excitatory and inhibitory synapses (Prange et al., 2004; Varoqueaux et al., 2004; Chih et al., 2005; Levinson et al., 2005; Budreck and Scheiffele, 2007). All mammalian neuroligins consist of a single extracellular domain which is homologous to acetylcholinesterase (AChE), but are catalytically inactive due to changes in several crucial amino acids (Ichtchenko, 1995). Through this AChE-like domain, neuroligins form dimers and bind to neurexins (Fig.1.1). All neuroligins contain one alternative splicing site in the AChE-like domain (neuroligin 1 contains an additional splicing site), which regulate their binding affinities to neurexins (Ichtchenko et al., 1996; Boucard et al., 2005). The AChE-like domain is linked to the transmembrane domain by a glycosylated linker sequence. Intracellularly, neuroligins contain the sites for interacting with other synaptic proteins. The tyrosine-based motif binds to gephyrin, a scaffolding protein at the GABAergic and glycinergic postsynaptic terminals (Poulopoulos et al., 2009). At the cytoplasmic C-terminal tail, all neuroligins contain a PDZ (PSD-95, Dlg, and ZO-1) domain binding motif which can interact with the third PDZ domain of PSD-95 (Irie et al., 1997; Nourry et al., 2003; Iida et al., 2004; Meyer et al., 2004). The first and second PDZ domain of PSD-95 can interact with glutamate receptors and K+ channels. PSD-95 can also bind to other adaptor proteins, including GKAP and SHANKS, which further link this complex with the cytoskeletal proteins (Kim and Sheng, 2004).

1.5 Neurexin

Neurexins are a family of single-pass transmembrane proteins that were first discovered as the receptors for α-latrotoxin, one of the components in the venom of the black widow spiders that leads to substantial neurotransmitter release from the presynaptic terminal (Ushkaryov et al., 1992). Unlike neuroligins, neurexins are mostly found in presynaptic terminals (Ushkaryov et al., 1992; Berninghausen et al., 2007). There are three neurexin genes reported in mammalian genomes, each containing two promoters driving the expression of α- and β-neurexins (Ushkaryov and Südhof, 1993; Tabuchi and Südhof, 2002). Extracellularly, α-neurexins contain six LNS (laminin, neurexin, sex-hormone-binding globulin) domains that are interspersed by three EGF-like domains, while β-neurexins only contain one LNS domain that are equivalent to the sixth LNS domain of α-neurexins. Although the binding properties are different, both α- and β-neurexins are capable of interacting with neuroligins through the last LNS domain of α-neurexins and the only LNS domain of β-neurexins in a Ca^{2+}-dependent manner. Through the LNS domain, two neurexins bind to the dimerized neuroligins, which form a trans-synaptic complex across the synaptic cleft (Ushkaryov and Südhof, 1993; Ichtchenko, 1995; Missler and Südhof, 1998; Boucard et al., 2005; Comoletti et al., 2006; Araç et al., 2007; Fabrichny et al., 2007). The extracellular region of α-neurexins contain five conserved sites for alternative splicing (β-neurexins contain only the last two sites) which could potentially give rise to thousands of isoforms, and thus these isoforms have been proposed as the codes to specify synapse formation among different pre- and postsynaptic partners (Missler and Südhof, 1998; Tabuchi and Südhof, 2002). This hypothesis is supported by the recent studies showing the differential binding affinities between various pairs of neuroligin and neurexin isoforms

(Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006). In addition to neuroligins, the extracellular region of neurexins can also bind to other postsynaptic adhesion molecules. LRRTMs (leucine rich repeat transmembrane proteins) have recently been identified as a binding partner of neurexins at excitatory synapses (de Wit et al., 2009; Ko et al., 2009). Similar to neuroligins, they can bind to both α- and β-neurexins. LRRTMs also interact with PSD-95 and they regulate the localization of AMPA receptors at the PSD and excitatory synaptic transmissions. In addition, neurexins can also interact with other postsynaptic binding partners, such as cerebellins, dystroglycan and neurexophilin (Missler et al., 1998; Sugita et al., 2001; Uemura et al., 2010). The cytoplasmic region of neurexins interacts with the synaptic vesicle protein synaptotagmin (Hata et al., 1993) and the CASK/MINT1/VELIs complex, which is coupled to synaptic vesicle exocytosis machinery and actin cytoskeletons (Hata et al., 1996; Butz et al., 1998; Biederer and Südhof, 2001).

1.6 Function of neuroligins and neurexins

Since the discovery of the trans-synaptic neuroligins and neurexins complex, many efforts have been contributed to unravel their role during synaptic development and maturation. Surprisingly, different types of assays led to very divergent hypotheses (Sudhof, 2008). The first clue came from a cell culture study in which neuroligin-overexpressed non-neuronal cells induced formation of presynaptic differentiation in the neighboring neurons (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004). Neurexins were also proved to have a similar synaptogenic feature (Graf et al., 2004; Nam and Chen, 2005). These results suggest that a trans-synaptic neurexin-neuroligin complex may play a role in the initiation of synapse formation (Dean and Dresbach, 2006). Consistent with this hypothesis,

overexpression of neuroligins or neurexins in cultured neurons results in increased synapse numbers (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). Specifically, overexpression of neuroligin 1 induces excitatory synapse formation, and neuroligin 2 induces inhibitory synapse formation (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). The above studies suggest the trans-synaptic neurexin-neuroligin complex may play an important role in the initiation of synapse formation (Dean and Dresbach, 2006; Chubykin et al., 2007; Missler et al., 2012). However, knockout mice studies show that neuroligins and neurexins may function at the mature synapse for proper synaptic transmission but are dispensable for the initial synapse formation (Missler et al., 2003; Varoqueaux et al., 2006; Chubykin et al., 2007). Neuroligin-1, 2 and 3 triple knockout mice have normal synapse density but display impaired synaptic function which leads to respiratory failure and postnatal lethality (Varoqueaux et al., 2006). A theory was then proposed to reconcile the discrepancies between the *in vivo* and *in vitro* studies: neuroligins and neurexins actually function to stabilize the transient immature synapses which then turn into long-lasting mature synapses (Sudhof, 2008; Missler et al., 2012). In support of this hypothesis, it was shown that the synaptogenic ability of neuroligins in cultured neurons is indeed activity-dependent (Chubykin et al., 2007) (Refer Table 1.1 for detail of the phenotypes). Interestingly, a recent article showed that reducing neuroligin-3, LRRTM-1 and 2 in cultured neurons from neuroligin-1 knockout mice decreases synapse numbers (Ko et al., 2011). As mentioned in the previous section, LRRTMs are postsynaptic binding partners of neurexins and play a very similar role as neuroligins (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010). This result suggests that there is functional redundancy between different pairs of synaptic adhesion molecules. In addition to the role in synaptic development and maturation,

neuroligins and neurexins have also been implicated in regulating synaptic plasticity and learning; however, further clarification is still needed to understand how they function during synaptic potentiation and what are the underlying mechanisms (Tabuchi et al., 2007; Kim et al., 2008; Etherton et al., 2009; Blundell et al., 2010; Dahlhaus et al., 2010; Choi et al., 2011; Shipman and Nicoll, 2012).

1.7 Implications in neurological disorders

Defects in human *NEUROLIGIN* and *NEUREXIN* genes have been associated with neural developmental disorders and cognitive disease, such as autism spectrum disorders (ASDs) and schizophrenia. ASDs are a heterogeneous group of developmental disorders with diverse neuropsychiatric conditions, characteristics and etiology. They are one of the most common neural development diseases. Approximately 1%-2.6% of the human population is diagnosed as ASDs (Kogan et al., 2009; Kim et al., 2011). The stereotypic symptoms of ASDs include impaired social interactions and communication skills, confined interests, and repetitive behavioral patterns. In some cases, these behavioral conditions are also accompanied by epilepsy and cognitive defects (Schmitz and Rezaie, 2008; Zoghbi and Bear, 2012). Based on the severity and pattern of the neurobehavioral symptoms of the patients, ASDs are further classified as autism, Asperger syndrome, pervasive developmental disorder not otherwise specified (Lord et al., 2000; Pardo and Eberhart, 2007).

The link between neuroligin and ASDs was first reported by Jamain et al. (2003). They found a missense mutation, R451C, in human *NEUROLIGIN-3* and a frameshift mutation in *NEUROLIGIN-4* in siblings with autism and Asperger syndrome. Two missense mutations in *NEUREXIN-1* were also identified in autism patients (Feng et al., 2006).

The association of *NEUROLIGIN-3* and *-4* and *NEUREXIN-1* with ASDs were then further demonstrated by many large-scale screenings. The genetic defects reported include point mutations, translocations, internal deletions, frameshifts, copy number variation, large-scale deletions (Auranen et al., 2002; Laumonnier et al., 2004; Yan et al., 2005; Szatmari et al., 2007; Lawson-Yuen et al., 2008; Daoud et al., 2009; Ching et al., 2010; Bottos et al., 2011; Sanders et al., 2011; Vaags et al., 2012; Schaaf et al., 2012; Steinberg et al., 2012; reviewed in Lisé and El-Husseini, 2006; Schaaf and Zoghbi, 2011).

Interestingly, shank3, the binding partner of neuroligins, has also been associated with ASDs and related neurodevelopmental disorders (Durand et al., 2007; Moessner et al., 2007; Lawson-Yuen et al., 2008; Walsh et al., 2008; Sykes et al., 2009; Awadalla et al., 2010; Gauthier et al., 2010; Waga et al., 2011; Boccuto et al., 2012). Together, mutations in *NEUROLIGIN-3* and *4*, *NEUREXIN-1* and *SHANK-3* as well as the chromosomal abnormalities at these loci can account for a significant proportion of hereditary ASDs (Sudhof, 2008; Schaaf and Zoghbi, 2011). A better understanding of the basic molecular mechanisms of these molecules may thus benefit the development of diagnostic and therapeutic strategies in ASDs.

To understand the etiology and pathology of ASDs and to test new treatments, the first step is to establish a mouse model that can recapitulate autism symptoms found in the patients. Many research groups have analyzed the synaptic and behavioral phenotypes of the mice carrying autism-associated mutations (Table 1.1). Neuroligin-3 R451C knock-in mice display increased inhibitory synaptic transmission in the somatosensory cortex, but show normal excitatory synaptic transmission (Tabuchi et al., 2007). Consistent with the common autistic symptoms, these mice have impaired social interactions and enhanced spatial

learning and memory. Therefore, it leads to the hypothesis that the behavioral symptoms associated with ASDs originate from the imbalance of excitatory and inhibitory synaptic function (Prange et al., 2004; Chih et al., 2005; Lisé and El-Husseini, 2006; Levinson and El-Husseini, 2007; Sudhof, 2008; Etherton et al., 2009, 2011; Yizhar et al., 2011). Similarly, neuroligin-4 knockout mice also show defects in social interactions and communication (Jamain et al., 2008), further supporting the idea of using neuroligin mutants as the model system to study ASDs.

1.8 Using *Drosophila* neuromuscular junction as a model system

Similar to the glutamatergic synapses in mammalian CNS, type I motor neurons of *Drosophila* NMJ also release glutamate as a neurotransmitter (Fig. 1.2) and therefore, has been a very well established model system to study synaptic structure and function (Jan and Jan, 1976a, 1976b; Ruiz-Canada and Budnik, 2006). Besides Dnrx and Dnlg1, many proteins at the *Drosophila* NMJ synapse have been identified as homologs of mammalian CNS synaptic proteins. For example, *Drosophila* Discs large (Dlg) is the homolog of mammalian PSD-95, the synaptic scaffolding protein interacting with mammalian neuroligins (Budnik et al., 1996). Interestingly, the *Drosophila* homolog of the p21-activated kinase (PAK), implicated in ASDs and Fragile X syndrome, is also expressed at the larval NMJ (Parnas et al., 2001; Hayashi et al., 2007). The above homologous players in *Drosophila* NMJ support the idea of using *Drosophila* to study the genetic and molecular aspects of the function of neuroligins in synapse development. Previous studies indicate that *Drosophila* genome contains four neuroligin homologs: Dnlg1 (CG31146), Dnlg2 (CG13772), CG34127, and CG34139 (Fig. 1.3) (Biswas et al., 2008; Banovic et al., 2010; Sun et al., 2011).

1.9 Overall Goal and Hypothesis

In this study, we aim to dissect the role of *Drosophila* Neuroligin (Dnlg2) at NMJ synapse and its interaction with other synaptic adhesion molecules. However, during the course of the study, Sun et al. (2011) also reported a null mutation of dnlg2 and concluded that loss of dnlg2 causes reduced bouton numbers at the larval NMJ and increased evoked junctional responses in the body wall muscle. Contradictory to this report, however, we found reduced synaptic transmission both in the *dnlg2* mutants that were generated previously by Sun et al. (2011) and in the *dnlg2* null mutants that we generated independently. We therefore determined to clarify the function of Dnlg2 by carefully examining the phenotypic consequences of both mutants and further studied the aspects of Dnlg2 function that remain unknown:

1. We have determined the role of Dnlg2 in NMJ bouton morphology, synaptic ultrastructure and synaptic transmission.
2. We have determined the mechanism of Dnlg2 function at NMJ synapse.
3. We have determined whether Dnlg2 and Dnrx are part of the same pathway.

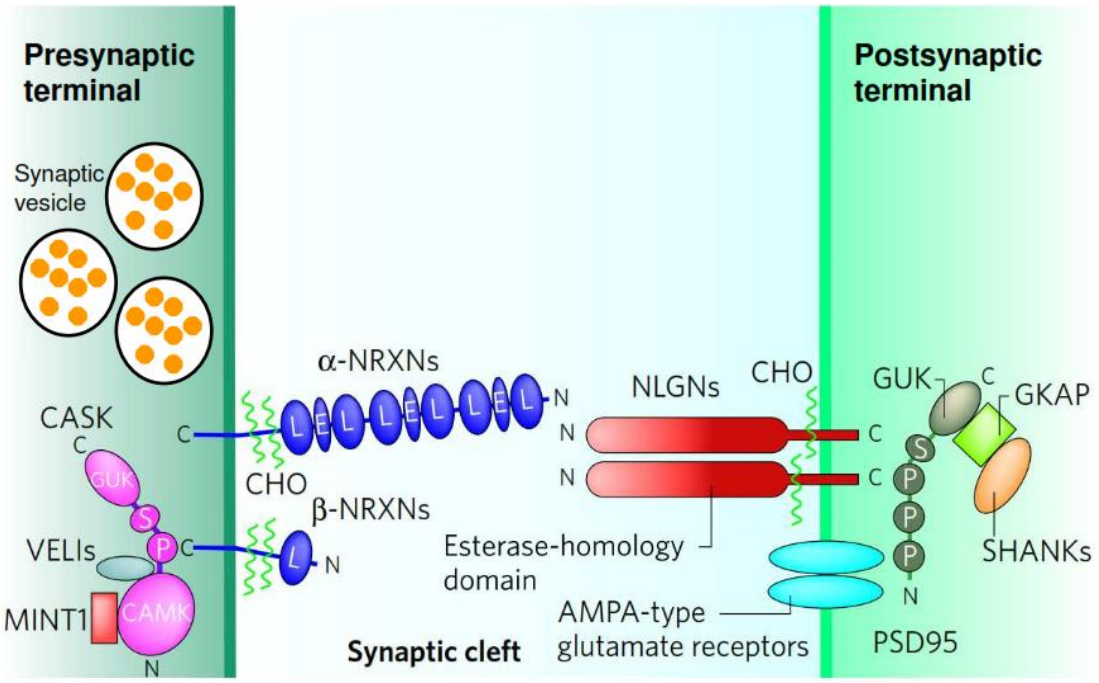


Figure 1.1 Diagram of trans-synaptic adhesion between neurexins and neuroligins. Neurexins interact with CASK, VELIs and MINT1 at the presynaptic terminal, and neurexins bind to the third PDZ domain of PSD-95 as well as GKAP and SHANKs at the postsynaptic side. The first PDZ domain of PDZ domain then in turn interacts with AMPA receptors. (adapted from Sudhof et al., 2008).

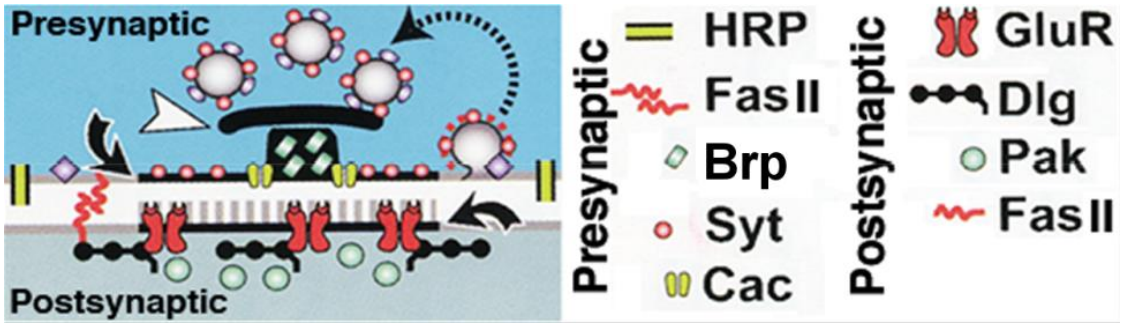


Figure 1.2 Schematic displaying known components at the synapse of Drosophila NMJ (modified from Prokop, 2006). Please refer text for detailed descriptions on the pre- and post-components. FasII: Fasciclin, Cac: Cacophony (a subunit of Calcium channel).

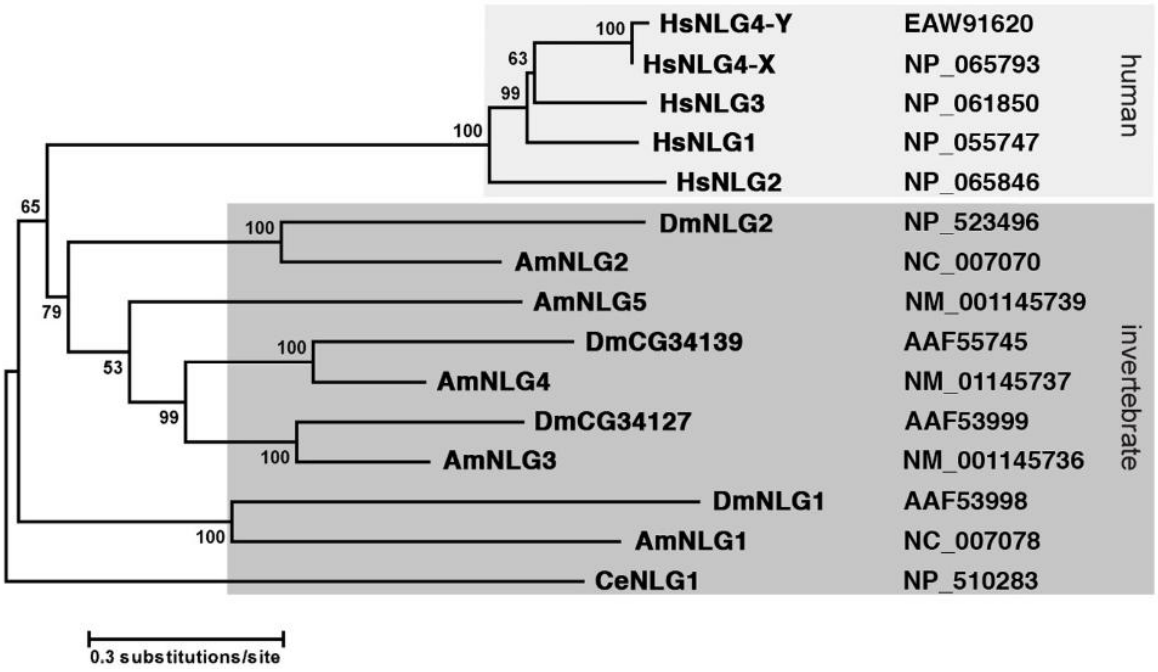


Figure 1.3 Phylogenetic analysis of human, Drosophila, Apis and Caenorhabditis Neuroligin proteins. Drosophila has four neuroligin genes. The branch lengths of the phylogenetic tree represent the evolutionary distances of Neuroligin homologs as the average number of amino acid substitutions. Numbers above the branch are the bootstrap confidence values. Each protein is marked with the species, name of the protein, and GenBank accession numbers (adapted from Biswas et al., 2008 and Banovic et al., 2010). (Hs: Homo sapiens, Am: Apis mellifera, Ce: Caenorhabditis *elegans*, Dm: *Drosophila melanogaster*)

Table 1.1 Phenotypes of neuroligin and neurexin mutant mice

Gene	Mutation	Synapse formation/ structure	Synapse function/ electrophysiology	Behavior	Reference
Nrx1-3	Nrx1-3α triple KO	Density of inhibitory synapses ↓	Calcium currents ↓ Release probability ↓ NMDA receptor function ↓	--	Missler et al., 2003 Kattenstroth et al., 2004
Nrx1	Nrx1α$^{+/-}$ heterozygous	--	--	Response to novelty ↑ Accelerated Habituation to novel environments (only in males, not females)	Laarakker et al., 2012
	Nrx1α$^{-/-}$ homozygous	--	mEPSC ↓ EPSC ↓	Prepulse inhibition ↓ Grooming behaviors ↑ Motor learning ↑	Etherton et al., 2009
Nlg1-3	Nlg 1-3 triple KO	Normal synapse density and size E/I synapse ratio ↑ GABAR clustering ↓	Basal synaptic transmission ↓ Ratio of E/I sPSC ↑	--	Varoqueaux et al., 2006
Nlg-1	Nlg1 KO	Normal synapse density and size	NMDAR/AMPAR ratio ↓ LTP ↓	Spatial learning and memory ↓ Repetitive, stereotyped grooming behavior ↑	Chubykin et al., 2007 Blundell et al., 2010
Nlg2	Nlg2 KO	Postsynaptic specializations (gephyrin) ↓	Inhibitory synaptic transmission ↓	Reactivity to handling ↑ Exploratory activity ↓ Ultrasonic vocalizations ↓ Anxiety-like behavior ↑ Pain sensitivity ↓	Wohr et al., 2012 Blundell et al., 2009 Chubykin et al., 2007 Poulopoulos et al., 2009
	Nlg2 overexpression	Synaptic terminal size ↑ E/I synapse ratio ↓ Vesicle reserve pool ↑	mIPSC frequency ↑	Stereotyped jumping behavior ↑ Exploratory activity ↓ Anxiety-like behavior ↑ Social interactions ↓	Hines et al., 2008
Nlg3	Nlg3 R451C KI	Synaptic terminal size ↓ Vesicle reserve pool ↓ Dendritic branching ↑ Altered NMDA receptor composition	Inhibitory synaptic transmission ↑ AMPA receptor-mediated excitatory synaptic transmission ↑ Altered kinetics of NMDA receptor-mediated synaptic responses LTP ↑	Social interactions ↓ Spatial learning ↑ (No defects in social behaviors in another independent line)	Tabuchi et al., 2007 Etherton et al., 2011 Chadman et al., 2008
	Nlg3 R704C KI	Normal synapse density and size	AMPA receptor-mediated synaptic transmission ↓	--	Etherton et al., 2011
	Nlg3 KO	Normal synapse density	No major synaptic phenotype	Ultrasonic vocalizations ↓ Fear conditioning ↓ Reversal learning ↑ Social memory ↓	Tabuchi et al., 2007 Radyushkin et al., 2009
Nlg4	Nlg4 KO	--	--	Ultrasonic vocalizations ↓ Social interactions ↓ Social memory ↓	Jamain et al., 2008

Nrx, neurexins; Nlg, neuroligin; KO, knockout; KI, knockin; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; E/I, excitatory versus inhibitory

CHAPTER 2

Drosophila Neuroligin 2 is Required Presynaptically and Postsynaptically for Proper Synaptic Differentiation and Synaptic Transmission

2.1 Introduction

Synapses are the fundamental units of neural networks and exhibit tightly apposed pre- and post-synaptic areas that are enriched in cell adhesion molecules (Giagtzoglou et al., 2009). A group of synaptic adhesion proteins thought to orchestrate formation of the pre- and postsynaptic structures are the Neuroligins (Nlgs) and their binding partners Neurexins (Nrxs) (Craig and Kang, 2007; Sudhof, 2008). A growing body of evidence associates these molecules with Autism Spectrum Disorders (ASD), as mutations in human *NLGs* were discovered in ASD patients (Jamain et al., 2003; Szatmari et al., 2007). Nlgs are a family of transmembrane proteins with an extracellular domain that displays homology to acetylcholinesterase (AChE) and localize to the postsynaptic membranes (Ichtchenko, 1995; Song et al., 1999). Nlgs form dimers and bind to Nrxs through this AChE-like domain. At the C-terminus, Nlgs have a PDZ (PSD-95, Dlg, and ZO-1) domain binding sequence motif which can interact with PDZ domain containing proteins (Song et al., 1999; Nourry et al., 2003) such as PSD-95 (Irie et al., 1997; Iida et al., 2004; Meyer et al., 2004).

Mammalian cell culture studies suggested that Nlgs play a role in synapse formation (Scheiffele et al., 2000; Dean et al., 2003; Chih et al., 2004; Nam and Chen, 2005). However, *in vivo* knockout studies of mouse Nlgs and Nrxs revealed normal synapse structure and

numbers but defective synaptic transmission pointing to their role in synapse function (Missler et al., 2003; Varoqueaux et al., 2006), as opposed to synapse formation. To further analyze the Nlg/Nrx function, recent studies utilized *Drosophila* to circumvent the functional redundancy issues and address the function of these proteins *in vivo* (Li et al., 2007; Zeng et al., 2007; Banovic et al., 2010).

Genome analyses in *Drosophila* identify four Nlg-like proteins (CG31146, CG13772, CG34127, and CG34139) (Biswas et al., 2008; Banovic et al., 2010; Sun et al., 2011). We have been attempting to determine the role of CG13772 [*Drosophila* Neuroligin 2 (Dnlg2)], but during the final stages of preparation of this work, Sun et al., (2011) reported the characterization of a null mutation in *dnlg2*. Here we report the generation of an independent null allele of *dnlg2*. We show that loss of Dnlg2 results in reduced synaptic development and neurotransmission. The synaptic function of Dnlg2 is only restored when Dnlg2 is expressed both pre- and post-synaptically at the NMJs, unlike what was reported (Sun et al., 2011). Furthermore, post-synaptic overexpression of Dnlg2 causes reduction in bouton growth, whereas combined pre- and post-synaptic overexpression leads to synaptic bouton overgrowth. We show that double mutants of *dnrx* (Li et al., 2007) and *dnlg2* are fully viable and display phenotypes that resemble *dnlg2* and *dnrx* single mutants. We therefore reach different conclusions than Sun et al. (2011). Our results reveal that Dnlg2 is required pre- and post-synaptically for synapse development and function at NMJs, and that both proteins largely affect the same biological processes *in vivo*, i. e., determining the proper number of active zones and the size of the presynaptic densities.

2.2 Experimental Procedures

Cloning of dnlg2 Full-Length cDNA

A PCR fragment was amplified from fly genomic DNA based on sequence homology with the vertebrate *Neuroligin-1*. The PCR fragment was radiolabeled to screen a *Drosophila* 0–20 hr embryonic cDNA library. Overlapping partial cDNA clones were isolated, sequenced, and compiled as into a full-length cDNA sequence of 4195 base pairs encoding an open reading frame of 1248 amino acids. This cDNA corresponds to *dnlg2*. The GenBank accession number of *dnlg2* sequence is AAF52450.

In Situ Hybridization

PCR amplified DNA fragments from the 3'region of *dnlg2* cDNA were amplified and labeled with digoxigenin-UTP (Roche) as sense and anti-sense probes and used for *in situ* hybridization following standard protocols (Kearney et al., 2004).

Production and purification of Dnlg2 Antibody

Guinea pig polyclonal antibodies against Dnlg2 were generated using a recombinant protein containing the cytoplasmic region of Dnlg2 fused with GST at the N terminus (GST-Dnlg2-CT). The serum was affinity purified after passing it through a GST-Sepharose column followed by binding with GST-Dnlg2-CT-Sepharose. The purified antibody was used at a dilution of 1:50 for immunostaining and 1:100 for immunoblot analysis.

Generation of *dnlg2* Mutants

dnlg2 null alleles were generated by targeted deletion using FLP-FRT recombination (Parks et al., 2004; Thibault et al., 2004). A *P* insertion upstream of the *dnlg2* genomic locus, *P{XP}d02251*, and a *piggyBac* insertion downstream of *dnlg2* locus, *PBac{WH}f04579* were selected. The males from *P{XP}d02251* and *PBac{WH}f04579* were individually crossed to virgin females bearing FLP recombinase. Male progeny carrying both *P{XP}d02251* and FLP recombinase were crossed to females carrying *PBac{WH}f04579* and FLP recombinase. After 2 days of egg laying, the parents and progeny were both heat-shocked at 37°C for 1 hour. On the 3rd day, the parents were removed and the progeny were heat-shocked for 1 hour each day for 4 more consecutive days. After eclosion, mosaic virgin females were mated with *yw;L/CyO* males. The red-eye progeny males were individually crossed to *yw;L/CyO* virgin females to obtain balanced stocks which were analyzed for *dnlg2* deletion by PCR. The following primers were used to verify the targeted deletion of *dnlg2* locus and to determine the breakpoints of the deletion: 5'-TGCTGAGCGCAACAAGGACCA-3', 5'-CGGGTGAATCTCTCCCACTAA-3', 5'-CCAAAGCTCCCGGATTTACC-3', 5'-CTACGTAAAGACTCGGCCCCATTCAGC-3', 5'-CTAACATCTCATCTGGGTCCTC-3', 5'-GACCAGGAGATCAAGATCCGC-3', 5'-CCGAGTCCAAGTCCAACTACA-3', 5'-CGGTTTTGGAATTCTCTAGAAATCTCTTTA-3'.

A *dnlg2* null allele was outcrossed to an isogenized w^{1118} *Canton-S* line (a gift from V. Budnik) for 7 generations and two independent lines $dnlg2^{CL2}$ and $dnlg2^{CL5}$ were balanced over a GFP balancer. For each set of experiment, homozygous *dnlg2/dnlg2* non-GFP wandering third-instar larvae were collected for experimental analyses.

Fly Stocks and Genetics

The same isogenized w^{1118} line used for outcrossing *dnlg2* null allele served as the control for all analyses. *P[acman]BAC CH322-173I20* (Venken et al., 2009), which carries the entire *dnlg2* genomic locus, was used to generate transgenic flies using *PhiC31* integrase-mediated site specific transgenesis (*attP* docking site at 68A4) (Bateman et al., 2006). The *UAS-dnlg2* flies used in rescue experiments were provided by G. Boulianne (Sun et al., 2011). The *dnrx* null allele, $dnrx^{273}$, was used for the genetic analyses in this study (Li et al., 2007). *Df(3R)5C1* (referred in the text as *Df*) which uncovers the *dnrx* locus, has been described previously (Li et al., 2007). *Gal4* lines used for Dnlg2 overexpression were: *C57-Gal4* (Budnik et al., 1996) and *24B-Gal4* (Luo et al., 1994) (expressed mainly in the musculature), *elav-Gal4* (expressed in all neurons) (Lin and Goodman, 1994) and tub^{P}*-Gal4* (expressed ubiquitously) (Lee and Luo, 1999). All stocks and crosses were raised at 21^{o}C. For each set of experiments, all genotypes and crosses were transferred to fresh culture at the same time to maintain consistency. Other fly stocks were obtained from the Drosophila Stock Center, Bloomington, IN.

Immunostaining, Confocal Microscopy, and Bouton Number Quantification

Preparation and antibody staining for whole-mount embryos and dissected wandering third-instar larvae were performed as described previously (Li et al., 2007). Dissected larval fillets were fixed in Bouin's fixative for 15 min. The following antibodies were used: guinea pig anti-Dnlg2 (1:50, this study), guinea pig anti-Dnrx (1:500; Li et al., 2007), mouse anti-GluRIIA (1:250), rabbit anti-GluRIII (1:2000) (Marrus et al., 2004); rabbit anti-Dlg (1:2000) (Woods and Bryant, 1991), mouse monoclonal anti-Brp (1:500) (Wagh et al., 2006). The

Dnrx signal at the NMJ was detected by using the VECTASTAIN ABC system (Vector Laboratories) and Tyramide Signal Amplification (TSA, Invitrogen-Molecular Probes) (Li et al. 2007). Secondary antibodies conjugated to Alexa 488, 568, and 647 (Invitrogen-Molecular Probes) were used at 1:400. Fluorescence-conjugated anti-HRP (Jackson Immuno Labs) antibodies were used at 1:50.

Samples for each set of experiments were processed simultaneously, stained in the same tube and imaged with the same parameters using Olympus FV1000 confocal microscope. Quantification of bouton numbers was performed at muscles 6/7 and muscle 4 of abdominal segment 3. Type Ib boutons at NMJ6/7 and at NMJ4 were visualized and quantified by staining of body wall muscle preparations with anti-HRP and anti-Dlg. Quantification for bouton numbers was normalized to wild type.

Quantification of fluorescence intensity

Control w^{1118} and all mutants were immunostained with anti-GluRIIA and anti-Brp or anti-GluRIII and anti-Brp. Terminal boutons at each branch of NMJ6/7 from five to seven animals of each genotype were scanned by confocal microscopy. Confocal stacks were acquired using the same settings with 0.25 μm steps through entire synaptic boutons. Images were processed using Volocity 5.3 (Improvision) software. The fluorescent intensity of GluRIIA or GluRIII in each terminal bouton was determined by integrating the fluorescent intensity of the areas with 15% to 100% intensity of the whole image. The integrated intensity of GluRIIA or GluRIII was then divided by the number of active zones in each bouton to obtain the level of GluRIIA or GluRIII fluorescence intensity per active zone. The total number of active zones at NMJ6/7 was quantified using the function “separate touching

objects" of Volocity. The areas with anti-Brp staining intensity at 10% to 100% were selected and the touching dots were separated using 0.03μm as the size reference.

Electron Microscopy and Morphometric Analysis

For ultrastructural NMJ studies, third-instar larval fillets were dissected at room temperature in ice-cold calcium free HL-3 medium (Stewart et al., 1994) containing 70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM Trehalose, 5 mM HEPES, 115 mM Sucrose; pH 7.2 and subsequently fixed overnight in 4% paraformaldehyde/1% glutaraldehyde/0.1 M cacodylic acid (pH 7.2). Microwave irradiation (MWI) with the PELCO BioWave® 34700 laboratory microwave system was used for subsequent EM processing steps. After overnight fixation, the fixed fillets were additionally fixed at 640W with a cycle of 10 sec on, 20 sec off, 10 sec on, followed by 4x water rinses at 150W for 40s each, post-fixed with 1% aqueous osmium tetroxide 2x at 90W with a cycle of 2 min on, 2 min off, 2 min on under vacuum and placed on ice in between changes with additional 1 hour incubation on rotator, dehydrated in increasing ethanol concentrations 1x at 150W for 40s each, followed by propylene oxide 2x at 250W for 40s each. Samples were gradually infiltrated with increasing resin to propylene oxide ratio up to full resin 2x at 250W for 3min each under vacuum. The samples were embedded in flat silicone mold with EMBED-812 resin and cured in the oven at 60^{o}C.

ImageJ 1.40g (National Institutes of Health, USA) was used for morphometric analyses of EM images. Only Ib boutons (diameter > 1.5μm) with clear SSR from muscles 6 and 7 in the third and fourth segments were examined and quantified. The bouton diameter was determined by bouton perimeter divided by π (~3.141593).

SSR width was quantified as described in Budnik et al. (1996). Three to four different measurements were made from postsynaptic density (PSD) to distal SSR for each bouton. The SSR width was then calculated by averaging these measurements. To reduce the effect of bouton size, the averaged SSR width was further normalized by the diameter of the bouton (averaged SSR width / bouton diameter). The postsynaptic area was defined as the area between the PSD and the SSR. Only those active zones which clearly showed postsynaptic area were measured. (N represents the number of boutons analyzed while n is the number of active zones).

Electrophysiology

Wandering third-instar larvae were dissected in ice-cold zero calcium HL-3 solution (Stewart et al., 1994). Dissected larvae were then rinsed three times with HL-3 with 0.5 mM Ca^{2+} and incubated in HL-3 with 0.5 mM Ca^{2+} for at least 3 min before recording. Body wall muscle 6 (abdominal segment A3 only) was used for intracellular recordings with sharp electrodes filled with a 2:1 mixture of 2 M potassium acetate and 2 M potassium chloride (32-40MΩ). Data were collected only when resting membrane potential was below -65 mV. The recording data were discarded when resting membrane potential shifted more than $\pm$ 5 mV during the course of experiment. In addition, only one muscle per larvae was recorded in each individual experiment. Excitatory junction potentials (EJPs) were recorded by directly stimulating the segmental nerve innervating each hemisegment A3 through a glass capillary electrode (internal diameter, ~10μm) at 0.2 Hz. The applied currents were 6 $\pm$ 3μA with fixed stimulus duration at 0.3ms. This is 50% more than that required to activate both Ib and Is boutons on the recorded muscles. Twenty to thirty evoked EJPs were recorded for each

muscle for analysis. Miniature EJPs (mEJPs) events were collected for 5 minutes. Both EJPs and mEJPs were amplified with an Axonclamp 2B amplifier in bridge mode under the control of Clampex 8.2 (Axon Instruments Inc). All experiments were performed at room temperature (20^{o}C–22^{o}C).

EJPs and paired-pulse stimulation were analyzed with pClamp 9.2 software (Axon Instruments). mEJPs was analyzed using the Mini Analysis Program (Synaptosoft Inc., Decatur, GA). Evoked EJP amplitude was corrected by using nonlinear summation (Feeney et al., 1998). The quantal content of evoked release was calculated from individual muscles by ratio of the averaged EJP and averaged mEJP amplitude. Statistical analyses of EJP and mEJPs between genotypes were made using Student's t test (SigmaPlot 10.0, Systat software Inc.).

Immunoprecipitation and Immunoblotting Analysis

The immunoprecipitation (IP) experiments were carried out as previously described (Banerjee et al., 2010). Briefly, fly heads of desired genotypes were homogenized using a glass homogenizer in a weight/volume ratio of 1:3 in ice cold lysis buffer containing 50mM HEPES (pH 7.2), 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and 1% NP-40 with protease inhibitors. The lysates were kept on ice for 10 minutes and centrifuged at 50,000× g for 30 minutes at 4°C, and used subsequently for IP and immunoblot analysis. For each IP reaction, 100µl of supernatant was pre-cleared with Protein A beads followed by incubation with primary antibodies at 1:20 dilution (anti-Dnlg2, anti-Dnrx) for 8 hours at 4°C. The supernatant-antibody mix was incubated with 25µl of pre-washed Protein A beads for 2 hours at 4°C. The beads were then washed three times in PBS followed by elution of the

immunocomplexes in 30µl of PBS/SDS buffer and resolved on SDS-PAGE for immunoblotting with respective antibodies. Anti-Dnlg2 was used at 1:100 and anti-Dnrx was used at 1:500 for immunoblot analysis.

2.3 Results

Generation of *dnlg2* null mutants

The domain structure of Dnlg2 is similar to that of mammalian Nlg1. The extracellular domain contains an N-terminal signal peptide and an acetylcholinesterase-like (AChE) domain. This is followed by a transmembrane domain (TM) and a cytoplasmic region with a PDZ binding motif (PBM) (Fig. 2.1A). The AChE domains of *Drosophila* Dnlg2 and human Nlg1 (NCBI Reference Sequence: NP_055747.1) (Saus et al., 2010) share ~36% amino acid sequence identity and ~56% similarity (Fig. 2.1A). To determine the *dnlg2* expression in the *Drosophila* we performed *in situ* hybridization in embryos. A *dnlg2* probe recognizing the transmembrane region revealed that *dnlg2* is primarily expressed in the ventral nerve cord (VNC) and the brain of stages 14-16 embryos (Fig. 2.1B). In addition, Dnlg2 expression is also observed at low levels in the embryonic musculature (data not shown).

To study the function of Dnlg2 *in vivo*, we generated *dnlg2* null mutants using a P-element and a PiggyBac that flank the gene and carry FRTs. Upon induction of FLP in the germline the DNA between the FRT sites is deleted (Parks et al., 2004; Thibault et al., 2004) (Fig. 2.1C and Fig. S2.1) resulting in a 32.6 kb deletion that includes the *dnlg2* locus and *CG13773* (Fig. 2.1C). To avoid issues with the genetic background, two *dnlg2* deletion alleles were outcrossed to an isogenized wild-type strain for seven generations and two independent excision stocks named $dnlg2^{CL2}$ and $dnlg2^{CL5}$ were established. Both $dnlg2^{CL2}$ and $dnlg2^{CL5}$ are homozygous viable. The endpoints of the deletions were established by PCR using the primers shown (Fig. 2.1D).

Next we generated antibodies against Dnlg2 to determine its subcellular localization in the third instar larvae. Immunostaining using anti-Dnlg2 and anti-Bruchpilot (Brp), a marker for presynaptic active zones (Wagh et al., 2006; Weyhersmüller et al., 2011) indicates that Dnlg2 and Brp are localized to CNS synapses of the VNC (Fig. 2.1E). No staining was observed in *dnlg2* mutants (Fig. 2.1F). To determine whether Dnlg2 is present pre- and/or post-synaptically at larval NMJs, we carried out immunostaining of 3rd instar larval musculature. Despite generating 11 antibodies against Dnlg2, we were unable to detect Dnlg2 at NMJs. Although Sun et al. (2011) reported that Dnlg2 localizes post-synaptically at the larval NMJs, we were not able to detect NMJ labeling using the anti-Dnlg2 with the protocol reported by Sun et al. (2011). We thus conclude that Dnlg2 levels at the larval NMJs are too low to be consistently detected.

To determine the relative molecular weight of Dnlg2 and to establish that *dnlg2*^{CL2} is indeed a null allele, we carried out immunoblot analysis of wild type and *dnlg2*^{CL2} adult heads lysates using a polyclonal guinea pig anti-Dnlg2 antibodies (this study). As shown in Fig. 2.1G, wild type lysates show the presence of a robust ~70 kDa band (Fig. 2.1Ga, red arrowhead) at a shorter exposure time which is absent in the *dnlg2*^{CL2} lysates. Upon longer exposure (30 minutes) a faint ~145 kDa band (Fig. 2.1Gb, red arrow, asterisk) is visible, which is absent from *dnlg2* lysates. In order to confirm the presence of the ~145 kDa band in the wild type lysates we split the blot and probed them separately with anti-Dnlg2 (Fig. 2.1H). We were able to detect both ~145 kDa (Fig. 2.1Ha) and ~70 kDa (Fig. 2.1Hb) bands in wild type lysates that were missing from the *dnlg2* lysates using this process. We conclude that the 145kDa band is specific to Dnlg2 and is only visualized when immunoblots are processed separately from the 70kDa band, most likely, as the levels of the 70kDa band are many folds

higher than that of the 145kDa band. The 145kDa molecular weight of Dnlg2 is slightly higher than that predicted from the open reading frame (~138kDa) and was not observed by Sun et al. (2011). These data show that *dnlg2* is indeed a null allele.

Since our immunohistochemical analysis could not detect the presence of Dnlg2 at the wild type larval NMJ (arrows, Fig. 2.1I), we overexpressed the full length *UAS-dnlg2* ubiquitously using *tub^{P}-Gal4* driver (Fig. 2.1J). Upon staining with anti-Brp (red) and anti-Dnlg2 (green), we were able to detect Dnlg2 at the NMJ synaptic boutons (Fig. 2.1J). In summary, our data indicate that Dnlg2 is a 145kDa protein and that it may undergo proteolytic processing or degradation to form a 70kDa isoform. It can easily be detected in the synaptic-rich areas of the larval VNC, but its abundance at NMJs is probably very low.

dnlg2 mutants exhibit a reduced number of boutons at larval NMJs

To determine if the NMJs were affected we performed immunostaining on the larval body walls of wild type and *dnlg2* mutants using anti-HRP to identify neuronal membranes and anti-Dlg to label type I boutons (Fig. 2.2A) (Budnik et al., 1990; Lahey et al., 1994). As shown in Fig. 2.2Ba-c, in *dnlg2* mutants, the number of boutons is severely reduced: they have fewer boutons at muscle 6/7 (NMJ6/7) (Fig. 2.2Ba-c) and muscle 4 (NMJ4) (Fig. 2.2Ea-c) when compared to wild type (Fig. 2.2A and D; quantified in Fig. 2.2F). This defect is caused by the loss of *dnlg2* and/or *CG13773* as this phenotype as well as other phenotypes (see below) are rescued with a genomic *BAC* (*P[acman]BAC CH322-173I20*; indicated by the green line in Fig.1D; Venken et al., 2009) that contains the entire genomic region of *dnlg2* and *CG13773* (Fig. 2.2C and 2.2F). However, *CG13773* is not implicated as ubiquitous expression of *UAS-Dnlg2* using *tub^{P}-Gal4* driver restores bouton number to wild-

type levels in the *dnlg2* excision mutants (Fig. 2.2F; also see later). The boutons in *dnlg2* mutants (Fig. 2.1E) are less defined when compared to the wild type (Fig. 2.1C). The wild type synaptic boutons have a rounded to oval morphology and are separated from each other by a distinct neural process giving a beaded appearance (Fig. 2.2Ea) whereas the *dnlg2* mutant boutons are not well separated (Fig. 2.2Eb). These data show that loss of Dnlg2 causes a reduction of boutons as well as an aberrant overall morphology.

To examine the distribution and localization of pre- and post-synaptic proteins at the *dnlg2* mutant synapses, we performed immunostaining using anti-Brp (pre-synaptic) and anti-GluRIII, (post-synaptic) which labels one of the subunits of *Drosophila* glutamate receptors (Marrus et al., 2004). Although all active zones have both Brp and GluRIII punctae juxtaposed to each other (Fig. 2.2G-I), the level of GluRIII is reduced in *dnlg2* mutants (Fig. 2.2Ha) compared to wild type (Fig. 2.2Ga). Quantification of the fluorescent intensity of GluRIII punctae suggests that there is a 30% decrease in *dnlg2* mutants (Fig. 2.2J). This phenotype is also rescued by genomic *BAC* construct or by ubiquitous Dnlg2 overexpression using tub^{P}*-Gal4* driver in *dnlg2* mutants (Fig. 2.2I, J; data not shown). However, staining with anti-Brp and anti-GluRIIA, another subunit of glutamate receptors, showed that there is a slight, but not statistically significant, increase in the level of GluRIIA in *dnlg2* mutants (Fig. 2.2K-M). These studies suggest that Dnlg2 is required for proper synaptic development and proper postsynaptic protein assembly at the NMJs.

dnlg2 and *dnrx* affect NMJ morphology and function in a similar manner

Studies in mice have indicated that Nrxs and Nlgs interact in trans to function at the synapse (Sudhof, 2008). Moreover, Banovic et al. (2010) recently presented data that

presynaptic DNRX affects Dnlg1 clusters in the postsynaptic densities. However, it has been argued that *dnrx* and *dnlg2* serve different functions at the NMJ as double mutants have a much more severe reduction in bouton number than either of the single mutants (Sun et al., 2011). To assess whether Dnlg2 and Dnrx serve similar or different functions at the NMJ synapses, we examined the morphology and the bouton numbers at the larval NMJs of *dnlg2* and *dnrx* single and double mutants. Both *dnlg2* (Fig. 2.3B) and *dnrx/Df* (Fig. 2.3C) single mutants are null mutations that display a significantly reduced number of boutons compared to their wild type counterpart (Fig. 2.3A, H). Larvae transheterozygotes for $dnlg2^{+/-};dnrx^{+/-}$ exhibit normal NMJ morphology (Fig. 2.3D, H) similar to the wild type (Fig. 2.3A, H). However, $dnlg2^{-/-};dnrx^{+/-}$ (Fig. 2.3E, H), $dnlg2^{+/-}$*;dnrx/Df* (Fig. 2.3F, H) and $dnlg2^{-/-}$*;dnrx/Df* (Fig. 2.3G, H) all display a similar reduction in bouton numbers as *dnlg2* (Fig. 2.3B, H) and *dnrx/Df* single mutants (Fig. 2.3C, H). The differences in bouton numbers between these mutant genotypes (Fig. 2.3B-G) do not reach any statistical significance. Furthermore, the total active zone numbers as visualized by anti-Brp staining at NMJ6/7 did not show any significant difference between the wild type and *dnlg2* mutants (Fig. 2.3I). In addition, whereas Sun et al. (2011) documented that $dnlg2^{-/-};dnrx^{-/-}$ are lethal, we find that our double null mutants are viable, further suggesting that loss of *dnlg2* and/or *dnrx* do not exacerbate the phenotype of the other, consistent with the conclusion that both proteins affect the same molecular events and cause very similar phenotypes at the NMJs.

dnlg2 mutants exhibit synaptic differentiation defects at the NMJs

The *Drosophila* larval NMJ synapse displays stereotypic ultrastructure including the pre-synaptic T-bars and densities as well as post-synaptic specializations, the SSR (Zhai and

Bellen, 2004; Fouquet et al., 2009). Since *dnlg2* mutants display synaptic growth defects at the NMJs (Fig. 2.2), we examined the ultrastructural features associated with the loss of Dnlg2 at synapses. We performed transmission electron microscopy (TEM) analyses on *dnlg2* mutants. Cross sections of the wild type boutons show several active zones with characteristic T-bars surrounded by synaptic vesicles (Fig. 2.4A) (Mendoza-Topaz et al., 2008; Fouquet et al., 2009). A wild type synapse at a higher magnification shows an active zone (AZ), the post-synaptic area (PSA) and SSR (Fig. 2.4B). These NMJ synaptic boutons are embedded in the muscle and surrounded by specialized membrane folds, the SSR. Several defects were observed in *dnlg2* mutants. *dnlg2* mutant boutons exhibit an increased number of active zones in each bouton (Fig. 2.4C). Interestingly, the space between postsynaptic density and the SSR, the PSA, is increased in *dnlg2* mutants (Fig. 2.4C, D; quantified in J). In addition, we find that the width of SSR is severely reduced in *dnlg2* mutants. All these phenotype are rescued by introduction of a *BAC* construct (*P[acman]BAC CH322-173I20*) that contains the genomic region of *dnlg2* (Fig. 2.4E; quantified in Fig. 2.4H-K).

The increase in active zone number per bouton is also observed in *dnrx* mutants (Fig. 2.4F) (Li et al., 2007) and double mutants of *dnlg2* and *dnrx* also exhibit a similar increased AZ numbers and defective PSAs (Fig. 2.4G). Consistent with an increase in number of active zones, we observed an increase in length of postsynaptic density per unit perimeter in all mutants. Together, these data indicate that Dnlg2 plays a critical role in proper post-synaptic differentiation and that Dnlg2 and Dnrx are jointly required for proper synapse organization and maturation.

Dnlg2 and Dnrx form a molecular complex

The morphological analyses presented in the preceding sections indicate that Dnlg2 and Dnrx function together to coordinate synaptic growth at the NMJs. To test if Dnlg2 and Dnrx are present in the same molecular complex, we performed immunoprecipitations (IP) followed by immunoblot analyses using anti-Dnlg2 and anti-Dnrx antibodies. When anti-Dnlg2 antibodies were used for IP in wild type adult fly head extracts, we were able to IP the 145 kDa Dnlg2 protein (Fig. 2.5A). When anti-Dnrx antibodies were used for IP in adult wild type and *dnlg2* fly heads, the anti-Dnlg2 antibody detected the 145kDa Dnlg2 protein in the IP complex (Fig. 2.5B) of wild type but not *dnlg2*. Interestingly, in the same blot, the 70kDa Dnlg2 could not be detected in both the wild type and *dnlg2* IP complex (Fig. 2.5C, arrowhead). These results show that Dnlg2 (145kDa) and Dnrx are present in the same molecular complex. When fly head lysates from wild type and *dnlg2* mutants were immunoprecipitated using anti-Dnlg2 antibodies, the Dnlg2 (70kDa) was abundantly detected in the wild type but not in the *dnlg2* mutants (Fig. 2.5D). To further determine whether loss of Dnlg2 had any effect on the protein stability and levels of Dnrx, we performed immunoblot analysis of equal amounts of wild type and *dnlg2* mutant adult head lysates. We found that the levels of Dnrx in *dnlg2* mutants are comparable with those in the wild type, suggesting that the stability of Dnrx is not affected in *dnlg2* mutants (Fig. 2.5E). Same amounts of lysates from wild type and *dnlg2* fly heads immunoprecipitated with anti-Dnrx antibodies showed the presence of Dnrx in both wild type and *dnlg2* mutants (Fig. 2.5F). These data indicate that the full-length 145kDa Dnlg2 is most likely the functional protein present in the Dnrx complex, while the 70kDa Dnlg2 might be a processed form that is not present in the Dnrx molecular complex.

Dnlg2 is required pre- and post-synaptically for synaptic development at NMJs

Vertebrate studies have shown that Nlgs that are expressed post-synaptically interact with Nrxs expressed exclusively pre-synaptically (Song et al., 1999; Chih et al., 2005; Nam and Chen, 2005; Sudhof, 2008; Wittenmayer et al., 2009). These conclusions were challenged as Nrxs were also observed to be expressed post-synaptically pointing to a complex mechanism of interactions between Nrxs and Nlgs in synapse function and modulation (Peng et al., 2004; Taniguchi et al., 2007). To establish whether Dnlg2 function is required pre- and/or post-synaptically at NMJs, we performed rescue analyses of *dnlg2* mutants (Fig. 2.6B, C) by driving *UAS-dnlg2* pre-synaptically (neurons), post-synaptically (muscle) or ubiquitously (Fig. 2.6D-N). When Dnlg2 was expressed post-synaptically using muscle specific drivers, *C57-Gal4* and *24B-Gal4*, the reduction in bouton number at *dnlg2* mutant NMJs could not be rescued in $dnlg2^{CL2}$ (Fig. 2.6D-G, quantified in Fig. 2.6O). Similarly, expression of Dnlg2 pre-synaptically using neuronal driver, *elav-Gal4*, also failed to rescue *dnlg2* NMJ phenotypes (Fig. 2.6H, quantified in Fig. 2.6O). However, when Dnlg2 was expressed in both neurons and muscles with a ubiquitous driver, tub^{P}*-Gal4*, the bouton number was restored to wild-type levels in $dnlg2^{CL2}$ mutants (Fig. 2.6I, J; quantified in Fig. 2.6O). These data show that Dnlg2 is required pre- and post-synaptically for proper bouton formation and growth at NMJs. We further confirmed that the ability to rescue the *dnlg2* phenotype using *tubP*-Gal4 compared to *24B*- or *C57-Gal4* is not due to higher expression of Dnlg2 in muscles. Interestingly, fluorescence signal intensity quantification showed that Dnlg2 expression in muscles under *24B-Gal4* is significantly higher than tub^{P}*-Gal4* while *C57-Gal4* is statistically comparable to that of *C57-Gal4* (data not shown).

Sun et al. (2011) previously reported that Dnlg2 functions post-synaptically and that *dnlg2* mutant phenotypes at the NMJs are fully rescued by post-synaptic expression of Dnlg2. However, we failed to rescue their $dnlg2^{K070}$ mutants (Sun et al., 2011) by post-synaptic expression of Dnlg2 using *24B-Gal4* and *C57-Gal4* (Fig. 2.6E, G, O). Together, our data indicate that pre- or post-synaptic expression alone of Dnlg2 is not sufficient to rescue *dnlg2* mutant NMJ phenotypes; rather Dnlg2 is required pre- and post-synaptically for proper bouton formation.

Several vertebrate studies have shown that overexpression of Nlgs is sufficient to promote synapse formation in cultured mammalian neurons (Scheiffele et al., 2000; Comoletti et al., 2003; Prange et al., 2004). We therefore assessed whether overexpression of Dnlg2 in the wild type animals affected normal bouton growth at NMJs. Surprisingly, post-synaptic overexpression of Dnlg2 using (*C57-Gal4* and *24B-Gal4*) reduced bouton numbers to levels similar to those observed in *dnlg2* mutants (Fig. 2.6K and L; quantified in Fig. 2.6P). However, pre-synaptic overexpression of Dnlg2 using *elav-Gal4* had no effect on bouton growth (Fig. 2.6M; quantified in Fig. 2.6P). In contrast, when Dnlg2 was overexpressed both pre- and post-synaptically using tub^{P}*-Gal4*, we observe an increase in bouton growth of about 27% when compared to wild type (Fig. 2.6N; quantified in Fig. 2.6O). Hence, Dnlg2 promotes bouton formation and synaptic growth at NMJs when expressed pre- and post-synaptically during development.

Synaptic transmission is reduced in *dnlg2* mutants

As shown in the preceding sections, loss of Dnlg2 results in the reduction of GluRIII levels at NMJs (Fig. 2.2J) and ultrastructural abnormalities at the synapse (Fig. 2.4). We next

examined the consequences of loss of Dnlg2 alone as well as the combined loss of Dnlg2 and Dnrx on synaptic transmission at the NMJs. We performed our electrophysiological analyses on muscle 6 of 3rd instar larval body walls and recorded the evoked excitatory junction potentials (EJPs) in 0.5 mM $[Ca^{2+}]_o$ at 0.2 Hz. Both *dnlg2*^{CL2} and *dnlg2*^{KO70} mutants exhibit a reduction in EJP amplitude which is rescued by the genomic *BAC* construct in *dnlg2*^{CL2} (Fig. 2.7A). Under identical conditions, *dnrx* mutants also have reduced EJP amplitudes, consistent with previous reports (Zeng et al., 2007; Ching et al., 2010). Interestingly, *dnlg2;dnrx* double mutants show a similar reduction in EJP amplitudes as *dnlg2* or *dnrx* single mutants, again suggesting that Dnlg2 and Dnrx function together at the synapse. We observed no significant changes in mEJP amplitudes in all mutant combinations when compared to control wild type (data not shown) and *dnlg2*^{CL2}*;BAC-Res* (Fig. 2.7B). All mutant combinations revealed severely decreased quantal contents compared to wild type (data not shown) and the genomic *BAC* rescue of *dnlg2* mutants (Fig. 2.7C). Interestingly, the total number of active zones at the NMJs on muscle 6/7 are comparable between wild type and *dnlg2* mutants (Fig. 2.3I), indicating that *dnlg2* mutants have a lower release probability due to synaptic structural alterations.

Next we determined the EJP, mEJP amplitudes and the quantal contents when Dnlg2 was expressed using neuronal, muscle and ubiquitous drivers in the *dnlg2*^{CL2} and *dnlg2*^{KO70} mutant backgrounds. Both *dnlg2* mutants showed a significant reduction in EJP amplitudes (Fig. 2.7A) and quantal content (Fig. 2.7C) when Dnlg2 was expressed either pre- or post-synaptically. The EJP amplitude, however, was similar to control levels (*dnlg2*^{CL2}*;BAC-Res*) in both *dnlg2* mutants when Dnlg2 was expressed both pre- and post-synaptically using *tub*^{P}*-Gal4* (Fig. 2.7A). The mEJP amplitudes in *dnlg2*^{CL2} mutants was not significantly different

upon Dnlg2 expression pre- and/or post-synaptically when compared to *dnlg2*^{CL2}*;BAC-Res* (Fig. 2.7B). However, in *dnlg2*^{KO70} mutants, post-synaptic expression of Dnlg2 resulted in a significant increase in the mEJP amplitude (Fig. 2.7B) when compared to wild type and mutant larvae expressing Dnlg2 pre-synaptically or ubiquitously. Together our data show that Dnlg2 is required both pre- and post-synaptically for proper synaptic transmission.

2.4 **Discussion**

Sequence analyses of the *Drosophila* genome revealed 4 *neuroligin* genes and mutational analyses of two of these genes *dnlg1* (Banovic et al. 2010) and *dnlg2* (Sun et al., 2011) revealed that Dnlg1 and Dnlg2 are required independently for synaptic growth and function. Dnlg1 functions post-synaptically and is required for proper synaptic development and differentiation (Banovic et al, 2010). Dnlg2 was also shown to function post-synaptically (Sun et al., 2011), however, some of the previously reported functions of Dnlg2 are inconsistent with the data presented here. We report the generation of mutations in *dnlg2* and characterization of the associated phenotypes. We find that loss of *dnlg2* causes a developmental defect at NMJs, with reduced bouton numbers. This phenotype is fully rescued when *dnlg2* was expressed pre- and post-synaptically, indicating that Dnlg2 is required in both pre- and post-synaptic compartments for normal synaptic growth. Ultrastructural analyses revealed that *dnlg2* mutants have significantly increased numbers of active zones and postsynaptic density length. However, the postsynaptic SSR width is reduced. Electrophysiological measurements revealed that *dnlg2* mutants have reduced EJP amplitude, but normal mEJP amplitude, indicating a reduced release probability. Furthermore, *dnlg2* and *dnrx* double mutants are viable and reveal phenotypes similar to *dnlg2* and *dnrx* single mutants, indicating that *dnlg2* and *dnrx* likely function in the same pathway to coordinate synaptic development and transmission. Finally, our phenotypic rescue data using the *Gal4/UAS* system (Brand and Perrimon, 1993) suggest that Dnlg2 is required both pre- and post-synaptically for proper NMJ bouton growth, synapse structure and neurotransmission.

Although some of our results are in agreement with published data on *dnlg2*, many of the results reported here are in disagreement with the data presented in Sun et al. (2011). First, it was reported that post-synaptic Dnlg2 expression alone is sufficient to rescue the *dnlg2* mutant phenotypes. Using the *dnlg2* mutant alleles reported in Sun et al. (2011) and post-synaptic Dnlg2 expression, we were unable to rescue the bouton growth phenotypes. Second, it was reported that EJP amplitudes are much increased in *dnlg2* mutants. However we find that EJP amplitudes in both *dnlg2* and $dnlg2^{KO70}$ mutants are decreased and that both mutants exhibit a reduction in neurotransmitter release probability. Third, our biochemical studies support the existence of a ~145 kDa molecular weight Dnlg2 that based on protein interaction data is most likely the functional form. This form was not reported in Sun et al. (2011). Fourth and perhaps most importantly, *dnlg2* and *dnrx* double mutants were reported to be lethal by Sun et al. (2011) and to display a more severe phenotype in bouton growth phenotype than individual mutants, suggesting that *dnlg2* and *dnrx* function in parallel pathways to affect the same biological process. We find that the double mutants are viable and have defects that resemble the *dnlg2* and *dnrx* single null mutants in overall NMJ morphology and at the ultrastructural level, strongly indicating that they do not function in parallel pathways. A possible explanation of the double mutant phenotypes documented by Sun et al. (2011) is that genetic background issues contribute to the lethality when combined with *dnrx* mutants. The potential effects of genetic background on NMJ morphology and function have been reported previously in a screen for synaptogenesis mutants (Liebl et al., 2006). Our phenotypic analyses revealed identical results from both *dnlg2* alleles [$dnlg2^{CL2}$ (this study) and $dnlg2^{KO70}$ (Sun et al. 2011)] ruling out any major contributions from the genetic backgrounds between the two independently generated *dnlg2* alleles. However, the

lethality observed in *dnlg2/dnrx* double mutants reported in Sun et al. (2011) could be attributed to contributions from the genetic background.

Drosophila Neuroligins and their role at the synapse

The functions of vertebrate Nrxs and Nlgs are thought to be important for synapse maturation and activity-dependent synaptic modulation, but dispensable for initiation of synaptogenesis (Missler et al., 1998, 2003; Sudhof, 2008). Several recent studies support these functional roles for Nrxs and Nlgs in synaptic plasticity (Kim et al., 2008; Etherton et al., 2009; Dahlhaus et al., 2010; Choi et al., 2011). These studies have raised interesting questions as to whether *Drosophila* Nlgs are involved in synaptic plasticity and modulation. Both Dnlg1 and Dnlg2 are similar in structure, but seem to function independently for synaptic development, organization and function (Banovic et al., 2010; Sun et al., 2011; this study). Although *dnlg1* and *dnlg2* null mutants display some similarities in their NMJ phenotypes, including reduced NMJ bouton numbers, reduced postsynaptic SSR thickness and reduced overall synaptic transmission, they also show key differences. *dnlg1* mutants have fewer active zones at muscle 6/7 and some mutant boutons are completely devoid of postsynaptic markers, which is not observed in *dnlg2* mutants. *dnlg1* mutants show mostly post-synaptic defects, but *dnlg2* mutants also display pre-synaptic defects, such as lower release probability, in addition to post-synaptic structural abnormalities. The similarities in mutant phenotypes suggest that they both are involved in bouton growth and SSR stability, and the differences in mutant phenotypes indicate that they have distinct functions in coordinating synaptic development and synapse differentiation. It is possible that Dnlg1 is involved in the recruitment or stabilization of the post-synaptic machinery, whereas Dnlg2

serves to fine tune and refine synapse organization as is revealed by ultrastructural analysis with increased number of active zones in the remaining boutons (Fig. 2.4). In the absence of Dnlg2 and Dnrx active zone number increase and the synaptic areas are significantly increased suggesting that the mutants fail to prune away ectopic active zones and are unable to refine densities. *dnlg1* mutants on the other hand lack post-synaptic differentiation at the synapses indicating that Dnlg1 and Dnlg2 perform distinct functions during synapse differentiation (Banovic et al., 2010). Interestingly post-synaptic expression of Dnlg1 and Dnlg2 repress bouton growth, implying that postsynaptic Dnlg1 and Dnlg2 may either interact and interfere with the functions of presynaptic proteins or dilute out functions of a key post-synaptic protein/s which is involved in normal bouton growth. How a single pre-synaptic Dnrx protein interacts with post-synaptic Dnlg1 and pre- and post-synaptic Dnlg2 to coordinate synaptic development remains unresolved.

Pre- and post-synaptic requirements of Neuroligins

Many studies have suggested that Nlgs primarily function as postsynaptic adhesion molecules and interact with pre-synaptic Nrxs (Song et al., 1999; Scheiffele et al., 2000; Berninghausen et al., 2007). However, there may be exceptions to the post-synaptic localization of Nlgs, as it was recently reported that a Neuroligin in *C. elegans* is present at both pre- and post-synaptic regions (Feinberg et al., 2008). Along similar lines, it was reported that Nrxs are also expressed in the post-synaptic areas, where they may play a role in controlling synaptogenesis by blocking the functions of post-synaptic Nlgs (Taniguchi et al., 2007). These observations suggest that Nrxs and Nlgs could modulate synapse formation by counteracting each other's functions during synapse formation and/or modulation. Our

data provide evidence in support of both a pre- and postsynaptic function of Dnlg2 in synapse formation. We show that a full complement of boutons at *dnlg2* mutant NMJs is only restored when Dnlg2 is expressed both pre- and post-synaptically. Expression of Dnlg2 only pre- or post-synaptically was not sufficient to restore bouton growth. Surprisingly, overexpression of Dnlg2 in the post-synaptic areas in the wild type animals also leads to a reduction in bouton growth, almost similar to *dnlg2* mutant levels. However, overexpression of Dnlg2 pre-synaptically did not result in such phenotypes. On the other hand, when Dnlg2 is expressed both pre- and post-synaptically in the wild type larvae, there is excess bouton growth at NMJs, similar to when Dnrx is overexpressed pre-synaptically (Li et al., 2007). These data suggest that a fine balance of the Dnlg2 protein levels is critical for normal bouton growth. It is possible that high levels of post-synaptic Dnlg2 may lead to an uncontrolled or untimely interaction with pre-or post synaptic proteins, such as Dnrx and DNlg1, respectively, and hinder bouton growth at NMJs, leading to phenotypes that are similar to *dnlg2* or *dnrx* mutants. A recent study also suggested that some neuroligin functions are neurexin-independent and that neuroligins can form complexes with other proteins at the synapses (Ko et al., 2009). This raises the possibility that pre-synaptic and post-synaptic Dnlg2 functions are dependent on formation of homophilic interactions with itself or heterophilic interactions with other synaptic proteins across the synaptic cleft to organize bouton growth at NMJs. It would be of significant interest to determine how loss of Dnlg2 leads to increased active zones and how mechanistically these functions of Dnlg2 are linked with Dnrx and other synaptic proteins.

In summary, our results show that Neuroligin functions is required pre- and post-synaptically for synapse development. Our observations in *Drosophila* and those of Feinberg

et al. (2008) in *C. elegans* suggest that Nlgs have pre- and post-synaptic functions that may be required to counter balance the functions of Nrxs or other proteins during synaptic growth and modulation. It was recently suggested that post-synaptic Nrxs counter the functions of Nlgs to ensure that synapses do not form at random places. However, in our model, antagonistic functions are unlikely given the similarity in phenotypes between the two mutants. Other synaptic adhesion molecules, such as LRRTM2 (Ko et al., 2009) and the recently identified Teneurins (Mosca et al., 2012) as new interacting partners of Dnrx and Dnlg1, respectively, further add to the complexity of trans-synaptic interactions and synapse organization. In this context, the requirement of Dnlg2 in both the pre- and post-synaptic compartments raises interesting questions about how synaptic organization is fine-tuned, and how signaling pathways regulate the expression of pre- and post-synaptic proteins during synaptic development and maturation. Deciphering the signaling role of Nrxs and Nlgs at the *Drosophila* synapses coupled with structure/function analyses should provide a better understanding of the underlying molecular mechanisms of synapse development and function. Such information will provide critical insights into how these molecules are involved in human health and diseases such as ASD.

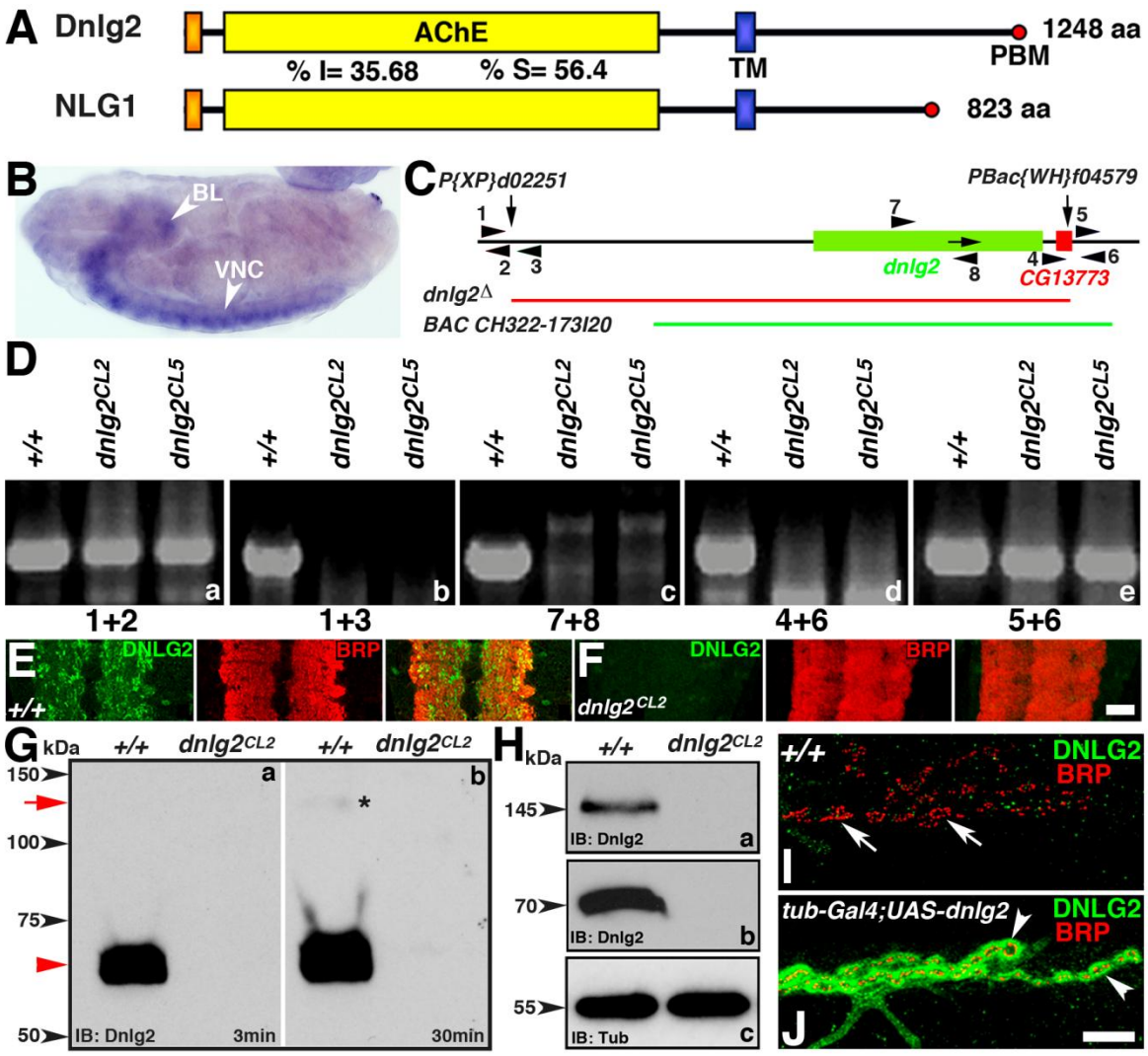


Figure 2.1. Generation of *dnlg2* mutants.
(**A**) Protein domain structure of *Drosophila* Dnlg2 and human NLG1. Similar to human NLG1, Dnlg2 is composed of a signal peptide, an acetylcholine esterase-like (AChE) domain and a transmembrane (TM) domain followed by a PDZ-domain-binding motif (PBM) at the C-terminus. The percent amino acid identity (I) and similarity (S) between Dnlg2 and NLG1 in the AChE domains are indicated. (**B**) In situ hybridization of wild-type embryo at stage 16 using a *dnlg2* labeled anti-sense probe shows mRNA expression in the ventral nerve cord (VNC, arrowhead,) and brain lobes (BL, arrowhead). (**C**) Genomic structure of *dnlg2* and the flanking insertions, *P{XP}d02251* in the 5'-end and *PBac{WH}f04579* in the 3'-end. The arrows pointing down indicate the sites of insertion. The arrow in the *dnlg2* locus shows the direction of transcription. *dnlg2* null mutant was generated using FRT-based recombination. The deleted genomic region is shown by the red line. A genomic *BAC* construct, *P[acman]BAC CH322-173I20*, spanning the region shown by green line was used to rescue the deletion. (**D**) PCR confirmation of the targeted deletion using different primer combinations. The PCR primer sets used are shown as numbers and arrowheads in (**C**). (**E, F**)

VNC sections from 3rd instar larvae of wild type (**E**) and *dnlg2*^{CL2} mutants (**F**) stained with anti-Dnlg2 (green) and anti-Brp (red) show expression of Dnlg2 in the synapse-rich regions of the VNC where Brp is expressed (merged image **E**). Dnlg2 expression is absent in *dnlg2* mutants (**F**). (**G, H**) Immunoblot analysis of Dnlg2. Adult fly head extracts from wild type (+/+) and *dnlg2* mutants immunoblotted with anti-Dnlg2 antibodies. A shorter (**Ga**) and a longer (**Gb**) exposure time reveal the presence of a strong ~70 kDa band in the wild type lysate (red arrowhead). The blot with the longer exposure time shows the appearance of a faint ~145 kDa band (**Gb,** red arrow, asterisk). Immunoblots with anti-Dnlg2 antibodies processed separately (**Ha, b**) detects the upper ~145 kDa molecular weight (arrowhead, **Ha**) and the bottom ~70 kDa band (arrowhead, **Hb**) in wild type lysates that are absent in the *dnlg2* lysates. For protein loading control, the blot was probed with anti-α-Tubulin (**Hc**, arrowhead). **(I, J)** Third instar larval NMJ from wild type (**I**) and *tub*^{P}*-Gal4/UAS-dnlg2* (**J**) show expression of Dnlg2 (green) and Brp (red) at the NMJ synaptic boutons. Scale bars E, F-20μm; I, J-10μm.

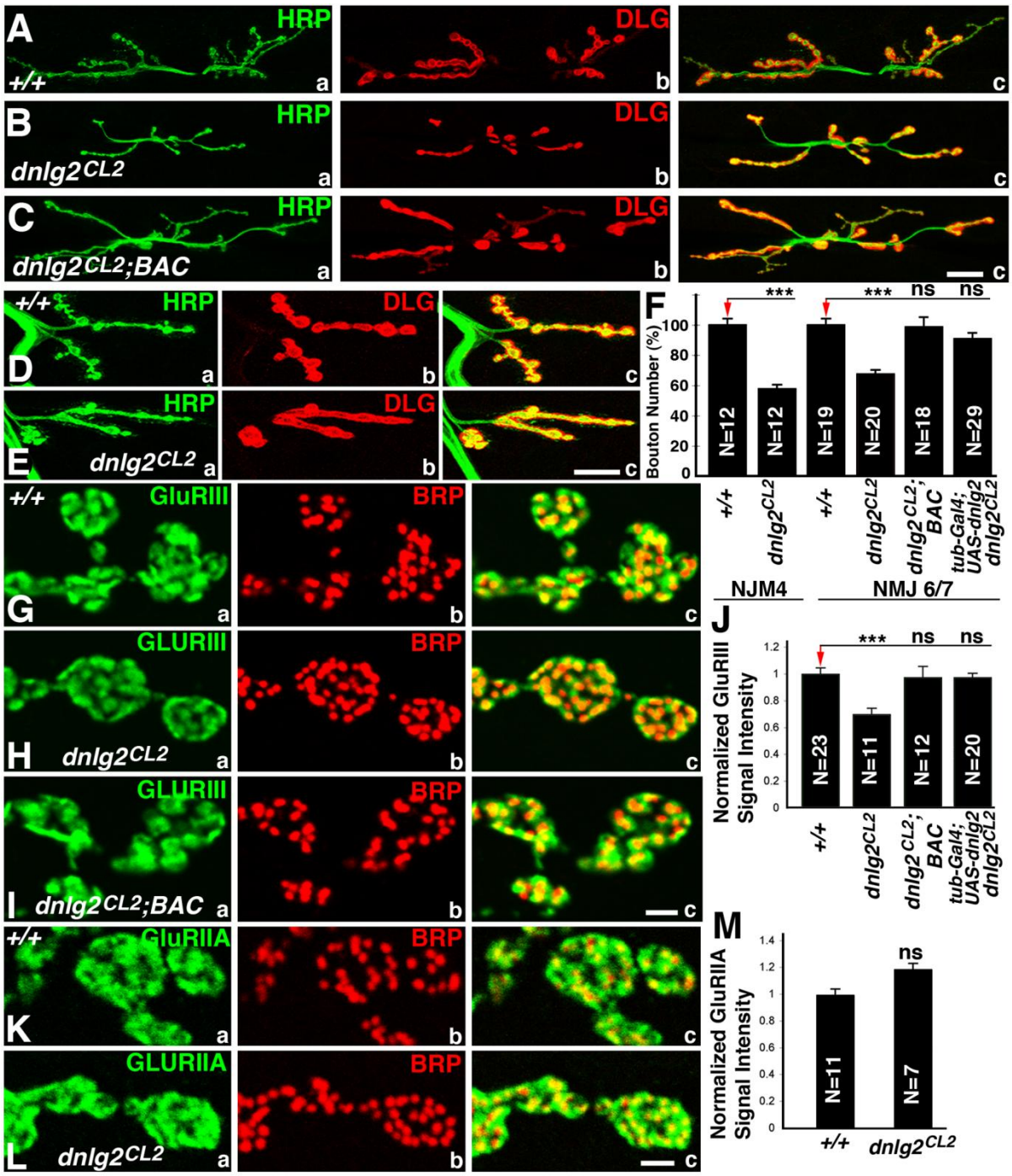


Figure 2.2. Synaptic bouton growth at NMJs is reduced in *dnlg2* mutants.
(**A-E**) Confocal images of NMJ6/7 (**A-C**) and NMJ4 (**D, E**) from abdominal segment 3 of 3rd instar larvae labeled with anti-HRP (green) and anti-Dlg (red). Compared to wild type NMJ6/7 (**A**), *dnlg2* homozygous mutants (**B**) show reduced NMJ expansion and fewer boutons. This phenotype is rescued by a *BAC* transgene containing *dnlg2* genomic region (**C**). At NMJ 4, compared to wild type (**D**), *dnlg2* homozygous mutants (**E**) have fewer boutons, which appeared to be merged. (**F**) Quantifications of type Ib and Is bouton number at NMJ6/7 and type Ib bouton number at NMJ4 adjusted to wild type bouton number. The

bouton number deficits in *dnlg2* mutants are rescued by *BAC* transgene or by ubiquitous Dnlg2 expression using tub^{P}*-Gal4*. (**G-I**) Confocal images of synaptic boutons at segment 3 NMJ6/7 labeled with postsynaptic marker, GluRIII (green) and active zone marker, Brp (red). The alignment of pre- and postsynaptic areas appears to be unaffected in *dnlg2* mutants (**Hc**). However, the levels of GluRIII in *dnlg2* mutants (**Ha**) are significantly reduced. This phenotype is rescued by the BAC transgene (**Ia**). (**J**) Quantification of GluRIII signal intensity with 3D reconstructed confocal images using Volocity software also reveals reduction in intensity in $dnlg2^{CL2}$ mutants. (**K, L**) Confocal images of synaptic boutons at NMJ6/7 labeled with postsynaptic marker, GluRIIA (green) and BRP (red). The alignment of GluRIIA with active zone and the levels of GluRIIA are unaffected in *dnlg2* mutants (**L**). (**M**) Quantification of GluRIIA signal intensity shows slight but not significant increase in *dnlg2* mutants compared to wild type. Error bar = SEM; n.s.: not significant; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (Student's t test). Scale bars A-E, 20μm; G-L, 2μm.

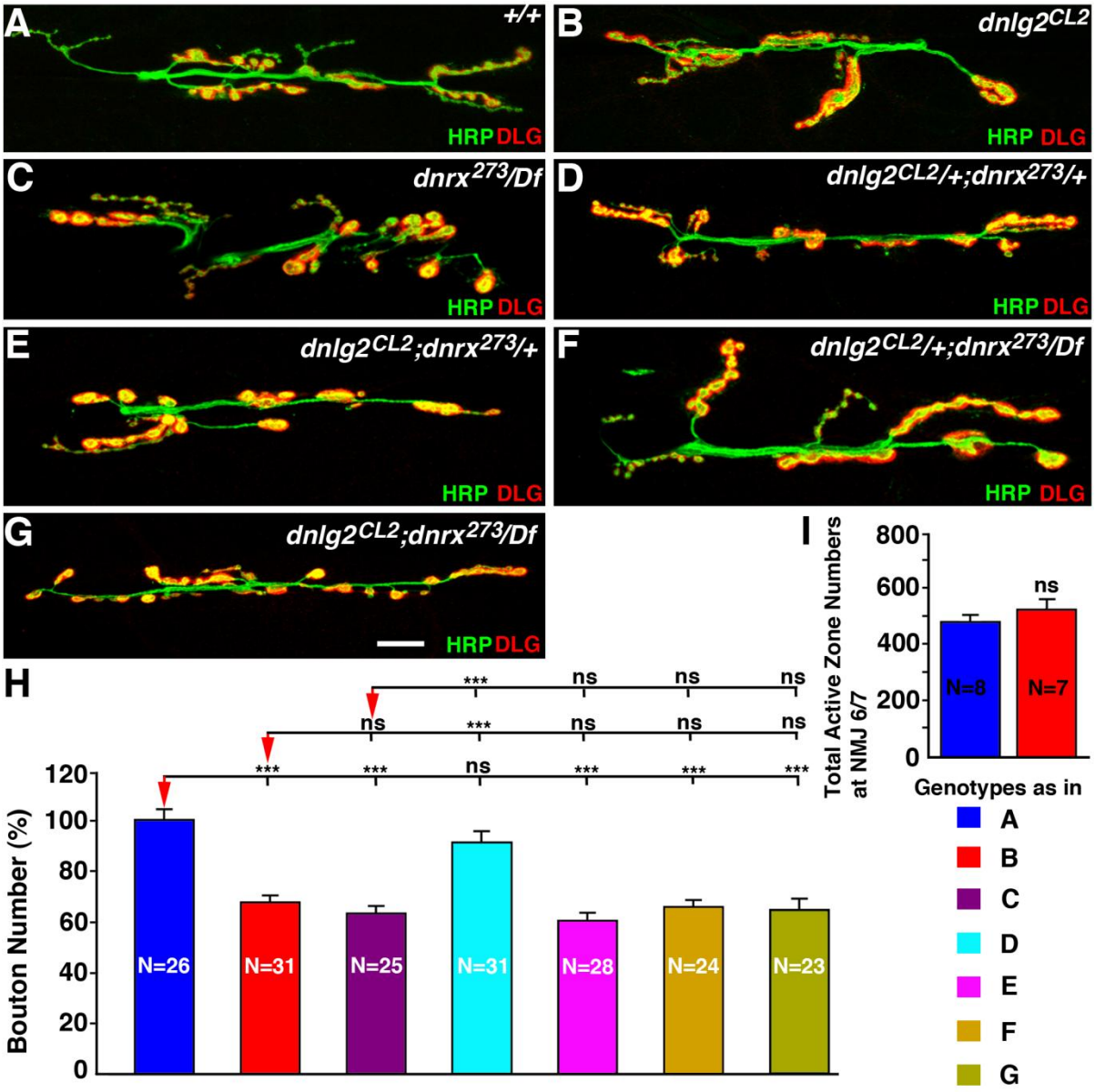
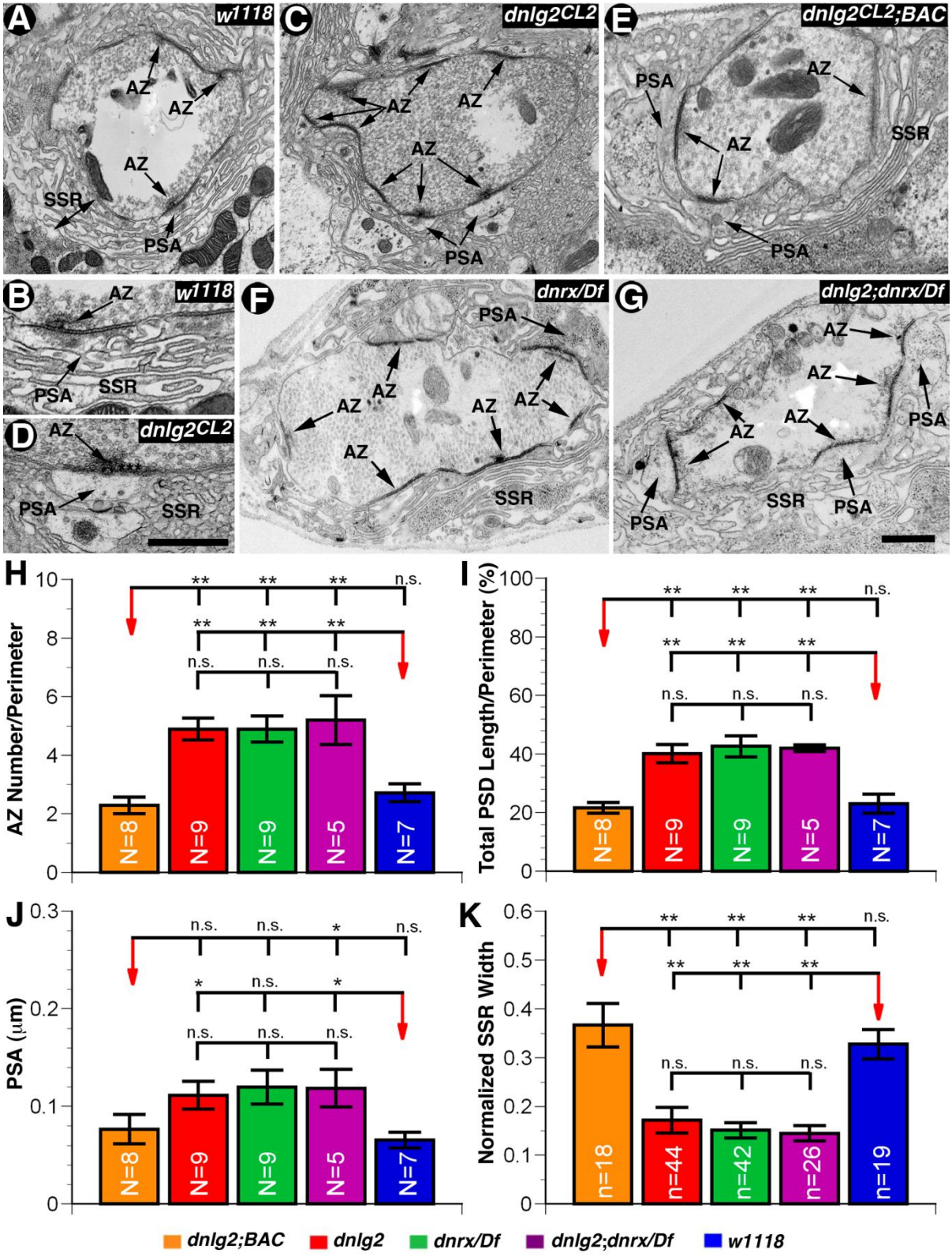


Figure 2.3. *dnlg2* and *dnrx* mutants display similar NMJ developmental defects. (**A-G**) Confocal images of NMJ6/7 from abdominal segment 3 in 3rd instar larvae labeled with anti-HRP (green) and anti-DLG (red). Compared to control (w^{1118}) (**A**), *dnlg2* mutants (**B**), *dnrx/Df* mutants (**C**), *dnlg2;dnrx*$^{+/-}$ (**E**), *dnlg2*$^{+/-}$*;dnrx* (**F**), *dnlg2;dnrx* double homozygous (**G**) show fewer boutons. The NMJ morphology of transheterozygous *dnlg2*$^{+/-}$*;dnrx*$^{+/-}$ (**D**) is unaffected. (**H**) Quantification of total bouton number at NMJ6/7 adjusted to control bouton number. *dnlg2*, *dnrx/Df* single mutants, *dnlg2;dnrx*$^{+/-}$, *dnlg2*$^{+/-}$*;dnrx/Df*, and *dnlg2;dnrx/Df* double homozygous have approximately 60-62% boutons compared to control. ***p < 0.001; **p < 0.01; *p < 0.05 (ANOVA, Tukey *post hoc* test). Scale bar= 20µm. (**I**) Quantification of total active zone numbers at NMJ6/7. *dnlg2* mutants have comparable number of active zones with those observed at the wild type NMJs (Student's t test).


A
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dnlg2CL2;BAC
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F
dnrx/Df
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dnlg2;dnrx/Df
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AZ Number/Perimeter
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Total PSD Length/Perimeter (%)
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n=44
n=42
n=26
n=19
dnlg2;BAC
dnlg2
dnrx/Df
dnlg2;dnrx/Df
w1118

Figure 2.4. *dnlg2* mutants display synapse differentiation defects with severely disorganized postsynaptic areas.
(**A-G**) TEM micrographs of wild type (**A, B**), *dnlg2* mutants (**C, D**) and *dnlg2* mutants with the genomic *BAC* construct (**E**), *dnrx/Df* (**F**) and *dnlg2;dnrx/Df* double mutants (**G**) showing the ultrastructural features of boutons at NMJ 6/7. Active zones (AZ), postsynaptic areas (PSA) and SSR are highlighted. SSR widths were measured from PSD to the distal fold. Note that the number of AZs (arrows) is increased and PSAs fail to differentiate properly in *dnlg2* mutants (**C, D**, PSA with arrows). (**H-K**) Quantification of ultrastructural morphometric analyses on all genotypes. (**H**) Compared to wild type, active zone number in boutons are increased in $dnlg2^{CL2}$ mutants. (**I**) The lengths of PSD adjusted to the perimeter in *dnlg2* mutants is also increased. Postsynaptic areas (PSA) are enlarged (**J**) and the widths of SSR are reduced (**K**) in *dnlg2* mutants. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (Student's t test). Scale bars= 0.5μm.

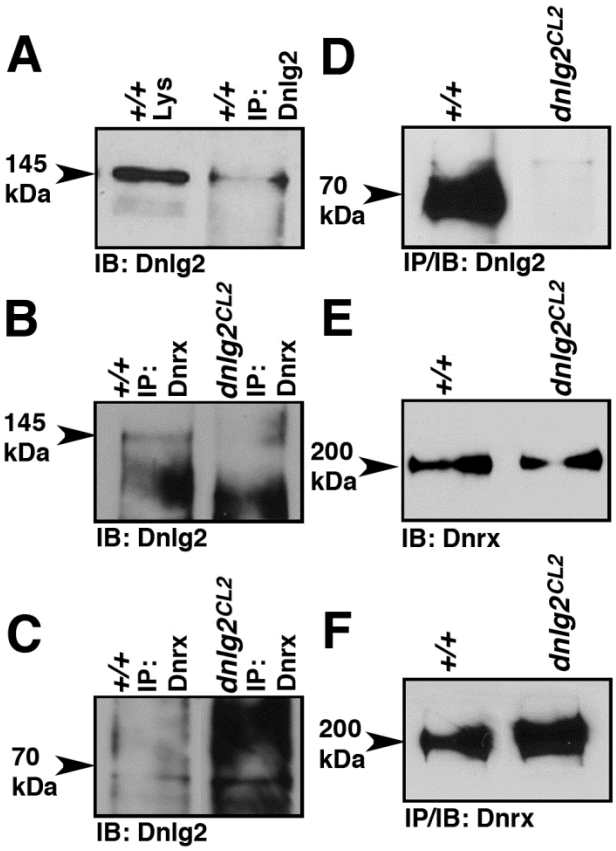

Figure 2.5. Dnlg2 forms a biochemical complex with Dnrx.
(A) IPs from wild type fly head lysates using anti-Dnlg2 antibodies show the presence of Dnlg2 (145 kDa, arrowhead). **(B, C)** IP from wild type fly head lysates using anti-Dnrx reveals the presence of Dnlg2 in the same complex (**B**, 145kDa, arrowhead). *The* 70kDa Dnlg2 does not associate with Dnrx. Only non-specific background bands are observed in the wild type and *dnlg2* lysates near where the 70kDa band is expected (**C**, arrowhead). Note that panels **B** and **C** are from the same protein blot probed separately. **(D)** IPs from equal amounts of wild type and *dnlg2* mutant fly head lysates using anti-Dnlg2 show presence of Dnlg2 (70kDa) in wild type but not in *dnlg2* mutants. (Note the break in between the lanes is due to removal of an empty lane). (**E**) Dnrx protein levels are unaffected in *dnlg2* mutants. **(F)** IPs from equal amounts of wild type and *dnlg2 mutants using anti-Dnrx show the presence of Dnrx in both wild type and dnlg2 mutants.*

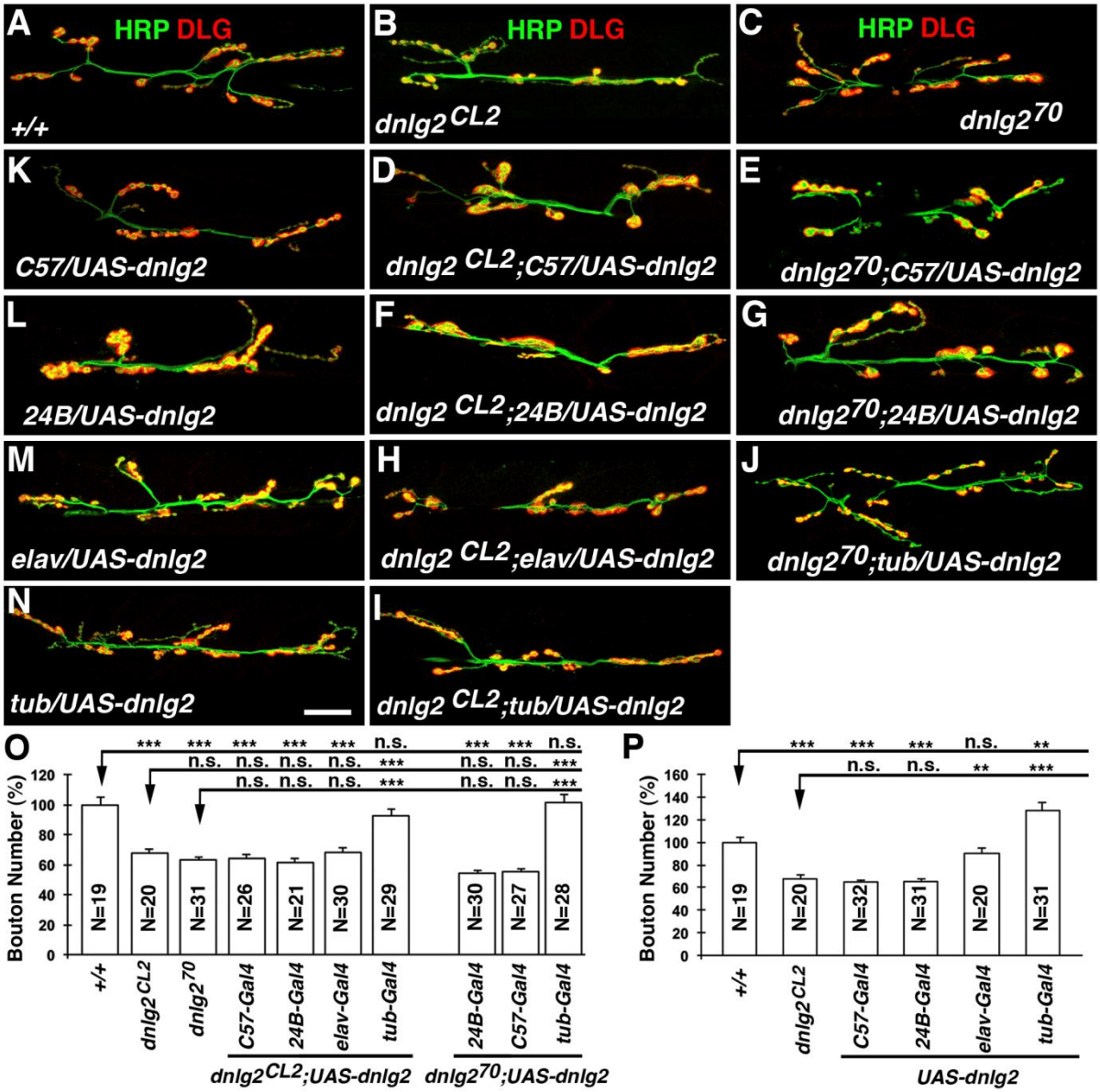

Figure 2.6. Dnlg2 is required pre- and post-synaptically for proper synaptic growth at NMJs.

(**A-J**) *dnlg2* cDNA transgene rescue analyses at NMJ 6/7. Compared to two *dnlg2* mutants, $dnlg2^{CL2}$ (**B**) and $dnlg2^{KO70}$ (**C**, Sun et al., 2011), expression of Dnlg2 in muscles with *C57-Gal4* (**D, E**) or *24B-Gal4* (**F, G**) failed to rescue bouton number deficits in both $dnlg2^{CL2}$ and $dnlg2^{KO70}$ mutants. Similarly, expression of Dnlg2 in neurons using *elav-Gal4* (**H**) also failed to rescue the NMJ phenotype. However, when Dnlg2 was expressed ubiquitously with *tub-Gal4* (**J, I**), the NMJ phenotype in both $dnlg2^{CL2}$ and $dnlg2^{KO70}$ mutants was restored to wild type levels. (**K-N**) *dnlg2* overexpression analyses in the wild type background. Overexpression of Dnlg2 in muscles using *C57-Gal4* (**K**) or *24B-Gal4* (**L**) adversely affected bouton growth. In contrast, overexpression of Dnlg2 in neurons (**M**) does not affect NMJ bouton growth or number dramatically. Simultaneous overexpression of Dnlg2 in muscles and neurons promotes bouton growth at NMJs (**N**). (**O, P**) Quantification of bouton number

at NMJ6/7 for rescue analyses (**O**) and overexpression analyses (**P**). Error bar = SEM; n.s.: not significant; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (ANOVA, Tukey *post hoc* test). Scale bar= 20μm.

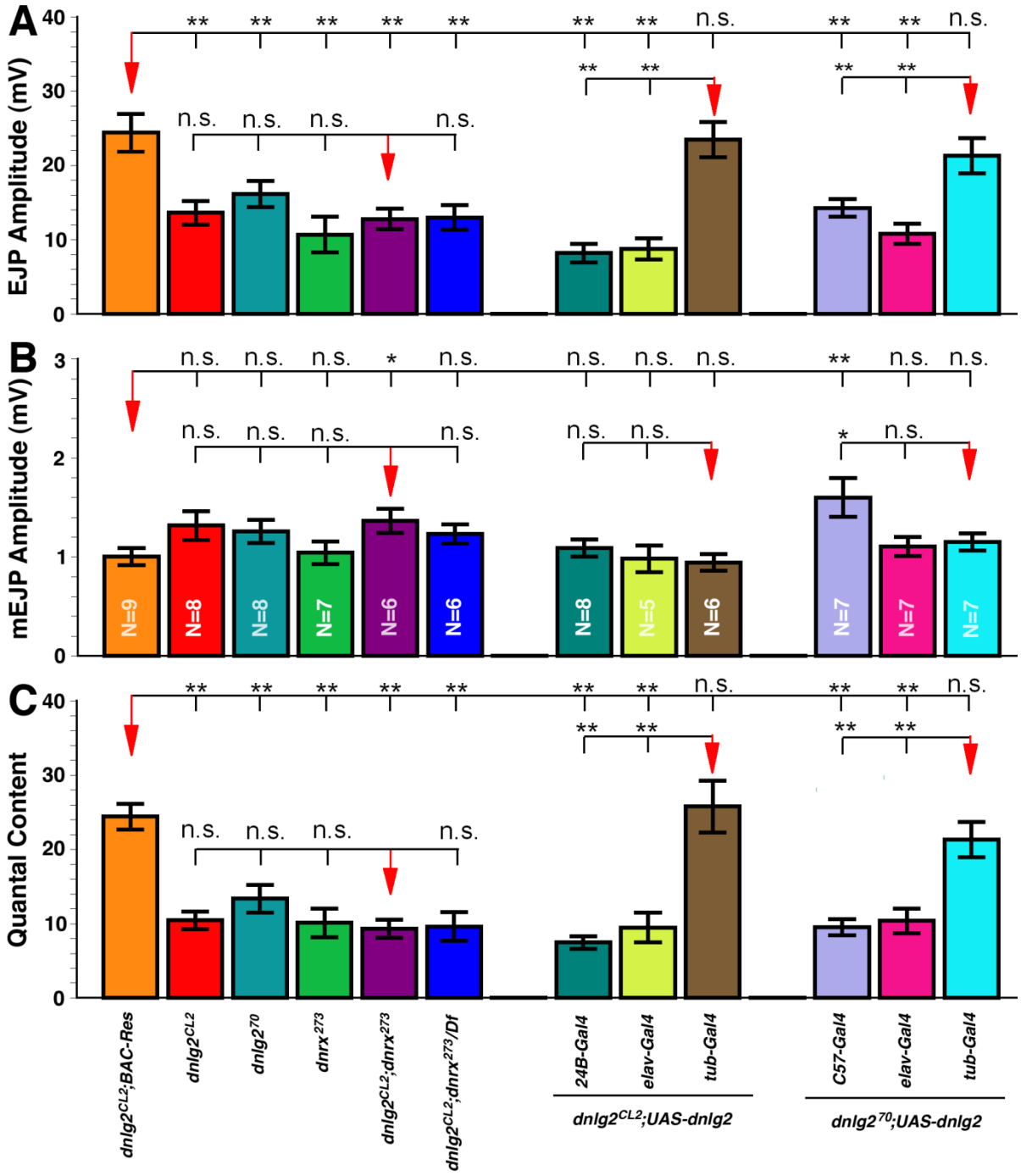
A
EJP Amplitude (mV)
B
mEJP Amplitude (mV)
C
Quantal Content
n.s.
**
*
N=9
N=8
N=8
N=7
N=6
N=6
N=8
N=5
N=6
N=7
N=7
N=7
dnlg2CL2;BAC-Res
dnlg2CL2
dnlg270
dnrx273
dnlg2CL2;dnrx273
dnlg2CL2;dnrx273/Df
24B-Gal4
elav-Gal4
tub-Gal4
C57-Gal4
elav-Gal4
tub-Gal4
dnlg2CL2;UAS-dnlg2
dnlg270;UAS-dnlg2

Figure 2.7. Dnlg2 expression is required in pre- and post-synaptic areas for rescue of synaptic transmission defects in *dnlg2* mutants.
(**A-C**) Quantification of electrophysiological analyses for *dnlg2* and $dnrx^{273}$ single and *dnlg2;dnrx* double mutants at muscle 6 of the third abdominal segment. *dnlg2* and $dnrx^{273}$ single and *dnlg2;dnrx* double mutants showed reduced EJP amplitudes (**A**) but normal mEJP amplitude (**B**). All the mutants have reduced quantal contents (**C**). Similarly, pre- (*elav-Gal4*) and post-synaptic (*24B-* or *C57-Gal4*) expression of Dnlg2 in $dnlg2^{CL2}$ and $dnlg2^{KO70}$ mutants failed to restore EJP amplitudes (**A**) and quantal content (**C**) deficits. Ubiquitous expression of Dnlg2 using tub^{P}*-Gal4* in both $dnlg2^{CL2}$ and $dnlg2^{KO70}$ mutants restores the EJP amplitude (**A**) to control levels. mEJP amplitudes (**B**) remain unchanged in $dnlg2^{CL2}$ mutants when Dnlg2 is expressed pre- and post-synaptically alone or in combination. However, an increase in mEJP amplitude is seen when Dnlg2 is expressed in muscles using *C57-Gal4* in $dnlg2^{KO70}$ mutants while no changes were observed when Dnlg2 is expressed using *elav-* or tub^{P}*-Gal4*. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Supplemental Figures

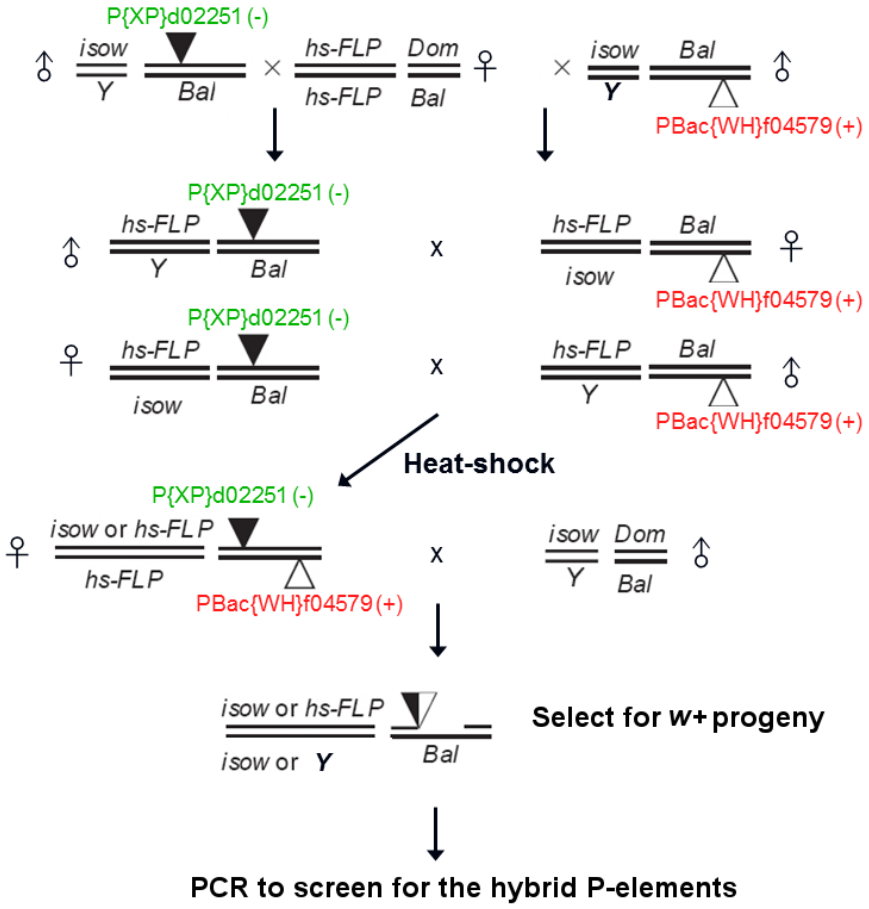

Figure S2.1 Genetic crossing scheme for the generation of *dnlg2* null mutants. FLP-FRT-based recombination was used to generate a site-specific deletion containing *dnlg2* genomic locus (Parks et al., 2004; Thibault et al., 2004). In the presence of the *hs-FLP* recombinase, two FRT-carrying transposable insertions, *P{XP}d02251* and *PBac{WH}f04579*, were brought *in trans*. Application of the heat shock drove FLP expression, resulting in the deletion of *dnlg2* locus and a hybrid *P*-element which is then confirmed by PCR. (*Dom*: dominant visible marker mutation; *iso*: isogenized chromosome.)

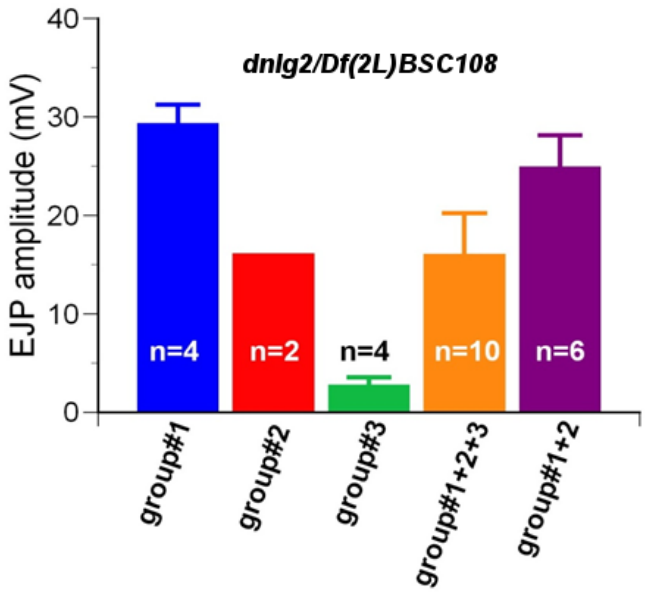

Figure S2.2 *dnlg2/Df* mutants display variable EJP amplitudes.
When placed over *Df(2L)BSC108*, the *dnlg2* mutants generated by FLP-FRT based deletion show a large variation in EJP amplitude, while the mEJP and the resting potentials remain normal. The EJP amplitude recordings were then grouped into three categories based on the level of the EJP amplitudes of the mutants. The average EJP amplitudes of the four larvae in Group#3 is significantly lower than Group#1.

CHAPTER 3

Conclusion and Future Directions

3.1 Conclusion

In this study, we characterized the *in vivo* function of *Drosophila neuroligin 2* and its interaction with *Drosophila neurexins* at the neuromuscular junction.

Multiple sequence alignments suggest that Dnlg2 is a homolog of mammalian neuroligins. At embryonic stage 16, the mRNA of Dnlg2 is localized at the central nervous system, the brain and the ventral nerve cord. Dnlg2 can be detected at the ventral nerve cord of the 3rd instar larvae, where a high density of synapses are localized, while the Dnlg2 at the NMJ bouton can only be detected when Dnlg2 is overexpressed. We identified two forms of Dnlg2, the 145kD full-length form and the 70kD cleaved form. Immunoprecipitation analyses suggest that only the 145kD full length Dnlg2 can interact with Dnrx.

In order to study the function of Dnlg2, we generated an independent line of *dnlg2* null mutants. Loss of Dnlg2 results in reduced bouton numbers at the NMJ6/7 and NMJ4 in 3rd instar larvae and at each bouton the level of postsynaptic glutamate receptor subunit III is decreased, but subunit IIA is unaffected. These results suggest that Dnlg2 is required for proper NMJ bouton growth and is responsible for the recruitment of a subset of postsynaptic proteins. Unexpectedly, the total number of active zones at NMJ6/7 is unaffected in *dnlg2* mutants, which leads to an increase in the number of active zones per bouton as observed in the electron micrographs. In addition, further analyses on the ultrastructure of the *dnlg2*

mutants revealed several structural defects, including increases in the length of postsynaptic densities and postsynaptic area as well as decreases in the width of the subsynaptic reticulum. These data indicate that although Dnlg2 is not required for the formation of active zones, it is responsible for proper organization of the synaptic structure.

Electrophysiological analyses show that *dnlg2* mutants have reduced evoked junctional potentials, but normal miniature junctional potentials; together, they suggest that the quantal content is decreased. Given that the total number of active zones at NMJ6/7 is normal, the reduced quantal content may result from a decrease in the release probability in response to presynaptic stimuli. Surprisingly, our rescue and overexpression analyses indicate that Dnlg2 function at both pre- and post-synaptic terminals and that overexpression of Dnlg2 only at the postsynaptic terminal reduces bouton numbers. These results may imply that the balance of Dnlg2 at the pre- and post-synaptic terminals is important in regulating bouton growth and synaptic function.

In order to study whether Dnlg2 and Dnrx interact genetically, we generated *dnlg2* and *dnrx* double mutants. Compared to *dnlg2* and *dnrx* single mutants, the double mutants have very similar morphological, electrophysiological and ultrastructural phenotypes, which suggest that Dnlg2 and Dnrx cooperate in the same complex to organize NMJ synaptic development and organization.

In summary, this study provides strong evidences on the role of Dnlg2 in bouton formation, assembly of postsynaptic proteins and synaptic transmission at the *Drosophila* neuromuscular junction. This is the first report to show a presynaptic function of neuroligin, which may provide novel insight in our knowledge of neuroligins and neurexins and the

underlying pathways which can in turn facilitate new diagnostic or therapeutic strategies for neural developmental disorders.

3.2 Future directions

Identifying functional domains of Dnlg2

It has been well established that mammalian neuroligins have an extracellular AChE-like domain that interacts with presynaptic neurexins, and a PDZ domain binding motif that interacts with PSD-95 (Ichtchenko, 1995; Irie et al., 1997). Recently, additional functional domains were also reported, such as a tyrosine-based motif that binds to gephyrin and a new non-PDZ intracellular domain (Poulopoulos et al., 2009; Shipman et al., 2011). Based on the protein multiple sequence alignment, Dnlg2 has an AChE-like domain and a potential PDZ domain binding motif, similar to the mammalian homologs. The next question would be whether these domains are functionally conserved in *Drosophila*. Although the prospective E-I-S-V sequence at the Dnlg2 C-terminus is not the typical PDZ binding motif reported previously (Nourry et al., 2003), it is possible that *Drosophila* has a different motif code for the interaction with PDZ domain proteins. Indeed, Dnlg1 also has an atypical C-terminal PDZ binding motif (T-T-N-I) (Banovic et al., 2010). In addition, Dnlg2 has a longer intracellular region compared to the mammalian homologs, and therefore, it would be also interesting to know whether Dnlg2 has additional intracellular functional domains. To answer these questions, transgenic flies expressing various truncated forms of Dnlg2 should be generated and to detect the expression of the truncated forms of Dnlg2, each construct should first be tagged with markers, such as GFP, Myc or His-tag to differentiate them from the

endogenous Dnlg2. The function of each domain can then be assessed by testing their ability to rescue the phenotypes when expressed in a *dnlg2* mutant background.

Cell culture studies tested the functions of the neuroligin domains by expressing truncated neuroligins in wild type neurons (Chih et al., 2005), although it is still not clear if the same mechanisms occur *in vivo*. This hypothesis can be easily tested by overexpressing different forms of truncated Dnlg2 in the wild type or the *dnrx* mutant background followed by NMJ phenotypic analyses. Similar studies have been performed for Dnlg1, in which the truncated Dnlg1, containing only the extracellular region, can disrupt the function of endogenous Dnlg1 in a dominant negative fashion (Banovic et al., 2010). Once all the truncations of Dnlg1, Dnlg2 and Dnrx are made, it will be interesting to test if Dnlg1, Dnlg2 and Dnrx interact with each other through their extracellular or intracellular regions by overexpressing truncated Dnlg1, Dnlg2 or Dnrx in the various combinations of *dnlg1*, *dnlg2* and *dnrx* single or double mutant backgrounds.

The physiological role of the cleaved Dnlg2

Out data indicate that in addition to the full-length 145kD Dnlg2, there is also a truncated form at 70kD. Immunoprecipitation analyses show that only the full-length Dnlg2 is capable of interacting with Dnrx. However, the intensity of the 70kD band is much higher than the 145kD band on the Western blot in wild type flies. Therefore, the key question arises: is the cleaved Dnlg2 functional or is it a by-product of the degraded Dnlg2? If the cleavage of Dnlg2 is functional, which portion of the cleaved Dnlg2 plays the role? Interestingly, two very recent studies show that mammalian neuroligin 1 can be cleaved by ADAM10 and MMP9 at the juxtamembrane region (Peixoto et al., 2012; Suzuki et al., 2012). The cleavage

is initiated by the activation of NMDA receptors and is dependent on synaptic activity. The resulting extracellular portion of neuroligin 1 is then released into the synaptic cleft which causes destabilization of neurexin1β, reduces presynaptic release probability, and decreases synaptic transmission. In contrast, blocking this proteolytic process can lead to an increase in synaptic spine formation. The other product of the cleavage is the membrane-bound intracellular region which is then processed by presenilin. It is possible that Dnlg2 also undergoes similar proteolytic cleavage as the mechanism to regulate synaptic development and activity. Interestingly, *Drosophila* also contains MMPs and therefore, the hypothesis could be tested by examining the Dnlg2 level and/or NMJ morphology when MMPs are reduced or inactive (Llano et al., 2000; Page-McCaw et al., 2003). Studying the mechanism in a model system that is easier to manipulate genetically could provide more novel insights.

In addition, the dominant negative effect of cleaved Dnlg2 may help explain the observation that postsynaptic Dnlg2 overexpression results in the reduction in bouton number in the wild type background. In this case, the enzymes responsible for this proteolytic process could be restricted to the postsynaptic membrane or the neighboring extracellular matrix because presynaptic Dnlg2 overexpression does not change the NMJ bouton number. Further biochemical and genetic analyses are required to test this hypothesis.

Potential modifiers of *dnlg2*

It has been established that genetic background can affect penetrance, dominance and expressivity of the phenotypes. To better understand these genetic effects, several studies have identified mammalian modifier genes that can affect transcription, modify gene expression, or affect the signaling pathways upstream or downstream of the target gene

(Gruneberg, 1950; Hummel, 1958; Bykhovskaya et al., 2000; Nadeau, 2001). The *dnlg2* mutants that are yet to be isogenized show variable EJP amplitudes (Fig. S2.2), implying that there are mutations or genetic variations in the modifier genes of Dnlg2. It is very likely that these mutations are present in the fly stocks carrying the transposable elements, *P{XP}d02251* and/or *PBac{WH}f04579*. At least some of the mutations appear to be dominant because the variation in EJP amplitudes was found in both *dnlg2/dnlg2* and *dnlg2/Df* (Fig. S2.2 and data not shown). In addition, the *dnlg2* mutants generated in this study are null mutants (Fig. 2.1), suggesting that these modifiers probably act downstream of Dnlg2. Therefore, performing a modifier screen will help identify the dominant enhancers or suppressors that affect the EJP amplitude of the *dnlg2* mutants as well as the signaling pathways downstream of Dnlg2 (Gruneberg, 1950; Chen et al., 1998; Bykhovskaya et al., 2000; Nadeau, 2001; LaJeunesse et al., 2001; Johnston, 2002; Ward et al., 2003; Bilen and Bonini, 2007; Kaplow et al., 2007; Kucherenko et al., 2008; Ma et al., 2009; Fernandes and Rao, 2011). The modifier screen can be performed by crossing the *dnlg2* mutants in various genetic backgrounds to the isogenized *dnlg2* mutants followed by screening for the progeny with higher or lower EJP amplitude. The modifiers can then be mapped by sequencing the genome of the stock and comparing to the single nucleotide polymorphism maps (Johnston 2002).

Interestingly, the observation of the EJP amplitude spectrum in *dnlg2* coincides with the heterogeneity of ASD symptoms and the spectrum of functional deficits in the patients. Therefore, the modifier screen for *dnlg2* may help uncover novel potential players that are involved in the etiology of ASD and thus, provide insights for new targets for diagnostic screens.

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