CRUCIAL ROLE OF *DROSOPHILA* NEUREXIN IN PROPER ACTIVE ZONE APPOSITION TO POSTSYNAPTIC DENSITIES, SYNAPTIC GROWTH AND SYNAPTIC TRANSMISSION

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ABSTRACT

JINGJUN LI: Crucial Role of *Drosophila* Neurexin in Proper Active Zone Apposition to Postsynaptic Densities, Synaptic Growth and Synaptic Transmission
(Under the direction of Manzoor Bhat)

Trans-synaptic adhesion molecules, a family of cell adhesion molecules that mediate the coordinated interactions between pre- and postsynaptic membrane, are thought to mediate target recognition, initiate synapse formation and alignment, maintain the integrity of synapse, and regulate synaptic function during synapse development and remodeling. Among these, neurexins—a family of highly conserved neuron-specific transmembrane proteins have been proposed to act as a key synapse organizer required for synapse formation and neurotransmitter release. However, their *in vivo* functions remain elusive, particularly due to the complexity and redundancy of mammalian *neurexin* genes.

Here, we report the cloning and characterization of the *Drosophila* homolog of neurexin genes. In contrast to the presence of 3 neurexin genes in mammals, we found that the *Drosophila* genome contains a single neurexin gene, which we named *Drosophila neurexin* (*dnrx*). In situ hybridization and immunohistochemical analyses revealed that *dnrx* is expressed in neurons of central nervous system (CNS) and localized to CNS synaptic regions, axons and glutamatergic neuromuscular junctions (NMJs) during development. At larval NMJ, DNRX is concentrated at active zones, but also extends into periactive zones within synaptic boutons.

We have obtained null mutations in the single *dnrx* gene. Using *Drosophila* NMJs, an excellent *in vivo* synapse model system, we demonstrate that *dnrx* loss of function prevents the normal proliferation of synaptic boutons, while *dnrx* gain of function in neurons has the opposite effect. Synaptic vesicle and active zone component markers are mislocalized along *dnrx* mutant axons, suggesting that DNRX is required for the proper recruitment and localization of key synaptic components during presynaptic differentiation. Postsynaptically, the distribution of postsynaptic density (PSD) proteins is enlarged. Conspicuously, *dnrx* null mutants display striking defects in synaptic ultrastructure with the presence of detachments between pre- and postsynaptic membranes, abnormally long active zones, and increased number of T-bars. These abnormalities result in corresponding alterations in synaptic transmission with reduced neurotransmitter release. Together, our results provide compelling evidence for an *in vivo* role of neurexins in the control of synapse growth, the modulation of synaptic architecture and adhesive interactions between pre- and postsynaptic compartments *in vivo.*

DEDICATION

This dissertation is dedicated to my father, whose strength, passion and perseverance always inspired me; to my mother, for her unending love and unconditional support on my pursuit of a career far away from home; to my husband, James—none of this work could have been completed without his unfailing love, understanding, encouragement, patience and support; and to our son, Alex, for every bit of joy he has brought since his arrival.

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PREFACE

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CHAPTER ONE

INTRODUCTION

Synapses are specialized intercellular junctions that link neurons and their target cells into functional network. Synapse development and function form the basis of many neuronal processes, including formation and function of neural circuitry, the ability to learn, and to store and recall memories. Synaptic dysfunction might be responsible for many neurological and behavioral disorders such as Alzheimer's disease and autism (Rieckhof et al., 2003; Selkoe, 2002; Zoghbi, 2003). Thus, elucidating the mechanisms by which synapses develop and are modified is a central question in neurobiology. Over the past few decades a number of factors have been identified that play major roles in synapse morphogenesis and synaptic plasticity. Among these, trans-synaptic cell adhesion molecules that mediate the coordinated interactions between pre- and postsynaptic membranes stand out.

Several families of cell adhesion molecules that interact in a homo- or heterophilic manner across the synaptic cleft, including (1) cadherins, (2) ephrins and their receptors Ephs, (3) neurexins and their ligands neuroligins, and (4) SynCAM have been shown to be required for the various aspects of synaptic development and function (Gerrow and El-Husseini, 2006a; Scheiffele, 2003; Yamagata et al., 2003). These trans-synaptic adhesion molecules have been proposed to mediate target recognition, induce pre- and postsynaptic specializations and their alignment during synapse formation, maintain the integrity of synapses, and regulate synaptic structure and function during synaptic development and remodeling. Of these, neurexins and their postsynaptic binding partners the neuroligins are emerging as key synapse organizing molecules.

The recognition of neurexins as a family of central organizing molecules for both excitatory and inhibitory synapses is based on the following evidence. First, neurexins are

widely expressed in nervous system and are neuron-specific (Ushkaryov et al., 1992). Second, in mammals, three neurexin genes, each of which has two promoters generating α- and β-neurexins, are subject to extensive alternative splicing. Theoretically, three genes, two promoters and multiple alternative splicing sites could give rise to about four thousand of neurexins variants. Thus, neurexins were proposed as candidate molecules to mediate target recognition and synaptic specificity (Missler and Sudhof, 1998a). Recent studies on the functional significance of a small subset of neurexin splicing variants support this idea (Comoletti et al., 2006; Chih et al., 2006; Rowen et al., 2002; Tabuchi and Sudhof, 2002; Graf et al., 2006; Boucard et al., 2005). Third, extensive protein-protein interaction studies indicate that the trans-synaptic interaction between neurexins and neuroligins may bridge the synaptic cleft by aligning the presynaptic neurotransmitter release machinery with PSD (Ichtchenko et al., 1995; Boucard et al., 2005; Song et al., 1999; Irie et al., 1997; Hata et al., 1993; Hata et al., 1996; Biederer and Sudhof, 2000). Fourth, a number of studies using cell culture systems demonstrate that neurexins and their ligands neuroligins can act bidirectionally to induce synapse formation (Scheiffele et al., 2000; Graf et al., 2004; Dean et al., 2003; Nam and Chen, 2005; Chih et al., 2005a; Prange et al., 2004; Levinson et al., 2005; Fu et al., 2003). Fifth, phenotypic analyses of α-neurexin triple knockout mice reveal that α-neurexins are required for Ca^{2+}-triggered neurotransmitter release (Missler et al., 2003; Zhang et al., 2005) and postsynaptic NMDA-receptor function (Kattenstroth et al., 2004). Lastly, recent genetic linkage studies have reported that mutations in neurexin genes are associated with autism spectrum disorders (Szatmari et al., 2007).

Despite the expanding evidence that highlights the essential role of neurexins in synaptic development and function, the key question still remains— what is the exact function of neurexins *in vivo*?

1.1 Neurexin genes and structure

Neurexins were first identified as primary receptors for α-latrotoxin, a component of black widow spider venom, which triggers massive neurotransmitter release (Ushkaryov et al., 1992). Mammalian genomes contain three neurexin genes. Each gene has two promoters. The upstream promoters generate longer transcripts encoding α-neurexins, whereas the promoters located in an intron of α-neurexins generate shorter transcripts encoding β-neurexins. Thus, β-neurexins are the truncated isoforms of α-neurexins with a unique N-terminus, but share identical transmembrane domain and cytoplasmic sequences with α-neurexins. The extracellular region of α-neurexins is composed of three LamG-EGF-LamG repeats, whereas β-neurexins contain a single LamG domain (Missler and Sudhof, 1998a) (Fig. 1.1). The extracellular sequences of neurexins share some homology to those in extracellular matrix proteins such as laminin and agrin, suggesting a possible adhesive function (Rudenko et al., 2001). The intracellular tail of neurexins contains a PDZ binding motif that mediates interactions with PDZ scaffolding proteins. The six principal transcripts of the three neurexin genes are subject to extensive alternative splicing, which arises from five canonical alternative splice sites in α-neurexins, and two in β-neurexins (Missler and Sudhof, 1998a)(Fig. 1.1). The expression of neurexin splicing isoforms is regulated both temporally and spatially during development, and alternative splicing regulates the protein-binding properties of

neurexins (see **The Protein Interaction Map of Neurexins**). Theoretically, neurexin genes could generate about four thousand isoforms which differ in extracellular sequences (Rowen et al., 2002; Tabuchi and Sudhof, 2002), therefore, raising the possibility that the expression of neurexin splicing variants could encode the specificity of synaptic circuits.

1.2 Expression and synaptic localization of neurexins

Northern blot and *in situ* hybridization studies revealed that neurexins are expressed throughout the CNS, exclusively in postmitotic neurons (Puschel and Betz, 1995; Ushkaryov et al., 1992). During development, the expression of neurexins is highly regulated (Gorecki et al., 1999; Patzke and Ernsberger, 2000; Puschel and Betz, 1995). Each of the six principal neurexin isoforms (Iα¸ Iβ, IIα, IIβ, IIIα and IIIβ) displays differential but overlapping expression pattern in various regions of brain, such that most neurons express multiple neurexins (Ullrich et al., 1995). Furthermore, at least a subset of the splicing variants are known to exhibit distinct and spatially separated distribution patterns at the cellular level (Ichtchenko et al., 1995).

The expression of neurexins including the levels and spatial patterns is also regulated by neuronal activity and extrinsic factors in environment (Rozic-Kotliroff and Zisapel, 2007). For instance, the expression of specific neurexins isoforms is unregulated in response to neurotrophins, BMP growth factors and ischemia (Patzke and Ernsberger, 2000; Patzke et al., 2001; Sun et al., 2000). The dynamic and inducible expression of neurexins indicates neurexins might be also involved in synaptic remodeling and plasticity.

Indirect evidence from protein-protein interaction studies and functional analyses of neurexins has suggested the presynaptic localization of neurexins (Graf et al., 2004; Missler et al., 1998; Missler et al., 2003; Scheiffele et al., 2000). In cultured neurons, antibody labeling showed that neurexins are concentrated in growth cones of isolated axons, and colocalized with synaptic markers (Dean et al., 2003; Graf et al., 2004). The current neurexin-neuroligin hypothesis depends on the pre- and postsynaptic localization of neurexins and neuroligins, respectively. However, in vivo, the precise subcellular localization of neurexins remains elusive. Several recent findings have suggested that neurexins might reside in the postsynaptic sites as well, thus challenging the current trans-synaptic neurexin-neuroligin model.

Kattenstroth et al found that NMDA-receptor function is impaired in postsynaptic cortical neurons of α-neurexin knockout mice via a cell-autonomous mechanism, indicating that α-neurexins might locate and function at postsynaptic sites as well (Kattenstroth et al., 2004). Using immunoelectron microscopy, Scheiffele and colleagues confirmed that endogenous neurexins are localized to axons and presynaptic terminals. Interestingly, they also revealed that neurexins are abundant in the PSD (Taniguchi et al., 2007). The distribution of anti-neurexin gold particles has a peak centered at the presynaptic active zone and PSD, but was also observed outside of active zone and PSD within presynaptic terminals and spines. The neurexin antibodies used in ultrastructural studies are pan-neurexin antibodies, which do not allow any conclusion regarding the localization of specific neurexin isoforms, i.e. whether specific neurexin isoforms target to presynaptic, postsynaptic, or both pre- and postsynaptic sites. To address this question, they generated transgenic mice overexpressing one specific neurexin Iβ isoform tagged

with GFP in neurons, and found a significant amount of this specific neurexin Iβ isoform appeared in the postsynaptic compartments. Furthermore, they demonstrated that postsynaptic expression of neurexins might inhibit neuroligin function via cis-interaction with neuroligins on the postsynaptic membrane in vitro. However, one caveat with overexpression conditions is that the overexpressed protein could be mis-sorted to dendritic spines. Indeed, a recent study using a novel biochemistry approach which has been validated by electron microscopy found that neurexin Iβ is principally presynaptic (Berninghausen et al., 2007).

Additional studies are required to define the precise synaptic localization of neurexin isoforms, particularly whether there are neurexin isoforms exclusively distributed to postsynaptic sites. Nevertheless, the unexpected postsynaptic localization of neurexins suggests that the function and the action modes of neurexins are more complicated and diverse than previously thought.

1.3 The protein interaction map of neurexins

Extensive protein-protein interaction and *in vitro* studies have established a protein-interaction map of neurexins (Fig. 1.2). Extracelluarly, neurexins bind to neuroligins, dystroglycan and neurexophilins. The interactions are selectively regulated by Ca^{2+}, alternative splicing, and posttranslational modifications including oligomerization and glycosylation. Intracelluarly, neurexins interact with synaptic vesicle protein synaptotagmin and PDZ scaffolding proteins CASK and Mints.

Extracellular binding partners

Neuroligins. Neuroligins, the most extensively studied neurexin binding partners, bind to both α- and β-neurexins in a Ca^{2+} -dependent manner (Ichtchenko et al., 1995; Ichtchenko et al., 1996). Five neuroligin genes are expressed in human, and four in rodents (neuroligin 1, 2, 3, and 4) (Ichtchenko et al., 1996; Jamain et al., 2003). Like neurexins, neuroligins are single transmembrane proteins that contain a large, alternatively spliced extracellular sequence and a PDZ binding motif at the intracellular tail. The major extracellular domain of neuroligins is an inactive acetylcholinesterase (AchE)-like domain, which mediates the binding of neurexins. Neuroligins are localized to postsynaptic densities (Song et al., 1999) and interact with several scaffold proteins such as PSD-95, thereby associated with neurotransmitter receptors, ion channels and signaling complexes (Hirao et al., 1998; Irie et al., 1997; Meyer et al., 2004). Interestingly, neuroligin 1 and neuroligin 2 is predominantly localized to excitatory and inhibitory synapses, respectively (Song et al., 1999; Varoqueaux et al., 2004).

The neurexin-neuroligin interaction is controlled by gene selection, the splicing codes of both neurexins and neuroligins, and oligomerization of neuroligins. For instance, biochemical assays showed that the binding affinity of different neuroligins (neuroligin 1-4) and neurexin 1β varies more than two orders of magnitude in range. Neuroligin 1 has the highest binding affinity to neurexin 1β, whereas neuroligin 2 displays the lowest affinity (Comoletti et al., 2006). Alternative splicing in neurexins and neuroligins also regulates binding affinity. Recent protein interaction assays and cell culture studies highlight the selectivity and functional significance of splicing codes conferred by splicing at site B in neuroligins (one of two splicing sites of neuroligins—splicing site A

and B) and at site 4 in β-neurexins (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006; Graf et al., 2006) . α-neurexins selectively bind to neuroligins lacking an insert at site B (-B neuroligins), whereas β-neurexins can bind to neuroligins regardless of splicing at site B in neuroligins. Splicing site 4 in β-neurexins modulates neuroligin binding. β-Neurexins containing an insert at site 4 interact preferentially with –B neuroligins, while β-neurexins lacking an insert at site 4 interact with –B neuroligins and +B neuroligins with equal affinity (Fig. 1.3). Such splicing codes that guide interactions between neurexins and neuroligins have been shown to affect the selective formation of excitatory or inhibitory synapse in cultured cells (see **Modulation of synapse specificity**).

Crystal structural studies on neurexin LamG domains that mediate the interaction of neurexins with their binding partners provide a structural framework and suggest how alternative splicing and Ca^{2+} might control ligand binding (Comoletti et al., 2007; Sheckler et al., 2006; Rudenko et al., 1999). The studies revealed that alternative splicing sites are located on the so-called hyper-variable surface, which is also the protein interaction surface, and reshapes as a result of alternative splicing. A Ca^{2+} binding site is located at the center of hyper-variable surface. Moreover, alternative splicing can affect Ca^{2+} binding sites.

Oligomerization of neuroligins is necessary for the binding of neuroligins to neurexins. An AchE-like domain mediates the dimerization of neuroligins, and mutations of residues in this domain results in loss of neuroligin 1 binding to β-neurexin (Comoletti et al., 2003; Dean et al., 2003).

Dystroglycan. In addition to the extracellular matrix proteins—laminin, agrin and perlecan, dystroglycan, a heavily glycosylated component of dystrophin-glycoprotein complex, interacts with both α- and β-neurexins. The binding is dependent on Ca^{2+}, alternative splicing of neurexins, and the glycosylation of dystroglycan (Sugita et al., 2001). Dystroglycan is expressed in muscle cells as well as a variety of cell types in the brain (Montanaro and Carbonetto, 2003). In cultured hippocampal neurons, it appears to be associated with GABAergic synapses, but not essential for GABAergic synapse formation (Levi et al., 2002). Brain-specific deletion of dystroglycan in mice led to brain malformations including defects in neuronal migration, and impaired long-term potentiation in hippocampal slices. It is notable that the basal synaptic transmission is not affected in dystroglycan deficient mice (Moore et al., 2002). Therefore, the exact localization and function of dystroglycan in synapses remain to be investigated.

Neurexophilins. Only α-neurexins, but not β-neurexins, interact with neurexophilins, a family of small neuropeptide-like protein, in a tight complex. The interaction is not Ca^{2+}-dependent (Missler and Sudhof, 1998b; Petrenko et al., 1996). In contrast to the wide expression of α-neurexins in CNS neurons, the expression of neurexophilins 1 and 3 (the only neurexophilins that bind α-neurexins in mice) is restricted to distinct brain regions and limited populations of neurons (Beglopoulos et al., 2005; Clarris et al., 2002; Petrenko et al., 1996). The brain morphology of single knockout mice is normal, but neurexophilin-3 deficient mice showed impaired sensory information processing and motor coordination, suggesting a functional role of neurexophilins in modulating selected neuronal circuits (Beglopoulos et al., 2005; Missler et al., 1998).

Intracellular binding partners

Intracelluarly, neurexins interact with PDZ domain proteins CASK (Hata et al., 1996) and Mints (Biederer and Sudhof, 2000), which can interact with synaptic vesicle fusion protein Munc18 (Borg et al., 1999; Hata and Sudhof, 1995; Okamoto and Sudhof, 1997) and presynaptic voltage-gated Ca^{2+} channels (Maximov et al., 1999). Consistent with the biochemical association of these scaffolding proteins with neurotransmitter exocytosis apparatus, the rate of spontaneous neurotransmitter release events is changed in CASK and Mints knockout mice (Ho et al., 2006; Ho et al., 2003; Atasoy et al., 2007). The C termini of neurexins can also directly bind to synaptotagmins, synaptic vesicle proteins that regulate neurotransmitter exocytosis (Perin, 1994). Therefore, the intracellular interactions of neurexins suggest that neurexins might recruit or organize synaptic vesicles and neurotransmitter release machinery.

Taken together, the protein-interaction map of neurexins have led to the hypothesis that the trans-synaptic interaction of neurexins and their relevant ligands, such as neuroligins, might bridge the synaptic cleft aligning the presynaptic neurotransmitter release machinery with postsynaptic neurotransmitter receptors and signaling complexes. However, whether certain protein-protein interactions, such as neurexins and dystroglycan, neurexins and the scaffolding protein CASK, are necessary for synaptic development and function in vivo is still uncertain. Studies on functional relevance of these interactions will provide insights into the underlying molecular mechanisms by which neurexins function.

1.4 Function of neurexins at synapses

The complexity and redundancy of neurexin genes in mammals pose a tremendous difficulty for understanding their function *in vivo.* A number of studies using cell culture systems have first demonstrated the functional significance of transynaptic neurexin-neuroligin complex in both excitatory and inhibitory synapse formation in vitro.

Neuroligin-neurexin trans-synaptic interaction and synaptogenesis in vitro

Using a coculture system that consists of non-neuronal cells and neurons, Scheiffele and colleagues (Dean et al., 2003; Scheiffele et al., 2000) first demonstrated the following important findings from the neuroligin point of view: (1) neuroligins can induce the assembly of presynaptic terminals via neurexins, (2) clustering of neurexins is sufficient to induce accumulation of synaptic vesicle proteins through interactions that require the cytoplasmic domain of neurexins (Dean et al., 2003). From the neurexin point of view, Graf et al (Graf et al., 2004) revealed that neurexin can induce postsynaptic specializations of both glutamatergic and GABAergic synapse via neuroligins. Lateral aggregation of neuroligins is sufficient to initiate postsynaptic organization. They also found that for glutamate receptors neurexins only induce clustering of NMDA but not AMPA receptors. Consistent with the findings in such artificial hemi-synapses formed by non-neuron and neuron cells, overexpression and knockdown of neuroligins in cultured neurons results in an increase and decrease in synapse number, respectively. Moreover, electrophysiological analysis revealed that knockdown of neuroligins leads to a predominant reduction of inhibitory synaptic function, suggesting that neuroligins might

also control the functional balance of excitatory and inhibitory synapses. In summary, the important findings in cultured cells suggest that neurexins and neuroligins can work together to induce synapse formation in a bidirectional manner, i.e., neurexin and neuroligins promote the recruitment and assembly of synaptic vesicle proteins and postsynaptic scaffolding proteins and receptors, respectively, in pre- or postsynaptic sites.

In vivo functions

The complete neurexin knockout mice are not available yet. Using α-neurexin triple-knockout mice, Missler and colleagues (Missler et al., 2003; Zhang et al., 2005) demonstrated that extracellular domains of α-neurexins are essential for Ca^{2+}-triggered neurotransmitter release. Intriguingly, mice deficient in all α-neurexins did not show any alterations in brain anatomical structure and synapse ultrastructure except for a significant reduction in the number of GABAergic synapses. However, synaptic transmission was severely impaired in triple-knockout mice due to a defect in Ca^{2+} channel function. These important findings raise a number of questions. To name a few, (1) how do α-neurexins couple or regulate Ca^{2+} channel function? Although the number of cell-surface Ca^{2+} channels appears normal in knockout mice, whether they are localized to the right place—the active zone, has not yet been examined; (2) the fact that α-neurexin triple knockout mice died on postnatal day1 (P1) due to respiratory failure, early before the peak of synaptogenesis in mice (P6-P13), prevents a thorough assessment of the role of α-neurexins in synapse development.

Similar to α-neurexin knockout mice, mice lacking neuroligins 1-3 (neuroligin 4 is expressed at a very low level in brain) die shortly after birth due to respiratory failure.

Synapses appeared to be normal in number and morphology. But glutamatergic and GABA-mediated neurotransmission were impaired, including a dramatic decrease in spontaneous GABAergic activity, a moderately reduced spontaneous glutamatergic activity, and a marked increase in the failure rate of evoked GABAergic transmission (Varoqueaux et al., 2006). Consistent with the central finding of in vitro studies, synaptic dysfunction in neuroligin knockout mice also leads to a shift in the excitation/inhibition (E/I) balance. Although the phenotype in synaptic transmission could be partly due to reduced postsynaptic clustering of GABA receptors and reduced levels of several synaptic vesicle proteins observed in neuroligin triple knockout mice, how neuroligins regulate synaptic function and how the neuroligin phenotype is related to neurexins are not clear. Particularly, whether the localization and expression level of neurexins is altered in neuroligin triple knockout mice is not known.

Together, initial knockout studies on the in vivo function of α-neurexins and neuroligins suggest that neurexins and neuroligins are essential for synaptic transmission but dispensable for the initial formation of synapses (the establishment of initial contact). One explanation for the discrepancy regarding the essential role of neurexins and neuroligins in initial synapse formation between cell culture and knockout mice studies is that other synaptogenic adhesion molecules might compensate the loss of neuroligins or α-neurexins in vivo. Further studies are required to assess the in vivo role of neurexins and neuroligins in synaptic development and function, and their functional relevance. This is more pressing for neurexins due to the lack of complete neurexin null mutant animal model. It is notable that the premature death of α-neurexin and neuroligin triple knockout mice make it impossible to identify the in vivo role of neurexins and

neuroligins in late stages of synapse development, synaptic plasticity, learning and memory. However, generating inducible triple knockout animal model will be a daunting task in mammals.

Modulation of synapse specificity

Difference in the excitatory vs. inhibitory synaptic localization and/or binding affinities between neurexin and neuroligin isoforms might contribute to the specification of excitatory or inhibitory synapses. Neuroligin 1 and neuroligin 2 are differentially localized to excitatory and inhibitory synapses, respectively. The functional significance of neuroligin 1 vs. neuroligin 2 in specification of excitatory vs. inhibitory synapses has recently been verified (Chubykin et al., 2007). In cultured neurons, neuroligin-1 overexpression specifically increased the number of functional excitatory synapses, whereas neuroligin2 specifically enhanced the number of inhibitory synapses. Accordingly, decreased excitatory and inhibitory synaptic responses were observed in neuroligin-1 and neuroligin-2 knockout mice, respectively. Moreover, alternative splicing regulates neurexin-neuroligin interactions, thus might selectively affect the development and function of excitatory and inhibitory synapses. For instance, it has been shown in artificial synapses that the presence of the S4 insert in neurexin 1-β preferentially reduces glutamatergic but not GABAergic synaptogenic activity of neurexin 1-β (Graf et al., 2006).

Why neurexins and neuroligins appear to have a stronger role in GABAergic synapse development is still an open question. One possibility is that there are more transynaptic cell adhesion molecules localized at glutamatergic than at GABAergic

synapses, which could compensate for the loss of neurexins and neuroligins (Craig and Kang, 2007).

1.5 Neurexin-neuroligin pathway and Autism Spectrum Disorders

Autism spectrum disorders (ASD) are a heterogeneous neurodevelopmental syndrome characterized by abnormal social interactions, impaired communication, and repetitive behavior. The concordance rate of ASD for identical twins is 90%, but less than 10% for fraternal twins and siblings, suggesting a strong genetic component (Geschwind and Levitt, 2007). Causes of ASD are not known. Among many hypotheses that have been postulated to cause ASD, mounting evidence supports that synapse abnormalities might be responsible for ASD (Zoghbi, 2003). In particular, an imbalance of E/I ratio in neural circuitry has been associated with autisms (Rubenstein and Merzenich, 2003; Polleux and Lauder, 2004). The direct evidence derives from the genetic findings that mutations in neurexin-neuroligin pathway are reproducibly associated with ASD. Jamain et al first reported that two pairs of Swedish brothers with ASD carry mutations in neuroligin genes—a missense mutation in neuroligin 3 (R451C) and nonsense mutation in neuroligin 4 (396X) (Jamain et al., 2003). Subsequently, two independent groups also found that nonsense and missense mutations in neuroligin 4 are associated with ASD and mental retardation (Yan et al., 2005; Laumonnier et al., 2004). Recently, mutations in Shank3, a scaffolding protein associated with neuroligins, have been identified in ASD families and individuals (Durand et al., 2007). More recently, the Autism Genome Project Consortium, a group of over 50 institutions in North America and Europe, carried out the largest linkage scan and copy number variation analyses to date by using samples from

more than 1100 ASD families with at least two affected individuals, found neurexin I gene associated with ASD (Szatmari et al., 2007). These findings led to the hypothesis that abnormalities in neurexin-neuroligin pathway are associated with ASD (Garber, 2007).

To directly test this hypothesis, Tabuchi et al introduced the R451C mutation into the endogenous neuroligin-3 gene in mice. The knockin mutant mice display increased inhibitory synaptic transmission and impaired social interactions, but enhanced abilities in spatial learning (Tabuchi et al., 2007). Although the behavioral abnormalities observed in neuroligin-3 knockin mice are only analogous to some diagnostic symptoms of ASD and autistics with enhanced cognitive abilities are extremely rare, neuroligin-3 knockin mice will be a very useful model to study ASD. The findings that R451C mutation resulted in an increase in inhibitory transmission without affecting excitatory transmission strongly support the hypothesis that a shift in E/I balance might contribute to the pathogenesis of ASD. Future studies on the mechanisms how abnormalities in neurexin-neuroligin pathway alter synapse development and function, shift the E/I balance in key neural circuits, and lead to autistic behavioral abnormalities will shed light on the pathogenesis and treatment of ASD.

1.6 *Drosophila* larval NMJ as an excellent in vivo model system to study synapse development and function

Drosophila body wall musculature has segmentally repeated pattern (Hoang and Chiba, 2001). In each embryonic/larval abdominal hemi-segment, there are 30 skeletal muscles innervated by about 40 motor neurons located within the ventral nerve cord. Each of the

30 muscle fibers can be easily identified by its position, orientation and shape. The innervating motor axons branch onto muscle surface forming a series of varicosities, called synaptic boutons. Based on their morphology, these boutons can be divided into three main types. Type-I boutons, the major class of terminals that innervate all body wall muscles, are glutamatergic. Type-II or type-III terminals innervate subsets of muscles and contain octopamine or peptide neurotransmitters as well as glutamate. At each developmental stage, type-I NMJs display a highly stereotypic morphology, including axon innervating and branching pattern, bouton number and size on each individual muscle.

Ultrastructurally, the morphology of *Drosophila* type I NMJs more resembles vertebrate central synapses than vertebrate NMJs. In contrast to vertebrate NMJs that show a 50- to 60-nm wide synapse cleft between the membranes of motor neuron and muscle and contain a basal lamina, the synapse cleft in *Drosophila* NMJs does not contain an evident basal lamina and is only 15- to 20-nm wide (Prokop and Meinertzhagen, 2006). At vertebrate NMJs, active zones and PSDs are not exactly aligned across the synapse cleft: PSDs are present mostly on the crests of junctional folds, and presynaptic active zones are located across the openings of junctional folds instead of the crests of junctional folds (Hughes et al., 2006). At *Drosophila* NMJ, however, PSDs are exactly juxtaposed to active zones. The active zone at *Drosophila* NMJs is characterized by so-called T-bars, due to their T-shaped appearance in cross sections of electron micrographs. The three-dimensional structure of T-bar resembles a pedestal table comprising a base surmounted by a platform. Clouds of synaptic vesicles surround the T-bar. Some vesicles appear physically attached to the T-bar, and some

fused to the presynaptic plasma membrane beneath the T-bar. The T-bar is thought to bridge synaptic vesicles in close proximity to release sites to facilitate neurotransmitter release (Prokop and Meinertzhagen, 2006). Functionally, the T-bar is analogous to the grid- or mesh-like dense structure formed by the cytomatrix at the active zone (CAZ) of vertebrate synapses (Dresbach et al., 2001).

Bruchpilot (BRP), as the first component of T-bars, has been recently identified. It is a homologue of mammalian CAZ component protein CAST, which binds RIM in a complex with Bassoon and Munc-13. *brp* mutants show reduced evoked vesicle release, altered short-term plasticity, but normal spontaneous transmission. At *brp* mutant active zones, T-bars are entirely lost and calcium channel densities are reduced. Thus, BRP is proposed to establish the proximity between Ca^{2+} channels and vesicles to allow efficient transmitter release (Kittel et al., 2006; Wagh et al., 2006).

Synapse formation at the NMJ starts during the late stages of embryogenesis. Motor neuron growth cones establish the initial contacts with specific muscle fibers, forming enlarged varicosities—synaptic boutons, containing the first active zones and postsynaptic densities (Featherstone and Broadie, 2000). The embryonic NMJs have only a few branches and boutons. However, during larval development, NMJs grow substantially in size and strength to match the continuous increase in muscle size, to maintain appropriate synaptic efficacy, and to achieve proper muscle depolarization (Griffith and Budnik, 2006)**.** The NMJ expansion involves the formation of new boutons and an increase in the number of active zones per bouton(Schuster et al., 1996; Zito et al., 1999). This coordinated development of pre- and postsynaptic specializations during synaptic growth is a good example of synaptic homeostasis. A number of genes have

been identified to control synaptic growth, including the cell adhesion molecule Fasciclin II (Schuster et al., 1996), the microtubule-associated protein Futsch (Roos et al., 2000), the cytoskeleton adaptor protein Nervous Wreck (Coyle et al., 2004), the ubiquitin ligase Highwire (Wan et al., 2000), the deubiquitinating protease Fat Facets (DiAntonio et al., 2001), and the components in BMP retrograde signaling pathways such as BMP type II receptor Wishful thinking.

In addition to highly-stereotyped morphology, well-studied ultrastructure and development, *Drosophila* larval NMJs offer a number of exceptional advantages as an excellent *in vivo* model system to study synapse development and function (Koh et al., 2000; Featherstone and Broadie, 2000): (1) The powerful *Drosophila* genetics has provided a variety of genetic tools and techniques to tackle the fundamental questions on synapse development and function, and to genetically dissect underlying mechanisms. Forward genetic screens have facilitated the discovery of new players in synapse development and function. (2) Type-I NMJs have many molecular features in common with central synapses in the mammalian brain. Many key synaptic molecules have shown striking conversation in regard to their functions and underlying mechanisms, but less gene redundancy in *Drosophila*. (3) Because of their large size, larval NMJs are easily accessible for a variety of cell biology techniques including electron microscopy and in vivo live imaging, and electrophysiological recordings.

1.7 Research Goals

Despite the expanding evidence suggesting that neurexins may act as a key player in synapse development and function, the exact in vivo function of neurexins still remains

exclusive, particularly due to the complexity and redundancy of neurexin genes in mammals. A potential strategy to tackle this longstanding question is to use a simpler model system, such as the fruit fly. We identified a single *Drosophila* neurexin gene (*dnrx*) that has striking conservation with mammalian neurexins. To understand the in vivo function of DNRX, I have accomplished the following specific aims: (1) characterize the *dnrx* gene and determine the precise subcellular localization of DNRX at larval NMJ; (2) generate *dnrx* loss-of-function mutants; and (3) identify the role of DNRX in synaptic development and function using *Drosophila* larval NMJ as a model system.

I present data demonstrating that DNRX is concentrated at the active zone of presynaptic terminals at glutamatergic NMJs. Phenotypic analyses of *dnrx* null mutants revealed that DNRX plays a crucial role in the control of synapse growth, the modulation of synaptic architecture and the regulation of synaptic transmission. The described studies provide novel insights into understanding the function of neurexins *in vivo*, and offer a strong basis for the interpretation of observations at mammalian central synapses.

Figure 1.1 Structure and Alternative Splicing of Neurexins

α-neurexins are composed of a large extracellular region containing a signal peptide (SP) and three LamG-EGF-LamG repeats, a single transmembrane region (TMR), and a short cytoplasmic region containing a PDZ binding motif. β-neurexins contain only one LamG domain, which is preceded by a N-terminal sequence specific for β-neurexins (βN). Alternative splicing sites (SS) are numbered and indicated by arrows. There are five alternative splicing sites present in α-neurexins, and two present inβ-neurexins.

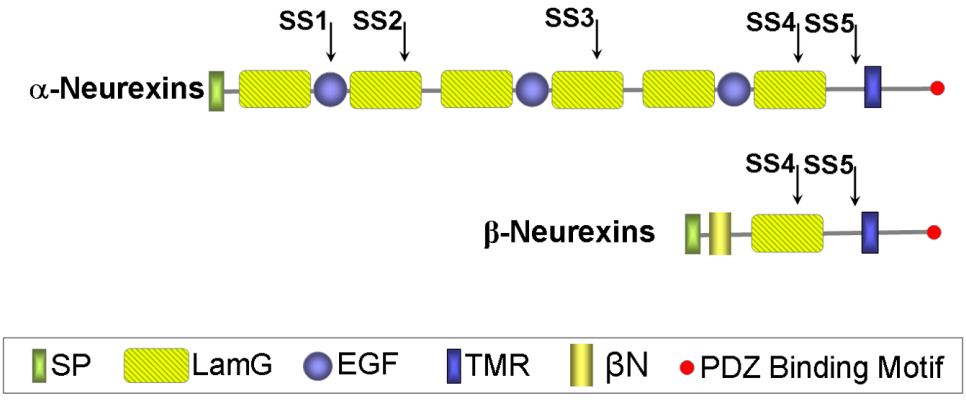


Figure 1.2 The Protein Interaction Map of Neurexins

The extracellular region of neurexins binds to neuroligins and dystroglycan in a Ca^{2+}-dependent manner. Neuroligins are localized to PSDs and associated with neurotransmitter receptors by interactions with scaffolding proteins such as PSD-95. Dystroglycan is an integral component of the dystrophin-associated glycoprotein complex. The glycosylation of dystroglycan is required for dystroglycan to bind to neurexins. Intracelluarly, neurexins interact with synaptic vesicle protein synaptotagmin and PDZ domain proteins Mints and CASK. Mints and CASK are linked to synaptic vesicle exocytosis machinery including presynaptic Ca^{2+} channels and Munc-18. Neurexophilins, a family of small neuropeptide-like protein, also directly bind to neurexins in a high affinity.

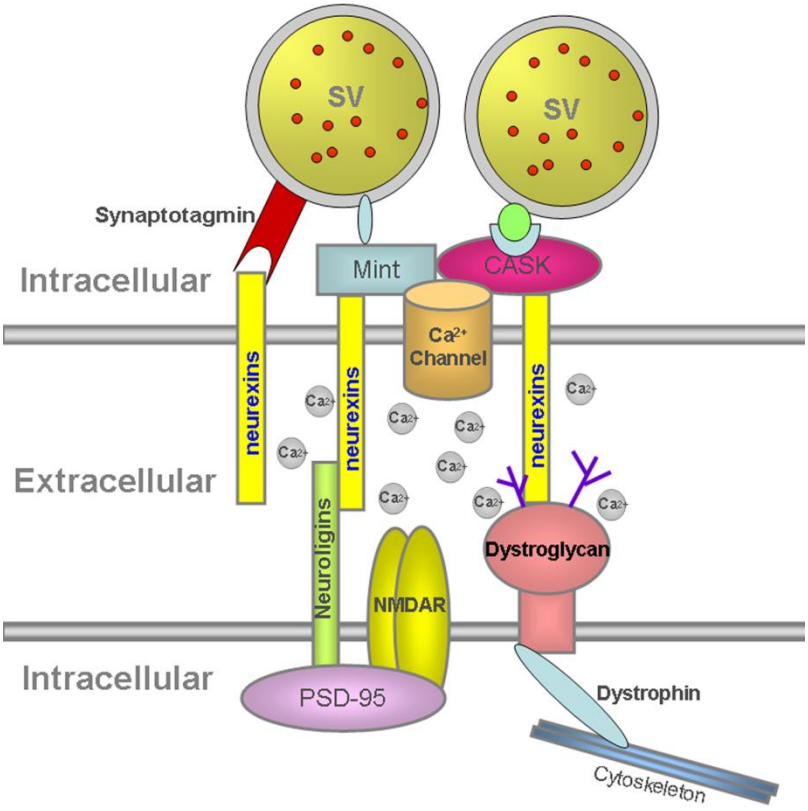
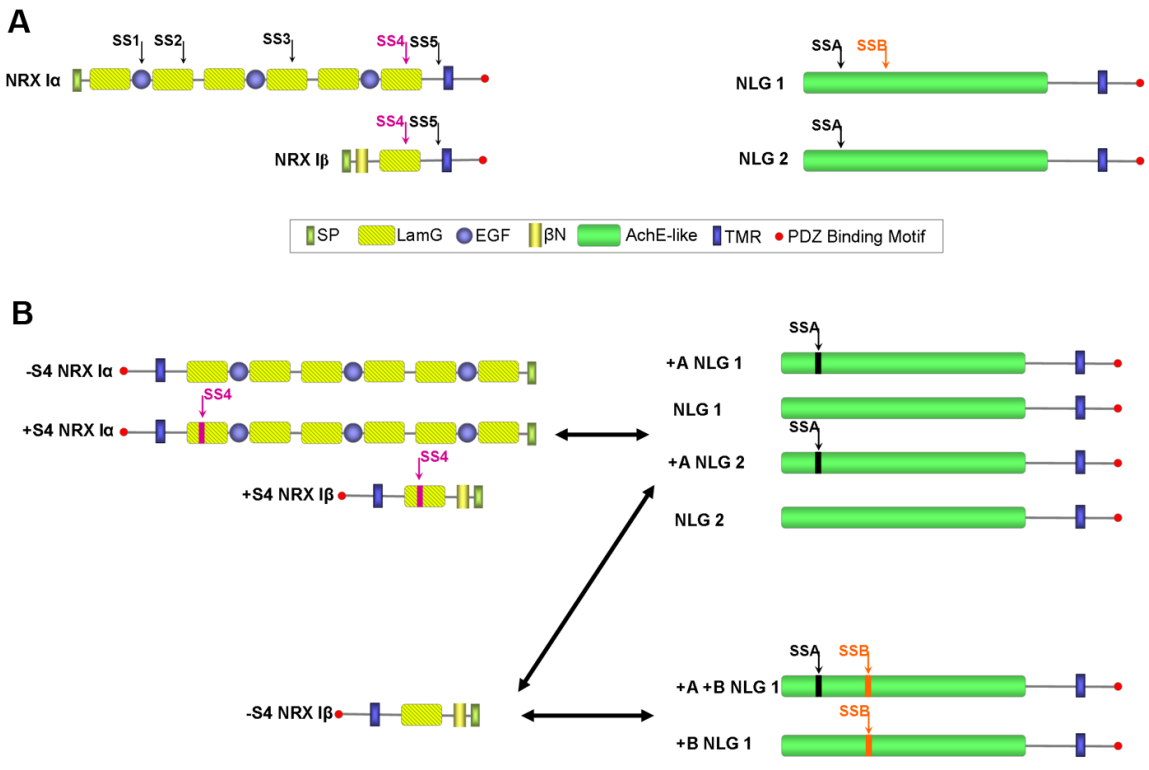


Figure 1.3 Neurexin-neuroligin Splicing Codes and Selective Interactions

(A) Potential splicing sites in neurexins and neuroligins. Arrows indicate the alternative splicing sites in α- and β-neurexins (NRX), neuroligin (NLG) 1 and 2.

(B) Splicing codes that guide the selective interactions of neurexins and neuroligins. α-neurexins selectively bind to neuroligins lacking an insert at SSB (-B neuroligins), whereas β-neurexins can bind to neuroligins regardless of splicing at SSB in neuroligins. β-Neurexins containing an insert at SS4 interact preferentially with –B neuroligins, while β-neurexins lacking an insert at SS4 interact with –B neuroligins and +B neuroligins with equal affinity.



CHAPTER TWO

DROSOPHILA NEUREXIN IS EXPRESSED IN CENTRAL NEURONS AND CONCENTRATED AT ACTIVE ZONES

2.1 Abstract

Neurexins, a family of highly conserved neuron-specific transmembrane proteins, have been proposed to act as a key synapse organizer required for synapse formation and neurotransmitter release. However, their *in vivo* functions remain elusive, particularly due to the complexity and redundancy of mammalian *neurexin* genes. Here, we report the cloning and characterization of the *Drosophila* homolog of neurexin genes. In contract to the presence of 3 neurexin genes in mammals, we identified a single neurexin gene in the *Drosophila* genome, which we named *Drosophila neurexin (dnrx)*. Toward a better understanding of DNRX function, we examined the expression and subcellular localization of DNRX during development. In situ hybridization reveals that *dnrx* expression starts before the differentiation of presynaptic terminals, and is highly enriched in the neurons of central nervous system. Consistent with this, immunocytochemical staining shows that DNRX localizes to CNS synaptic regions, axons, and glutamatergic neuromuscular junctions (NMJs) during development. At larval NMJ, DNRX is concentrated at active zones, but also extends into periactive zones within synaptic boutons. These findings indicate the potential roles of DNRX in synapse development and function, particularly the assembly and organization of active zone, synaptic transmission, and/or synaptic growth.

2.2 Introduction

Synapses are specialized cell-cell connections that link neurons and their target cells into functional network through neurotransmission. Neurotransmission depends on two ultrastructurally defined synapse components— the presynaptic active zone and postsynaptic density (PSD). The active zone, which is defined as the presynaptic region specialized for the release of neurotransmitter-filled vesicles, is juxtaposed against the PSD, which contains neurotransmitter receptors and signaling complexes to respond to neurotransmitters (Waites et al., 2005). Recent evidence has highlighted the importance of trans-synaptic cell adhesion molecules in synapse development and function (Dalva et al., 2007; Gerrow and El-Husseini, 2006b; Scheiffele, 2003; Yamagata et al., 2003). Of these, neurexins—a family of highly conserved neuron-specific transmembrane proteins, have been proposed to act as a key synapse organizer required for synapse formation and neurotransmitter release (Dean and Dresbach, 2006; Craig and Kang, 2007).

Mammalian genomes contain three neurexin genes, each of which has two promoters generating α- and β-neurexins. The extracellular region of α-neurexins is composed of three LamG-EGF-LamG repeats, whereas β-neurexins contain a single LamG domain (Missler and Sudhof, 1998a) and a unique N-terminus. α-neurexins and β-neurexins share identical transmembrane domain and cytoplasmic sequences. Neurexins are subject to extensive alternative splicing, generating a large number of variants (Missler and Sudhof, 1998a; Rowen et al., 2002; Tabuchi and Sudhof, 2002), which may mediate synaptic specificity. (Comoletti et al., 2006; Chih et al., 2006; Rowen et al., 2002; Tabuchi and Sudhof, 2002; Graf et al., 2006; Boucard et al., 2005). The

extracellular region of neurexins binds to neuroligins (Ichtchenko et al., 1995; Boucard et al., 2005) and dystroglycan (Sugita et al., 2001). Neuroligins are localized to PSDs (Song et al., 1999) and associated with neurotransmitter receptors by interaction with scaffolding proteins (Irie et al., 1997; Meyer et al., 2004; Hirao et al., 1998; Irie et al., 1997). Intracelluarly, neurexins interact with the synaptic vesicle protein synaptotagmin (Hata et al., 1993), and PDZ domain proteins CASK (Hata et al., 1996) and Mints (Biederer and Sudhof, 2000), which are linked to the synaptic vesicle exocytosis machinery (Atasoy et al., 2007; Ho et al., 2003). Thus, the trans-synaptic interaction between neurexin and neuroligin may bridge the synaptic cleft aligning the presynaptic neurotransmitter release machinery with PSDs.

Important findings from cell culture studies suggest that neurexins and neuroligins could act bidirectionally to induce pre- and postsynaptic assembly, thus controlling synapse formation (Scheiffele et al., 2000; Graf et al., 2004; Dean et al., 2003; Nam and Chen, 2005; Chih et al., 2005; Prange et al., 2004; Levinson et al., 2005; Fu et al., 2003). Interestingly, phenotypic analyses of α-neurexins triple knockout mice demonstrate that α-neurexins are required for Ca^{2+} channel function and thus neurotransmitter release but are dispensable for synapse formation (Missler et al., 2003; Zhang et al., 2005). Recently, a large scale genetic linkage scan and copy number variation study found neurexin I gene associated with autism (Szatmari et al., 2007). However, despite the expanding evidence indicating that neurexins may play a key role in synaptic development and synaptic transmission, the in vivo function of neurexins still remain exclusive, particularly due to the complexity and redundancy of neurexin genes in mammals.

A potential strategy to resolve these issues is to use a simpler model system, such as the fruit fly, to investigate the *in vivo* function of neurexins. However, the first neurexin-related gene isolated in *Drosophila*, *neurexin IV* (*nrx IV*), is primarily expressed in epithelial and glial cells, where it is required for the organization and function of septate junctions (Baumgartner et al., 1996; Banerjee et al., 2006; Faivre-Sarrailh et al., 2004). Nrx IV has an identical domain structure to Contactin-associated protein 1 (Caspr1), a member of the Caspr family which is distantly related to the neurexin family and mediates neuron-glia interactions (Bellen et al., 1998; Bhat et al., 2001; Peles et al., 1997; Poliak et al., 1999).

Taking advantage of the near completion of the *Drosophila* genome project (Adams et al., 2000), we identified a single gene with striking conservation with mammalian neurexins, resurrecting the initial idea of using the *Drosophila* system to understand neurexin function at synapses. In the goal of understanding the in vivo function of *Drosophila neurexin* (*dnrx*), we have examined the expression and the subcellular localization of DNRX during development. *dnrx* expression starts before the differentiation of presynaptic terminals, and is highly enriched in central neurons. DNRX localizes to synaptic regions, including glutamatergic neuromuscular junctions (NMJs), where DNRX is concentrated at the active zone within synaptic boutons. Our results indicate a potential role of DNRX in the formation and development of active zone, synaptic transmission, and/or synaptic growth. In addition, anti-DNRX antibody would be of general use as an active zone marker to study synapse development and function.

2.3 Materials and Methods

Drosophila Strains

Flies were raised at 25 °C on standard medium. All flies used in this work are the wild-type strain Canton-S.

Cloning and Sequencing of *dnrx* Full Length cDNA

A radio-labeled *dnrx* EST (*LP03809*) was used to screen a *Drosophila* 0-20hr embryo cDNA library. Overlapping partial cDNA clones were isolated, sequenced and compiled as a cDNA sequence of 5738 base pairs (GenBank accession number EF460788), which comprises an open reading frame of 5520 base pair with 5' and 3' UTR.

Bioinformatics

Rat neurexin 1α cDNA sequence was used to perform BLAST search against the *Drosophila* genome and EST databases, and *dnrx* (CG7050) was identified with the highest degree of similarity. The domain organization of DNRX was analyzed using SMART (Schultz et al., 1998). Megs 3.1 (Kumar et al., 2004) was used for sequence alignment (ClustalW) and phylogenetic analysis (neighbor-joining method).

In situ Hybridization

A HindIII-SalI fragment of *dnrx* cDNA clone encoding the 2nd LamG-EGF-LamG-domain repeat, and a PstI-NotI fragment encoding the last-LamG-domain-cytoplasmic-region were cloned into pBluescript vector. The two resulting clones were linearized. Antisense and sense RNA probes were synthesized by using T7 and T3 RNA

polymerases, labeled with digoxigenin-UTP (Roche), and used for in situ hybridization following standard protocols (Kearney et al., 2004).

Protein Expression and Purification of DNRX **Antibody**

A cDNA fragment encoding the cytoplasmic region of DNRX was cloned into pET-28a and pGEX vector, respectively. His-tagged DNRX fusion protein was expressed in *E.coli BL21(DE3)* and purified using Ni-NTA agarose affinity resin (Novagen). Guinea pigs were immunized with this protein at Cocalico Biologicals, PA. Recombinant glutathione S-transferase (GST) fusion protein GST-DNRX was expressed in *E.coli BL21,* purified with glutathione-sepharose (GE Phamacia), and coupled to a NHS-activated Sepharose column (GE Phamacia). Antisera were affinity purified using this column. The column was washed in 500mM NaCl, 20mM Tris-HCl, pH 7.5, and eluted with 500mM NaCl, 100mM glycine-HCl, pH2.7. To minimize denaturation of the antibody at low pH, the eluted fractions were immediately mixed with the neutralization buffer (1M Tris-HCl, pH8, and 10 mg/ml BSA).

Immunohistochemistry and Confocal Microscopy

Preparation and antibody staining for whole-mount embryos and dissected wandering 3rd instar larvae were performed as described by Bellen and Budnik (2000). Dissected larval NMJs were fixed in Bouin's fixative (15:5:1 mixture of saturated picric acid, 37% formaldehyde and glacial acetic acid) for 15 min. The following antibodies were used: guinea pig anti-DNRX (1:500), rabbit anti-DPAK (1:2000, N. Harden, Simon Fraser University, Canada; (Harden et al., 1996), rabbit anti-NWK (1:2000, B. Ganetzky,

University of Wisconsin, Madison; (Coyle et al., 2004), monoclonal anti-discs-large (1:500), anti-BRP (1:500), and anti-FASII (1:100) from Developmental Studies Hybridoma Bank, University of Iowa. Secondary antibodies conjugated to Alexa 488 and 568 (Invitrogen-Molecular Probes) were used at 1:400. Fluorescence conjugated anti-HRP (Jackson Immuno Labs) antibodies were used at 1:50.

DNRX signal at the NMJ was detected by using the VECTASTAIN ABC system (Vector Laboratories) and Tyramide Signal Amplification (TSA, Invitrogen-Molecular Probes). Double-staining of NMJ with anti-DNRX and a second primary antibody was performed as following. Briefly, fixed larvae were washed in PBT (PBS containing 0.3% Triton X-100) and blocked for 1hr with 1% blocking reagent (TSA kit, Invitrogen-Molecular Probes). Blocked samples were incubated with anti-DNRX and a second primary antibody overnight at 4 °C. After washes with PBT, the samples were incubated with biotinylated goat anti-guinea pig antibody (1:400, Vector Laboratories) and a fluorescent secondary antibody. The incubation was followed by washes with PBT and one-hour incubation in ABC reagent (1:250 in 2% BSA /PBT, diluted 30 min before use, Vector laboratories). Six subsequent washes with PBT and one with PBS were followed by Tyramide labeling according to the manufacturer's instructions.

Confocal images were acquired using a Bio-Rad Radiance 2000 confocal microscope with LaserSharp2000 software.

2.4 Results

DNRX **is the Single *Drosophila* Homolog of Vertebrate Neurexins**

To identify a neurexin homolog in *Drosophila*, the rat neurexin 1α cDNA sequence (Ushkaryov et al., 1992) was blasted against the *Drosophila* genomic and EST databases. We identified an EST (*LP03809*) with significant homology to the C-terminal sequences of vertebrate neurexins. This EST was subsequently used to screen a 0-20 hr old embryonic cDNA library to obtain a full-length *dnrx* cDNA (Gen Bank accession number EF460788). In contrast to the presence of three neurexin genes in mammals, our genome-wide search revealed only a single *neurexin* gene (*CG7050*) in *Drosophila* (also reported by Tabuchi and Sudhof, 2002; Zeng et al., 2007), which we named ***Drosophila n****eu****r****ex****i****n*** (*dnrx*). The DNRX protein has an identical domain structural organization to mammalian α-neurexins (Fig. 2.1A). The large extracellular region consists of an N-terminal signal peptide and three LamG-EGF-LamG repeats. Although the cytoplasmic region of DNRX is longer than the mammalian counterparts, the PDZ binding motif at the C-terminus is highly conserved (Fig. 2.1C). Overall, DNRX is 36-37% identical to human α-neurexins, and shares high amino acid sequence identity with mammalian neurexins within each individual protein domain (Fig. 2.1A).

Previously, the *Drosophila* gene *neurexin IV* (*nrx IV*) was identified and proposed to belong to the neurexin family (Baumgartner et al., 1996). However, the domain arrangement of NRX IV differs from that of classical neurexins (Fig. 2.1A). Furthermore, our phylogenetic analysis shows that DNRX is the closest homolog of vertebrate neurexins, whereas NRX IV is only distantly related to neurexins (Fig. 2.1B).

dnrx Is Highly Expressed in Central Neurons

To determine where DNRX function might be required during development, we performed *in situ* hybridization to examine *dnrx* expression during embryonic stages. *In situ* hybridization in embryos using two independent RNA probes revealed that *dnrx* expression was enriched in the neurons of the brain and ventral nerve cord (Fig. 2.2A, 2.2B, and2.2C), but undetectable in muscle cells (Fig.2.2D). *dnrx* mRNA first appeared in subsets of central neurons at late stage 14 (Fig.2.2A), when axon pathfinding is nearly complete and the differentiation of presynaptic terminals is about to begin (Featherstone and Broadie, 2000). This expression reached its highest levels at stages 16 and 17, when a larger number of neurons in the brain and ventral nerve cord expressed *dnrx* at elevated levels (Fig. 2.2B and 2.2C). Low levels of *dnrx* expression could also be detected in small subsets of peripheral nervous system (PNS) neurons (Fig. 2.2A).

DNRX localizes to Axons, Central Synaptic Regions and Glutamatergic NMJs

The subcellular localization of DNRX was determined by immunocytochemical analysis using an affinity-purified polyclonal antibody generated against the cytoplasmic region of DNRX. In embryos DNRX was concentrated in neuropil regions of the brain and ventral nerve cord, axon tracts of the ventral nerve cord, and motor axons (Fig. 2.3A and 2.3B). The immunoreactivity was absent in homozygous embryos of *Df(3R)5C1* which uncovers the *dnrx* gene, demonstrating the specificity of the DNRX antibody (Fig. 2.3C). A similar DNRX distribution was observed in 3rd instar larvae. DNRX is expressed in the ventral ganglion and subsets of neurons in brain lobes, and labels synaptic regions and motor axons (Fig. 2.3D). In addition, DNRX was present at the glutamatergic type I boutons of

the larval body wall muscles. This was confirmed by double staining with an antibody against the scaffolding protein Discs-large (DLG) (Fig. 2.3E-2.3E"), which labels type I boutons, but not other bouton types (Fig. 2.3E'; (Lahey et al., 1994).

DNRX Is Concentrated at Active Zones within Synaptic Boutons

Confocal microscopy analysis revealed that within synaptic boutons DNRX immunoreactivity did not have a uniform distribution, but displayed discontinuous patches (Fig. 2.4A-A", 2.4B-B", 2.4C and 2.4D). To define the precise subcellular localization of DNRX in these patches, we used a number of synaptic markers. A presynaptic marker, Bruchpilot (BRP; (Wagh et al., 2006; Kittel et al., 2006), is thought to label at least one component of the active zone, the T-bar. Bright DNRX patches were juxtaposed and slightly overlapped with almost every BRP spot, as revealed by examining the labels in thin consecutive confocal slices (Fig. 2.4A-A", arrows and insets). In addition, DNRX appeared to surround BRP immunoreactivity. Notably, the postsynaptic density marker *Drosophila* p-21 activated kinase (DPAK; (Sone et al., 2000), also appeared juxtaposed to DNRX patches, but in contrast to BRP, minimal overlap between the labels was observed (Fig. 2.4B-B", arrows and insets).

We next double labeled NMJ preparation with DNRX and the so-called periactive zone markers, such as the cell adhesion molecule fasciclin II (FASII) (Sone et al., 2000) and the SH3 adaptor protein nervous wreck (NWK) (Coyle et al., 2004). In addition to FASII and NWK, many synaptic proteins that regulate synaptic bouton growth including the ubiquitin ligase highwire (Wan et al., 2000), the guanine-nucleotide exchange factor still life (Sone et al., 2000), and the scaffolding protein for synaptic vesicle endocytic

machinery Dap 160 (Marie et al., 2004; Koh et al., 2004), are also localized to this region. In general, DNRX showed no overlap with NWK, although occasionally colocalization between less intense DNRX regions and NWK was observed (Fig. 2.4C-C"). In the case of FASII, there are significant regions of nonoverlap with some regions displaying partial overlap (Fig. 2.4D-D"). Thus, within synaptic boutons DNRX appears to be concentrated at active zones, but also extends into periactive zones. The abundance of DNRX at active zones suggests its potential role in the assembly and organization of active zone and neurotransmission, while the extended distribution into the peri-active zone suggests additional function during synapse development such as synaptic growth.

2.5 Discussion

Trans-synaptic cell adhesion molecules mediate the coordinated interaction between pre- and postsynaptic membrane, affect the development and function of synapses at multiple levels. Among these, neurexins and neuroligin are perhaps the most extensively studied, and are emerging as central synaptic organizing molecules during synapse development and in synaptic transmission. However, their in vivo function and functional relevance are still unclear. The complexity and redundancy of neurexin genes in mammals have posed a tremendous difficulty in understanding their function in vivo. In this study, we have identified a single neurexin gene in *Drosophila* (*dnrx*), and characterized the expression pattern of DNRX during development, and defined its precise synaptic localization, thus providing a strong basis for the interpretation and a better understanding of DNRX function in vivo.

In contrast to three neurexin genes in mammals, we found that *dnrx* is the only neurexin gene in *Drosophila*. The DNRX protein has an identical domain structure to vertebrate α-neurexin. We have not been able to identify β-neurexin or additional isoforms from the cDNAs we isolated and available EST sequences. Moreover, our Western blotting analysis with the affinity-purified antibody against the cytoplasmic region of DNRX revealed a single ~200 kDa band (data not shown), suggesting that no β-neurexin is present in *Drosophila* (also reported by Zeng et al., 2007). Although we could not rule out the alternative splicing isoforms with a similar molecular weight, and/or the soluble isoforms that is unable to be detected by our DNRX antibody; all available cDNA and EST sequences so far have not showed any splicing isoform. Therefore, it appears unlikely that *dnrx* is subject to extensive alternative splicing. The single neurexin gene and lack of polymorphism in *Drosophila* would offer a great advantage for genetic dissecting of neurexin function in vivo, and simplify the interpretation of the results.

Mammalian neurexins are expressed throughout the CNS and exclusively in neurons (Ushkaryov et al., 1992; Ullrich et al., 1995; Puschel and Betz, 1995). In cultured neurons, neurexins are concentrated in growth cones of isolated axons, and colocalized with synaptic markers (Dean et al., 2003; Graf et al., 2004). Using immunoelectron microscopy, Scheiffele and colleagues have recently confirmed the synaptic localization of neurexins, which have long been postulated by protein interaction studies. Unexpectedly, in addition to the presynaptic active zone, neurexins are abundant in the PSD as well (Taniguchi et al., 2007). The distribution of anti-neurexin gold particles has a peak centered at the active zone and PSD, but was also observed outside of active zone and PSD. It is notable that the neurexin antibodies used in this study are pan-neurexin

antibodies, which do not allow any conclusion regarding the localization of specific neurexin isoforms. That is, whether specific neurexin isoforms target to presynaptic, postsynaptic or both pre- and postsynaptic sites are unclear.

Our study shows that DNRX is widely expressed in central neurons, localized to synaptic regions in CNS, and concentrated at the active zone at NMJs. The expression pattern and the synaptic localization of DNRX show remarkable parallels with mammalian neurexins. Moreover, we found that the proteins that have been shown to interact with mammalian neurexins also have homologs in *Drosophila*. Therefore, we propose that the function of neurexins and underlying signaling mechanism are evolutionarily conserved. The active zone localization of DNRX indicates its potential role in the assembly and organization of the active zone, and/or synaptic transmission. Moreover, no detectable expression in muscles and minimal overlap with the PSD marker DPAK suggest that DNRX might primarily function at the presynaptic site at NMJ.

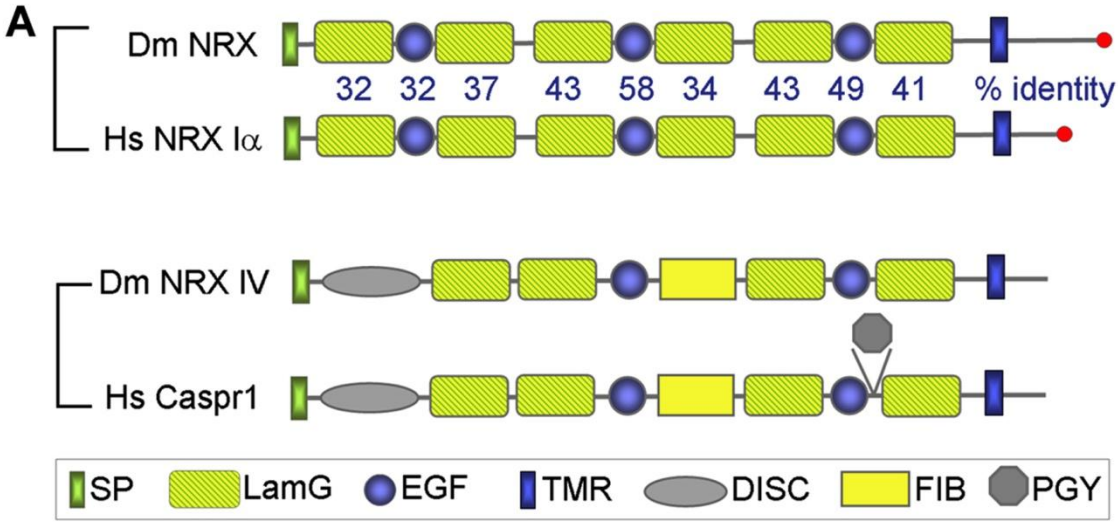
The periactive zone is referred as the synaptic region that is immediately adjacent to active zone (Sone et al., 2000). In addition to the endocytosis machinery, cell adhesion molecules such as N-cadherins and signaling molecules that necessary for synapse development, maintenance, and plasticity also reside in the periactive zone (Sudhof, 2004; Dustin and Colman, 2002; Sone et al., 2000; Tai et al., 2007). However, little is known about the organization and coordinated functions of the periactive zone. Interestingly, a few molecules that have been shown to regulate synaptic bouton growth localize to this region, including the cell adhesion molecules FASII(Sone et al., 2000), the cytoskeleton adapter protein NWK (Coyle et al., 2004), the ubiquitin ligase highwire (Wan et al., 2000), and the scaffolding protein for synaptic vesicle endocytic machinery

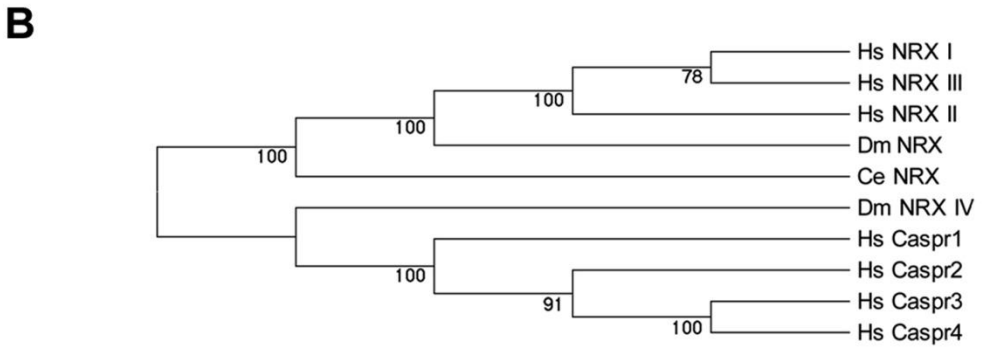
Dap 160 (Marie et al., 2004; Koh et al., 2004). Thus, it is hypothesized that the periactive zone is specialized for synaptic growth regulation (Coyle et al., 2004)**.** The extended distribution of DNRX into this region indicates its potential role in synaptic growth.

In summary, our results demonstrated that *dnrx*, the single *Drosophila* neurexin gene, is expressed in central neurons and concentrated at the active zones within synaptic boutons at NMJ. The similar expression pattern and synaptic localization of neurexins in *Drosophila* and mammals, and the existence of highly conserved neurexin-interacting protein homolog in *Drosophila* suggest that the function of neurexins and underlying mechanism might be evolutionarily conserved. Our functional study of DNRX using larval NMJ in vivo model synapse system would provide insights into many aspects of DNRX function in synapse development and function.

Figure 2.1 Molecular Analysis of DNRX

(A) Domain structure of DNRX, neurexin Iα, neurexin IV and Caspr1. The percent amino acid identity between DNRX and human neurexin Iα in specific domains is indicated. SP, signal peptide; LamG, laminin G domain; EGF, EGF repeat; DISC, discoidin-like domain; FIB, a region similar to fibrinogen; PGY, PGY repeat; TMR, transmembrane region. Dm, *Drosophila* melanogaster; Hs, Homo sapiens; Ce, C. elegans.
(B) Phylogenetic analysis of human, *Drosophila,* and *C. elegans* neurexins, *Drosophila* neurexin IV and human Caspr proteins using the neighbor-joining method (Mega 3.1; Kumar et al., 2004). DNRX and NRXIV belong to neurexin and Caspr subfamily, respectively. Numbers along each branch are the bootstrap confidence value.
(C) Multiple sequence alignment (ClustalW) of cytoplasmic sequences of *Drosophila, C. elegans* and human neurexins. The PDZ binding motif at the C-termini of neurexins is boxed.


A
Dm NRX
32 32 37 43 58 34 43 49 41 % identity
Hs NRX Iα
Dm NRX IV
Hs Caspr1
SP
LamG
EGF
TMR
DISC
FIB
PGY


B
Hs NRX I
Hs NRX III
Hs NRX II
Dm NRX
Ce NRX
Dm NRX IV
Hs Caspr1
Hs Caspr2
Hs Caspr3
Hs Caspr4
78
100
100
100
100
91
100

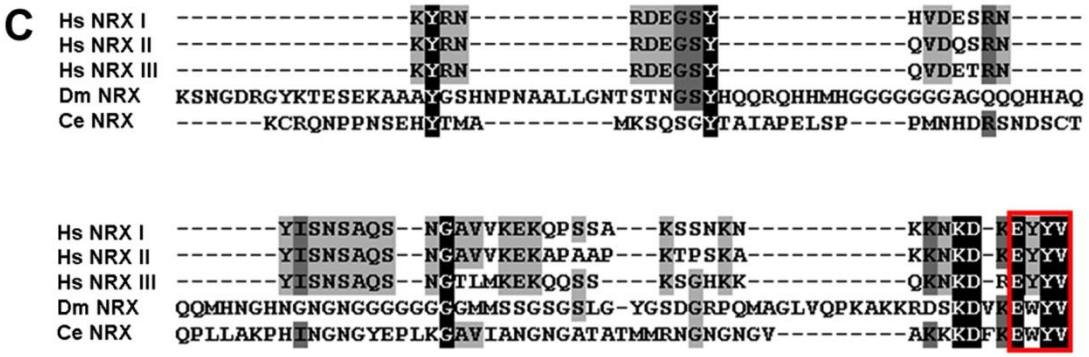

C
Hs NRX I
Hs NRX II
Hs NRX III
Dm NRX
Ce NRX

Figure 2.2 Developmental and Cellular Distribution of *dnrx* Expression Revealed by In situ Hybridization to 0-22-hour Embryos

(A and B) Lateral view of whole-mount embryos at late stage 14 (A) and stage 17 (B), showing that *dnrx* is abundantly expressed throughout the brain (Br) and ventral nerve cord (VNC). Low levels of *dnrx* expression were also detected in small subsets of peripheral nervous system neurons ([A], white arrows).

(C) Ventral view of a stage-17 whole-mount embryo showing *dnrx* mRNA distribution in CNS neurons.

(D) Higher magnification of a dissected embryo showing that *dnrx* mRNA is enriched in central neurons, but undetectable in muscle cells (black arrows).

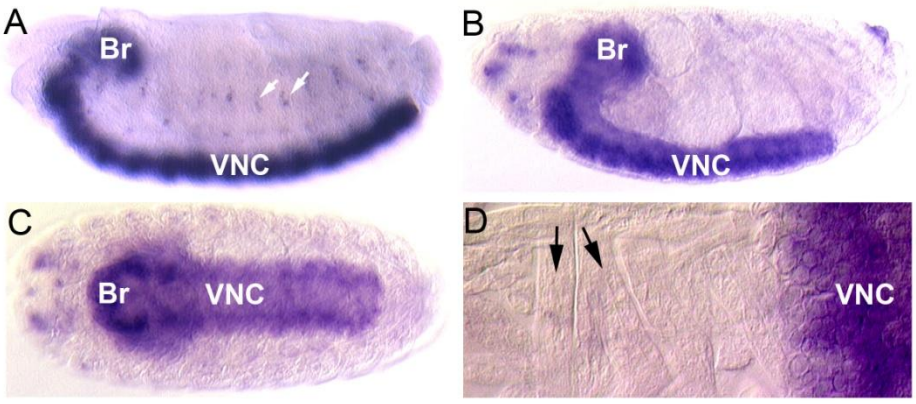


Figure 2.3 DNRX Localizes to Axons, CNS Neuropil Regions, and Glutamatergic NMJs

(A and B) Lateral and ventral view of wild-type embryos stained with anti-DNRX antibody, showing that DNRX is localized in the CNS neuropil and axonal tracts (arrows), and motor axons ([B], arrowheads).

(C) A *Df(3R)5C1* homozygous embryo stained with anti-DNRX, showing the specificity of antibody.

(D) 3rd instar larval CNS stained with anti-DNRX, showing that DNRX is concentrated in the synaptic regions of brain lobes and ventral ganglia.

(E-E'') Double-staining of wide-type 3rd instar larval NMJ 6/7 with anti-DNRX (green) and anti-DLG (red), which labels glutamatergic type I boutons. The merged DNRX and DLG images show that DNRX is located at type I boutons.

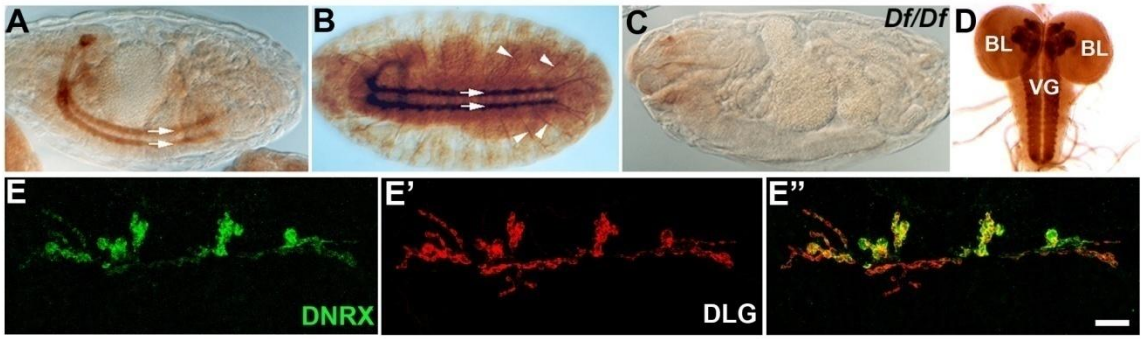


Figure 2.4 DNRX Is Concentrated at Active Zones within Synaptic Boutons

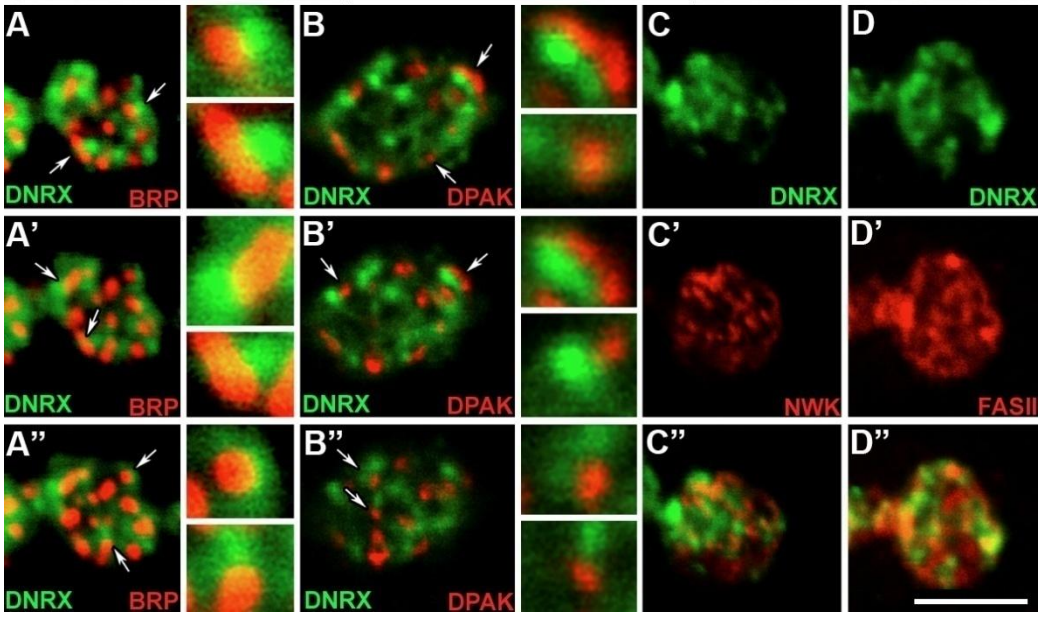
(A-A") Three consecutive single confocal slices from NMJs labeled with anti-DNRX and anti-BRP which labels a component of active zone, showing that bright DNRX spots are juxtaposed and slightly colocalized with BRP spots (Given that DNRX spots were associated to one side of BRP spots, such juxtaposition could not be seen in every slice, but could be verified in a sequence of single slices).

(B-B") Three consecutive single confocal slices from NMJs labeled with anti-DNRX and anti-DPAK which labels the PSDs, showing that DPAK and DNRX largely do not colocalize, and DPAK appears juxtaposed to DNRX patches.

Columns to the right side of A-B" are high magnification views of BRP-DNRX or DPAK-DNRX spots indicated in the low magnification panels by arrows.

(C-C'') Single confocal scan of synaptic boutons double-stained for DNRX and NWK, which localizes to the periactive zone of presynaptic terminals, shows that DNRX and NWK localize to distinct areas and do not display any significant overlap.

(D-D'') Single confocal scan of synaptic boutons double-stained for DNRX and FASII, a cell-adhesion molecule defines the periactive zone of pre- and postsynaptic membrane shows that most of DNRX staining regions do not overlap with that of FAS II. Scale bar, 5 μm (D").


A
DNRX
BRP
B
DNRX
DPAK
C
DNRX
D
DNRX
A'
DNRX
BRP
B'
DNRX
DPAK
C'
NWK
D'
FASII
A''
DNRX
BRP
B''
DNRX
DPAK
C''
D''

CHAPTER THREE

CRUCIAL ROLE OF *DROSOPHILA* NEUREXIN IN PROPER ACTIVE ZONE APPOSITION TO POSTSYNAPTIC DENSITIES, SYNAPTIC GROWTH AND SYNAPTIC TRANSMISSION

3.1 Abstract

Neurexins, a family of synaptic adhesion molecules, have been proposed to function as a major mediator of the coordinated pre- and postsynaptic apposition. However, key evidence for this role in vivo has been lacking, particularly due to the complexity and redundancy of mammalian *neurexin* genes. Here, we have obtained null mutations in the single *Drosophila* neurexin gene (*dnrx*). Using *Drosophila* larval neuromuscular junctions (NMJs), we demonstrate that *dnrx* loss of function prevents the normal proliferation of synaptic boutons at glutamatergic NMJs, while *dnrx* gain of function in neurons has the opposite effect. Conspicuously, *dnrx* null mutants display striking defects in synaptic ultrastructure with the presence of detachments between pre- and postsynaptic membranes, abnormally long active zones, and increased number of T-bars. These abnormalities result in corresponding alterations in synaptic transmission with reduced quantal content. Together, our results provide compelling evidence for an *in vivo* role of neurexins in regulating synapse growth, and the modulation of synaptic architecture and adhesive interactions between pre- and postsynaptic compartments.

3.2 Introduction

Synapse development and function form the basis of many neuronal processes, including formation and functioning of neural circuits, the ability to learn, and to store and recall memories. Thus, elucidating the mechanisms by which synapses develop and are modified is a central question in neurobiology. Over the past few decades a number of factors have been identified that play major roles in synapse morphogenesis and synaptic plasticity. Among these, trans-synaptic cell adhesion and signaling molecules that mediate the interactions between pre- and postsynaptic membranes stand out. They are thought to mediate target recognition, initiate synapse formation and alignment, maintain the integrity of synapse, and regulate synaptic function (Scheiffele, 2003; Yamagata et al., 2003). In particular, neurexins and their postsynaptic binding partners the neuroligins are emerging as key synapse organizing molecules.

Neurexins were first identified as primary receptors for α-latrotoxin, a neurotoxin that triggers massive neurotransmitter release (Ushkaryov et al., 1992). There are three *neurexin* genes in mammals, each of which has two promoters generating α- and β-neurexins. Neurexins are subject to extensive alternative splicing, generating a large number of variants (Missler and Sudhof, 1998a; Rowen et al., 2002; Tabuchi and Sudhof, 2002), which may mediate target recognition and synaptic specificity (Comoletti et al., 2006; Chih et al., 2006; Rowen et al., 2002; Tabuchi and Sudhof, 2002; Graf et al., 2006; Boucard et al., 2005). The extracellular region of neurexins binds to neuroligins (Ichtchenko et al., 1995; Boucard et al., 2005) and dystroglycan (Sugita et al., 2001). Neuroligins are localized to postsynaptic densities (PSDs) (Song et al., 1999) and associated with neurotransmitter receptors by interaction with scaffolding proteins (Irie et

al., 1997; Meyer et al., 2004; Hirao et al., 1998). Intracelluarly, neurexins interact with the synaptic vesicle protein synaptotagmin (Hata et al., 1993), and PDZ domain proteins CASK (Hata et al., 1996) and Mints (Biederer and Sudhof, 2000), which are linked to the synaptic vesicle exocytosis machinery (Atasoy et al., 2007; Ho et al., 2003). Thus, the trans-synaptic interaction between neurexin and neuroligin may bridge the synaptic cleft aligning the presynaptic neurotransmitter release machinery with postsynaptic densities. Important findings from cell culture studies indicate that neurexins and neuroligins could act bidirectionally to induce pre- and postsynaptic assembly, thus controlling synapse formation (Scheiffele et al., 2000; Graf et al., 2004; Dean et al., 2003; Nam and Chen, 2005; Chih et al., 2005; Prange et al., 2004; Levinson et al., 2005; Fu et al., 2003). Interestingly, phenotypic analyses of α-neurexins triple knockout mice suggest that α-neurexins are required for neurotransmitter release but dispensable for synapse formation (Missler et al., 2003; Zhang et al., 2005). Thus, despite the expanding evidence indicating that neurexins may act as synaptic recognition and organizer molecules in synapse development and function, the complexity and redundancy of neurexin genes in mammals pose a tremendous difficulty in understanding their function *in vivo.*

A potential strategy to resolve these issues is to use a simpler model system, such as the fruit fly, to investigate the *in vivo* function of neurexins. Our previous studies have identified the single *Drosophila* neurexin gene (*dnrx*) and characterized its expression pattern during development. DNRX is expressed in central neurons and concentrated at the active zone of glutamatergic neuromuscular junctions (NMJs), indicating its potential role in synaptic development and function, especially the formation and development of active zone.

Here we report the isolation of *Drosophila neurexin* (*dnrx*) null mutants and the characterization of its function during synapse development. Our results demonstrate that *dnrx* plays a critical role in synaptic growth, the cytoarchitecture of synapses, and the regulation of synaptic function. These studies provide a better understanding of neurexin function in an intact organism, and offer a strong basis for the interpretation of observations at mammalian central synapses.

3.3 Materials and Methods

Generation of *dnrx* Mutants

XP d08766 (Thibault et al., 2004), a P-element inserted ~200bp upstream of *dnrx*, was used to carry out an excision screen. To increase the frequency of large deletions, the excision screen was carried out in *mus309* background (Adams et al., 2003; McVey et al., 2004). Briefly, *XP d08766* was first recombined to $mus309^{N1}$ and $mus309^{N1}$*,XPd08766/TM6 Tb* recombinants were identified by single fly PCR using $mus309^{N1}$- and *XPd08766*- specific primers. Next, $mus309^{N1}$ *XPd08766/TM6 Tb* males were massively mated with $mus309^{D3}$ *Sb* Δ*2-3/TM6 Tb* females. The $mus309^{N1}$ *XPd08766/*$mus309^{D3}$ *Sb* Δ*2-3* males were individually crossed with *D/TM6 Tb* females. w^- male progeny was selected and individually crossed with *Df(3R)5C1/TM3 Sb Kr::GFP*. Single Δ*XP/ Df(3R)5C1* male flies were subject to PCR to select imprecise excisions, and Δ*XP/ TM3 Sb Kr::GFP* were used to establish stock lines. *dnrx* deletions were obtained by selecting imprecise excisions downstream of the P-element insertion site using primer pairs covering the whole *dnrx* locus. The following primer pairs were used to confirm $dnrx^{273}$ and $dnrx^{241}$ alleles with a common 5'primer (5'-ACGCGTCGCGCTAAAATCCAGCCCG-3'); and separate 3' primers ($dnrx^{273}$, 5'-

CGTATGAGTGCTTGGAGCGGAA-3'; and *dnrx*^{241}, 5'-AGCCGGTGCCGATGTCTATGACGAA-3').

Fly Stocks and Genetics

dnrx mutant alleles *dnrx*^{273}, *dnrx*^{241} *and Df(3R)5C1* (a deficiency that removes *dnrx*) were balanced over *TM3 Sb Kr::GFP*. *dnrx* mutants were identified by selecting 3rd instar larvae without GFP expression. A precise excision of *XPd08766* and/or wild-type (Canton-S) were used as control lines. *UAS-dnrx* transgenic flies were generated by cloning the entire *dnrx* full-length cDNA into *pUASP* vector for germ line transformation. Gal4 lines used for DNRX overexpression and rescue experiments were *C380*, which drives Gal4 expression mainly in motoneurons (Budnik et al., 1996), and *elav*, which drives Gal4 expression in all neurons (Lin and Goodman, 1994). DNRX overexpression experiments were performed by crossing Gal4 homozygous females to y *w/Y; UAS-dnrx /UAS-dnrx; +/+* and *y w/Y; UAS-dnrx /UAS-dnrx; UAS-dnrx /UAS-dnrx* males, respectively. For *dnrx* cDNA rescue studies, either *C380/Y; +/+; Df(3R)5C1/TM6 Tb* or *C380/Y; +/+; dnrx*^{273}*/TM6 Tb* were crossed to *+/+; UAS-dnrx /UAS-dnrx; dnrx*^{273}*/TM6 Tb.*

Larval Locomotor Assay

The larval locomotor assay was performed as described by Connolly and Tully (1998). Individual larvae were placed in the center of a 145-mm diameter Petri dish, with 3% agar covering the bottom and a 0.5X0.5 cm square grid marked on the lid. The number of grid line crossings within a 30 second time window was recorded six times.

Immunohistochemistry, Confocal Microscopy, and Morphological Quantification

Preparation and antibody staining for whole-mount embryos and dissected wandering 3rd instar larvae were performed as described by Bellen and Budnik (2000). Dissected larval NMJs were fixed in Bouin's fixative for 15 min. The following antibodies were used: guinea pig anti-DNRX (1:500), rabbit anti-DPAK (1:2000, N. Harden, Simon Fraser University, Canada; (Harden et al., 1996), rabbit anti-NWK (1:2000, B. Ganetzky, University of Wisconsin, Madison; (Coyle et al., 2004), rabbit anti-Syt (1:500, H. Bellen, Baylor College of Medicine, Houston; (Littleton et al., 1993a); rabbit anti-GluRIII (1:2000, A. DiAntonio, Washington University, St. Louis; (Marrus et al., 2004); guinea pig anti-Dap160 (1:2000, H. Bellen, Baylor College of Medicine, Houston; (Koh et al., 2004), monoclonal anti-discs-large (1:500), anti-BRP (1:500), anti-GluRIIA (1:50), and anti-FASII (1:100) from Developmental Studies Hybridoma Bank, University of Iowa. 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.

Confocal images were acquired using a Bio-Rad Radiance 2000 confocal microscope with LaserSharp2000 software. Samples for each experiment were processed simultaneously, and imaged using the same settings. For Cac-GFP live imaging, larvae were mounted on the slides and the images were acquired from live body wall muscle preparations with an Improvision spinning disc confocal microscope.

Quantification of bouton number was performed at muscles 6/7 and muscle 4 of abdominal segment 3. Total boutons at NMJ6/7 and type Ib boutons at NMJ4 were

visualized by staining of body wall muscle preparations with anti-HRP. For quantification of axonal Syt accumulation, the length of SNb axon innervating muscles 12/13 or ISN axon and its primary branches innervating muscle 4 in abdominal segment 3 or 4 was measured; the number of Syt puncta along these axons was counted and further divided by the axon length. Statistical analyses were performed using InStat 3.00 (Graphpad).

Quantification of GluR IIA Intensity

NMJ staining of *dnrx* mutant and wild-type larvae with monoclonal anti-GluRIIA and FITC anti-HRP was performed in the same tube. Several regions of NMJ4 from 3 animals for each genotype were scanned by confocal microscope. Confocal stacks were acquired using the same settings that prevented pixel saturation with 0.25 μm steps through entire synaptic boutons. Images were processed using Volocity 4.1 (Improvision). Total volumes of synaptic boutons outlined by HRP staining, and the fluorescence of GluRIIA staining were calculated. Total GluRIIA fluorescence divided by total volumes of boutons represented GluR intensity.

Electron Microscopy

Body wall muscles were prepared for transmission electron microscopy (TEM) as previously described (Torroja et al., 1999). Synaptic boutons were serially sectioned and photographed at 10,000-30,000X using a JEOL 100S TEM. For morphometric analysis, the cross section corresponding to the bouton midline (cross-section of largest diameter) was identified, the negative scanned at 60,000X, and used for quantification using Image

J as in Budnik et al., 1996. The number of samples used were 3 wild type controls (14 boutons), 3 *dnrx/Df* (11 boutons), and 3 rescue animals (15 boutons). For analysis of synapse length and synapse ruffles, sections other than the midline section were also considered for quantification, as long as pre- and postsynaptic densities as well as the synaptic cleft were clearly visualized, had consistent thickness, and lacked the blurry appearance of membranes cut at a tangential plane. Statistical analysis was performed using a two-tailed Student t-test.

Electrophysiology

Electrophysiology recordings were performed as in Ashley et al., 2005. Briefly, third instar larvae were dissected under cold 0.3mM Calcium HL-3 saline (Stewart et al., 1994) and then perfused continuously with 0.5mM Calcium HL-3 saline at 22°C. Muscle 6 in segment A3 was then impaled with a 15-20 MΩ glass electrodes. Only samples with resting membrane potentials between -60 mV and -63 mV were used for analysis. Data was collected using an Axoclamp2A (Molecular Devices, Union City, CA), filtered at 1 kHz, and digitized with an Instrutech (Port Washington, NY) ITC-16 computer interface using Pulse software (HEKA Electronik, Lambrecht/Pfalz, Germany). Spontaneous and evoked events were then measured using Mini Analysis software (Synaptosoft, Decatur, GA.). Statistical analysis was performed using the Student's t test.

3.4 Results

Genetic Analysis of *dnrx*

To determine the role of DNRX in synaptic development and function, we generated *dnrx* null mutants. The *dnrx* gene is predicted to comprise 13 exons and 12 introns, spanning ~13.4 kb. To disrupt the *dnrx* locus, we carried out an excision screen with a P-element (*XPd08766*) located ~200 base pairs upstream of *dnrx* (Fig S3.1). Our RNA interference study using double strand RNAs corresponding to two different regions of DNRX indicated that DNRX loss of function might not result in lethality (data not shown). Therefore, we used a single fly PCR strategy rather than screening for the lethality to select *dnrx* deletions. Two large *dnrx* deletion alleles, $dnrx^{273}$ and $dnrx^{241}$ (Fig. 3.1A), were isolated from the screen. The break points of the deletions were molecularly determined by PCR and sequence analysis. The $dnrx^{273}$ allele had an ~8-kb deletion within the *dnrx* locus, which removed most of the coding sequence for the extracellular region of DNRX, from the start codon to the 4th LamG domain. $dnrx^{241}$ uncovered the entire *dnrx* gene and the upstream region of an adjacent transcription unit (Fig. 3.1A). Western blot analysis with the DNRX C-terminus specific antibody showed that ~200 kDa DNRX band in wild-type was absent in $dnrx^{273}/Df(3R)5C1$, and there was no detectable truncated protein in *dnrx* mutants (Fig. 3.1B). Immunostaining with this antibody also showed no detectable protein in homozygous mutant embryos (data not shown) and NMJs (Fig. 3.1C-C') from both *dnrx* alleles, confirming that $dnrx^{273}$ and $dnrx^{241}$ are null alleles. In this study, the following allelic combinations were used for phenotypic analysis: $dnrx^{273}/Df(3R)5C1$, $dnrx^{273}/dnrx^{273}$, *and/or* $dnrx^{273}/dnrx^{241}$. A precise excision of the P-element and a wild type strain were used as controls.

***dnrx* Mutants Display Abnormal NMJ Morphology with Fewer Synaptic boutons**

α-neurexin triple knockout mice and most double-knockout mutants die prematurely due to respiratory problems (Missler et al., 2003; Zhang et al., 2005). In contrast, 10% of the *dnrx*^{273}*/Df(3R)5C1* progeny died at pupal stages while the remaining progeny survived to adulthood and were fertile. This partial lethality was rescued by expressing a *dnrx* full-length cDNA in neurons using the *C380-Gal4* driver (Budnik et al., 1996).

Given that DNRX localizes to larval NMJs, we first examined whether the NMJ morphology altered in *dnrx* mutants. Compared to controls (Fig. 3.2A and 3.2E), *dnrx* mutants (Fig. 3.2B, 3.2C, 3.2F and 3.2G) had less NMJ expansion, shortened axon branches with fewer boutons. In addition, mutant NMJ branches often contained long intervening axon stretches devoid of synaptic boutons. Further quantification revealed that *dnrx* mutants had a 40-60% decrease in bouton number (Fig. 3.2I and 3.2J). The reduced bouton number was fully rescued by expression of the *UAS-dnrx* full length cDNA transgene in neurons (Fig. 3.2D, 3.2H, 3.2I and 3.2J). Thus, these results suggest that DNRX is required for proper proliferation of synaptic boutons during larval development, which is necessary for coordinated matching of pre-and postsynaptic compartments during this period of intense growth (Griffith and Budnik, 2006).

In addition to synaptic regions, DNRX was localized to axons as well. Therefore, we also investigated whether axon guidance and pathfinding were altered in *dnrx* mutants. The specificity of axon pathfinding was examined by immunostaining with BP102 and FASII antibodies which stain all CNS axons and both a subset of CNS axonal fascicles and peripheral motor axons, respectively. No apparent defects were observed in

both CNS axonal pathways and muscle innervation patterns in *dnrx* mutants (data not shown).

Neuronal Expression of DNRX **Promotes Proliferation of Synaptic Boutons**

Studies in cell culture have indicated that neurexins and their postsynaptic binding partners, the neuroligins, induce synapse formation (Graf et al., 2004a; Chih et al., 2005; Scheiffele et al., 2000; Dean et al., 2003). Therefore, we asked whether DNRX could similarly induce the formation of new synaptic boutons *in vivo*. Expression of a full-length *dnrx* transgene in wild-type background, using two different neuron-specific Gal4 drivers, the pan-neural driver *elav* (Lin and Goodman, 1994) and neural driver *C380*, significantly enhanced the formation of synaptic boutons (Fig. 3.3 C, compare with 3.3A and 3.3B). Increasing *dnrx* gene dosage in all neurons by one copy resulted in over a 30% increase in the number of synaptic boutons, while increasing it by two copies resulted in more than 40% enhancement (Fig. 3.3D). A similar trend was observed by using *C380-Gal4* (Fig. 3.3D).

Recent studies on murine central synapses suggest that postsynaptic neurexins affect glutamate receptor function, and may also inhibit neuroligin function via cis-interaction with neuroligins on the postsynaptic membrane (Kattenstroth et al., 2004; Taniguchi et al., 2007). To explore the potential functional relevance of DNRX in the postsynaptic compartment at NMJs, we examined whether muscle expression of DNRX had any effect on synaptic growth. Compared to genetic background matched controls, expression of either one or two copies of a *dnrx* transgene in muscle cells, using the *C57* Gal4 driver (Budnik et al., 1996), did not result in any significant change in bouton

number (Fig.3.5E). Taken together, the DNRX loss- and gain-of-function analyses demonstrate that DNRX is necessary to promote the proliferation of synaptic boutons, and further suggest that for this function DNRX is required in the pre- but not in the postsynaptic compartment.

DNRX Is Required for Pre- and Postsynaptic Differentiation

We also explored whether the expression and localization of synaptic proteins were affected in *dnrx* mutants. The abundance and localization of periactive zone proteins, such as the cell adhesion molecule fasciclin II (FASII), the cytoskeleton adaptor protein nervous wreck (NWK), and the scaffolding protein for synaptic vesicle endocytic machinery Dap 160 (Roos and Kelly, 1998; Roos and Kelly, 1999; Koh et al., 2004; Marie et al., 2004), appeared unchanged in *dnrx* mutants (Fig. S3.2). Several other synaptic proteins, including the scaffolding PDZ-protein DLG and the microtubule-binding protein Futsch (Roos et al., 2000; Hummel et al., 2000), also appeared unaffected in *dnrx* mutants (data not shown). However, profound abnormalities in the distribution of synaptic vesicle and active zone proteins, as well as glutamate receptor (GluR) clusters were observed in *dnrx* mutant NMJs.

In wild-type, synaptotagmin (Syt), a synaptic vesicle protein, is efficiently transported to presynaptic terminals, therefore it is seldom observed within motor axons (Fig. 3.4A-A' and 3.4D; Littleton et al., 1993). In *dnrx* mutants, however, intense punctate Syt staining was often observed in motor axons (Fig. 3.4B-B', 3.4C, and 3.4D). Similarly, the active zone protein, Bruchpilot (BRP) (Wagh et al., 2006; Kittel et al., 2006), which is rarely seen in wild-type axons was also mislocalized to mutant axons

(Fig. 3.4C'). Notably, BRP immunoreactivity colocalized with Syt at these accumulations along the motor axons (Fig. 3.4C"). The abnormal accumulations of synaptic vesicle and active zone proteins in *dnrx* mutant axons suggests that DNRX is involved in the proper recruitment, localization, or transport of key synaptic components during presynaptic differentiation.

At mammalian synapses it has been hypothesized that neurexins are required for the alignment of pre- and postsynaptic compartments (Graf et al., 2004; Yamagata et al., 2003**).** Therefore, we next examined whether pre- and postsynaptic apposition was affected in *dnrx* mutants by double-labeling synaptic boutons with the presynaptic active zone marker BRP and the PSD markers *Drosophila* p-21 activated kinase (DPAK) (Sone et al., 2000) and GluRIII (Marrus et al., 2004). No gross defects in pre- and postsynaptic alignment were observed (Fig. 3.5A", 3.5B", 3.5C'' and 3.5D"). DPAK and GluRIII clusters were exactly juxtaposed to active zones in *dnrx* mutants. However, the size of DPAK and GluRIII clusters was markedly enlarged in mutant boutons (Fig. 3.5B' and 3.5D' [compare with 3.5A' and 3.5C']). Enlargement of GluRIIA clusters was also observed, while the GluRIIA intensity in boutons was not statistically different between mutant and wild-type (Fig. S3.3). Taken together, these results suggest that although the juxtaposition of pre- and postsynaptic components is not altered at least at the light microscopy, the distribution of PSD proteins is affected in *dnrx* mutants.

Active Zones and PSDs Are Altered in *dnrx* Mutants

To determine the significance of the light microscopic phenotypes described above, we carried out ultrastructural and functional analyses of NMJs in *dnrx* mutants. For the

ultrastructural studies, *dnrx* mutant and control NMJs were serially sectioned and subject to a morphometric analysis. *dnrx* mutants had striking structural abnormalities in active zones and PSDs of type Ib boutons. Wild type boutons are characterized by the presynaptic compartment containing synaptic vesicles, mitochondria and endosomes (b in Fig. 3.6A). At the presynaptic membrane, active zones are composed of regulatory electron dense structures, the T-bars (arrow in Fig. 3.6A and 3.6C) containing BRP/CAST (Kittel et al., 2006; Wagh et al., 2006), and the pre-synaptic densities (PRDs), the likely sites for synaptic vesicle fusion (Fig. 3.6C). The PRDs are exactly juxtaposed to PSD (between arrowheads in Fig. 3.6C and 3.6E), which contain GluRs in high-density clusters (Prokop and Meinertzhagen, 2006). Separating both membranes is the synaptic cleft which has a uniform size, and is filled with material that differs in electron density and structure from the rest of the bouton extracellular space (Fig. 3.6A, 3.6C and 3.6E).

Several features of the active zones and PSDs were altered in *dnrx/Df* mutants. PRDs and apposed PSDs were over 60% longer in the mutants compared to controls (Fig. 3.6D and 3.6G). This is in agreement with the light microscopic studies showing that DPAK and GluR clusters were increased in size in *dnrx* mutants. In addition, the number of T-bars per bouton was increased by more than 2-fold (Fig. 3.6B and 3.6G). Most strikingly, the PRD showed signs of detachment from the PSD, a phenotype that is rarely seen in wild type (Fig. 3.6D, 3.6F and 3.6G). In these *dnrx* mutant synapses, PRDs showed bleb-like invaginations at several points, and at these sites the typical electron density of PRDs was lost (Fig. 3.6F). The material at the synaptic cleft also appeared altered, but no defects were observed at the corresponding sites of the PSDs (Fig. 3.6F).

Thus, in *dnrx* mutants, synapses within synaptic boutons are dramatically altered, showing sites of presynaptic membrane detachment, abnormally long active zones, and increased number of T-bars. The increase in the number of T-bars and the aberrant detachment of PRD were completely or almost completely rescued by expressing a *dnrx* transgene in the mutant background, respectively (Fig. 3.6G). However, the increased length of the active zones was only partially rescued by the transgene, suggesting that this phenotype might be highly sensitive to DNRX dosage. Our ultrastructural analysis of *dnrx* mutant synaptic boutons provides the first direct *in vivo* evidence supporting the model that neurexins are involved in adhesion between the pre- and the postsynaptic cells.

dnrx Mutants Have Defects in Synaptic Transmission which Correlate with the Alterations in Synapse Ultrastructure

To assess the functional consequences of the reduced bouton number, the enlarged GluR clusters, the increase in the size of active zones and PSDs, and the abnormal detachments of PRDs, we carried out an electrophysiological analysis of NMJs (Fig. 3.7). For these experiments, the amplitude of spontaneous miniature excitatory potentials (mEJPs) was measured by intracellular recordings of muscles at low Ca^{2+} concentrations. In addition, the amplitude and kinetics of evoked excitatory potentials (EJPs) were measured by stimulating the segmental nerve containing the motor axons. Several defects were observed in *dnrx* mutants. Evoked synaptic transmission was reduced, as manifested by a small, but significant decrease in the amplitude of EJPs. In addition, the frequency and amplitude of mEJPs was dramatically increased, suggesting both a pre- and a

postsynaptic defect. Overall, quantal content was reduced, indicating defective synaptic transmission. The defect in EJP amplitude was completely rescued by expressing a *dnrx* transgene in neurons using *C380-Gal4* or ubiquitously using *T80-Gal4*. However, no rescue of the mEJP amplitude or frequency was observed, consistent with the ultrastructural studies, in which the size of the active zones and PSDs was only partially rescued by the *dnrx* transgene. This partial rescue is not surprising given that neuronal expression of a *dnrx* transgene in wild-type background also has deleterious effects on quantal content (Fig. 3.7C). It is notable that the functional abnormalities were observed in homozygous $dnrx^{273}$ mutants, $dnrx^{273}/dnrx^{241}$ combinations, as well as *dnrx* over a deficiency chromosome. Thus, they are unlikely to result from any genetic background effects.

Mutations in *dnrx* Have Abnormal Calcium Sensitivity without Altering the Distribution of Presynaptic Calcium Channels

Studies in mammals have suggested that neurexins are involved in coupling Ca^{2+} channels to synaptic vesicle release apparatus (Missler et al., 2003). Therefore, we next examined whether the Ca^{2+} sensitivity of neurotransmitter release could be altered in *dnrx* mutants. For these experiments, the amplitude of evoked responses was measured at three Ca^{2+} concentrations, 0.5, 0.75, and 1 mM. As previously described, there was a significant decrease in EJP amplitude in *dnrx* mutants at 0.5 mM Ca^{2+}. Surprisingly, however, this defect was completely restored when recordings were performed at 1 mM Ca^{2+} (Fig. 3.8A).

The change in the slope of Ca^{2+} dependency of release could be due to either changes in Ca^{2+} coupling to exocytosis, or an abnormality in the distribution of presynaptic Ca^{2+} channels. To address this issue we examined the distribution of Cacophony (Cac), the presynaptic N-type Ca^{2+} channel at these synapses (Smith et al., 1996; Littleton and Ganetzky, 2000). Ca^{2+} channels are extremely sensitive to fixation. Therefore, we used a GFP-tagged Cac transgene (*Cac-GFP*) to visualize Ca^{2+} channel *in vivo*. Previous studies have demonstrated that expression of this transgene faithfully replicates endogenous Cac distribution and function (Kawasaki et al., 2004; Kittel et al., 2006). *Cac-GFP* transgene was expressed in neurons using *elav-Gal4*, and samples were imaged live in a spinning disk confocal microscope. There were no statistically significant differences either in the size or intensity of Cac-GFP clusters between controls and *dnrx* mutants (Fig. 3.8B, 3.8B', 3.8C and 3.8 C'), indicating that the defect in Ca^{2+} sensitivity is most likely the result of changes in coupling rather than Ca^{2+} channel distribution.

dnrx Mutants Exhibit Decreased Locomotor Activity

dnrx mutants exhibited severely impaired behavior in larval stages, being uncoordinated and sluggish. Locomotor activity was reduced in all *dnrx* mutants, including the two *dnrx* alleles over deficiency and in allelic combinations. For the $dnrx^{273}/Df(3R)5C1$ line, this phenotype was quantified in a larval locomotor assay. In this assay, the number of grids on a horizontal agar surface entered by individual 3rd-instar wandering larvae within a 30 sec time window over a test period of 180 sec was counted**.** While control animals passed about 5 grids on average, $dnrx^{273}/Df(3R)5C1$ mutants entered less than 2 grids (Fig. 3.9).

This phenotype was completely rescued by expressing DNRX in neurons using the *C380-Gal4* driver (Fig. 3.9). Thus, decreased locomotor activity is due to *dnrx* loss-of-function in neurons.

3.5 Discussion

Although cell adhesion molecules have long been postulated and in several cases, shown to be major participants in synapse development and plasticity, the impact of their function and the molecular mechanisms that they activate remain a puzzle. Particularly intriguing is the function of neurexins, which may provide clues to our understanding of synapse organization. We have isolated null mutants in the single *Drosophila dnrx* gene. We show that *dnrx* mutants have striking abnormalities in synapse development and function. A recent study reported that *Drosophila* neurexin is required for synapse formation in the adult CNS (Zeng et al., 2007). In the current study, we not only demonstrate a primary role of DNRX in regulating synapse formation during NMJ expansion, but also reveal the crucial role of DNRX in the proper development of active zones and regulating synaptic function in an intact organism, thus providing novel insights into understanding the function of neurexins *in vivo*.

Function of DNRX during Synapse Development

Our studies provide compelling evidence that DNRX plays a prime role during the expansion of the NMJ and in particular, in defining the cytoarchitecture of the active zones within synaptic boutons. First, in *dnrx* mutants synaptic bouton proliferation is severely disrupted, and therefore NMJ expansion is significantly stunted. Second, DNRX gain of function promotes the formation of new boutons in a gene dosage-dependent

manner. Third, the ultrastructural analyses show that PRDs are not apposed normally to PSDs displaying signs of abnormal adhesion to the PSD, although every PRD is exactly juxtaposed to the PSD. Fourth, in *dnrx* mutants critical components of the presynaptic compartment, such as synaptic vesicle proteins and active zone components are ectopically localized within axons. Fifth, the distribution of GluRs at the PSD is abnormally large, although this phenotype may arise as a consequence of the presynaptic defects observed in *dnrx* mutants (discussed below).

The great majority of abnormal phenotypes in *dnrx* mutants could be completely rescued by expressing a wild type *dnrx* transgene in neurons; although in some instances the rescue was partial. However, even in the later case expressing *dnrx* in both muscles and neurons did not further improve the residual abnormalities, suggesting that *dnrx* functions primarily if not exclusively in the presynaptic compartment. This is consistent with our findings from in situ hybridization and immunostaining studies, which showed that DNRX mostly localizes to the presynaptic terminals.

The partial rescue of some of the phenotypes, such as the defects in mEJPs and the morphology of active zones, might be due to the high sensitivity of these processes to the right levels and correct temporal expression of *dnrx*, which is not completely mimicked by the UAS/Gal4 system. This view is supported by the observation that overexpression of *dnrx* in a wild type background also decreased quantal content, suggesting that increased *dnrx* dosage may have detrimental effects on synapse structure and/or function. However, the data strongly support that the abnormal phenotypes arise from the lack of *dnrx*. First, all our experiments were carried out in mutants over a deficiency chromosome in an independent genetic background. Second, a precise

excision of the P-element did not show any of the mutant phenotypes. Together these data establish a specific role for DNRX in proper synaptic development.

Role of DNRX in Active Zone Morphogenesis

One of our important findings is that *dnrx* mutants displayed defective active zones with larger PRD, and especially containing regions of detachment from the PSD. These detachment sites implicate DNRX as a mediator of cell adhesion between the pre-and the postsynaptic cell, in accordance with previous suggestions in mammalian neurons (Scheiffele et al., 2000; Graf et al., 2004; Dean et al., 2003). While a complete detachment of active zones is not observed, *dnrx* mutants have a significant decrease in the number of boutons. This raises the possibility that the phenotypes we observe are from those boutons that are maintained, and that a more drastic consequence is a failure to form synaptic boutons. Nevertheless, the lack of complete detachment of active zones in *dnrx* null mutants suggests that DNRX, although an important synapse organization molecule, is not sufficient for trans-synaptic cell adhesion.

Another notable phenotype in *dnrx* mutants was the presence of enlarged PRDs and increased number of T-bars. A major feature of *Drosophila* larval NMJ is its ability to compensate for decreased postsynaptic responses by upregulating neurotransmitter release. For instance, a decrease in the number of postsynaptic GluRs results in an increase in neurotransmitter release, thus maintaining the amplitude of evoked responses (Petersen et al., 1997). It is plausible that the enlarged PRDs and increase in number of T-bars in *dnrx* mutants are a compensatory mechanism to adjust for defective presynaptic cell adhesion and/or reduced neurotransmitter release (Murthy et al., 2001; Stewart et al.,

1996). In support of this notion, in *dnrx* mutants there was a 50% decrease in synaptic bouton number, but this was accompanied by a 2-fold increase in the number of T-bars, such that the total number of T-bars/NMJ remained constant, despite the changes in bouton number. Similarly, defective presynaptic cell adhesion and/or reduced neurotransmitter release could lead to an increase in GluR accumulation (O'Brien et al., 1998). In our studies we found that the length of the PSD was enlarged in *dnrx* mutants as well as the distribution of GluR clusters.

Functional Consequences of Altering DNRX Function

The above structural abnormalities were accompanied by corresponding functional deficits. In *dnrx* mutants the frequency of mEJPs was strikingly increased. Further, although the T-bars were rescued by expression of a *dnrx* transgene, the length of the PRDs was not, and a similar lack of rescue was observed for mEJP frequency. Thus, there appears to be a notable correlation between the size of the PRD and mEJP frequency perhaps due to increased probability of synaptic vesicle release with increased synapse size. In addition, we also observed a substantial increase in mEJP amplitude. Two factors may contribute to this change. First, the distribution of GluR clusters was enlarged while the GluR intensity remains unchanged, suggesting that more GluRs were accumulated at mutant synapses. An additional contributing factor is that mEJP frequency was increased, and we observed instances of summation.

Overall, despite the increase in PRD size and the maintenance of overall T-bar number, evoked events had a decrease in amplitude, and quantal content. Recent studies have suggested that a major constituent of the T-bars is BRP/CAST (Kittel et al., 2006;

Wagh et al., 2006). In *brp* mutants T-bars fail to form, but PRDs appear unaltered. Further, although EJP amplitude is decreased, mEJP amplitude and frequency are normal. This has led to the model that T-bars *per se* are not required for synaptic transmission, but that they regulate the efficiency of transmission. In *dnrx* mutants, PRDs are disproportionately large, which could result in asynchronous release, leading to an EJP with decreased amplitude. It is also possible that the presynaptic membrane detachments observed in *dnrx* mutants could contribute to the functional impairment of neurotransmitter release.

A recent study demonstrated that in *dnrx* mutant larvae associative learning is impaired in an olfactory choice paradigm (Zeng et al., 2007). However, in this study larval locomotion was not assessed. Our study showing that locomotor behavior is impaired in *dnrx* mutants raises the possibility that the poor performance of mutant larvae in the conditioning assay might also result from the locomotor abnormalities. Zeng *et al* also reported that the number of T-bars in the calyx of the mushroom bodies, the learning centers of the fly, was reduced in adult flies. In contrast, we found a significant increase in the number of T-bar per bouton, and since *dnrx* mutants have fewer boutons, this translated in the maintenance of T-bar number per NMJ. The differing results might be due to different mechanisms regulating T-bar formation in the two tissues.

DNRX Function in Relation to Mammals

The presence of a neurexin in *Drosophila* strengthened the view that neurexins are highly conserved across species (Tabuchi and Sudhof, 2002). The synaptic DNRX expression pattern and its function show remarkable parallels with mammalian neurexins. Moreover,

the proteins that have been shown to interact with mammalian neurexins also have homologs in *Drosophila*, which further supports the idea that the function of neurexins and underlying signaling mechanism are evolutionarily conserved. Among these, *Drosophila* neuroligin and/or dystroglycan (Dg) (Deng et al., 2003) might be potential DNRX ligands. *Drosophila* neuroligin transcription exhibits almost an identical temporal and spatial expression pattern as *dnrx* during embryonic stages (Li, J. et al., unpublished data). *dg* is highly expressed in the somatic musculature of embryos (BDGP gene expression report). *dg* mutants are embryonic lethal, and perturbation of Dg function by RNAi as well as genetic interaction studies suggest an involvement of Dg in muscle maintenance and axonal pathfinding in adult flies (Shcherbata et al., 2007). Future studies on the identification and characterization of DNRX binding partners in *Drosophila* should provide additional insights into the mechanisms by which neurexins function in synapse development and function.

Extensive cell culture studies of neurexins and neuroligins and functional studies using α-neurexin knockout mice have established a central role for neurexins as synaptic adhesive and organizing molecules. Our studies on DNRX provide novel evidence in an intact organism that neurexin is required for important aspects of synapse development and function. Our DNRX gain-of-function analysis reveals overexpression of DNRX is sufficient to promote the formation of synaptic boutons *in vivo*, in agreement with the findings from cell culture studies suggesting that neurexin-neuroligin trans-synaptic complexes can induce pre- and post-synaptic differentiation and synapse formation (Graf et al., 2004; Scheiffele et al., 2000; Dean et al., 2003; Chih et al., 2005). Moreover, the accumulations of synaptic vesicle and active zone proteins along axons of *dnrx* null

mutants further support the notion that neurexins may recruit or organize synaptic proteins or organelles during presynaptic differentiation. Phenotypic analyses of α-neurexin knockout mice demonstrated that α-neurexin is essential for synaptic transmission in a process that depends on presynaptic voltage-dependent Ca^{2+} channels (Missler et al., 2003; Zhang et al., 2005). However, triple knockout mice have normal surface expression of Ca^{2+} channels. These findings have led to the hypothesis that neurexins regulate the coupling between Ca^{2+} channels and the neurotransmitter release machinery. Similarly, in *dnrx* null mutants we found that the Ca^{2+} sensitivity of evoked responses was abnormal, but the distribution or levels of presynaptic Ca^{2+} channel Cac was unchanged, consistent with the above hypothesis. Notably, Syt I, a synaptic vesicle protein that binds Ca^{2+} and has been proposed to function as a Ca^{2+} sensor (Geppert et al., 1994; Yoshihara and Littleton, 2002; Brose et al., 1992) during synaptic vesicle exocytosis , was partly mislocalized to axons in *dnrx* mutants. Furthermore, the structure of active zones was impaired in these mutants. Therefore, the organization of active zone proteins including the assembly of neurotransmitter release machinery might be affected in *dnrx* mutants.

In conclusion, our studies in *Drosophila* demonstrate that DNRX is required for both synapse development and function, and in particular for proper formation of active zones. Our studies provide compelling evidence for an *in vivo* role of neurexins in the modulation of synaptic architecture and adhesive interactions between pre- and postsynaptic compartments.

Figure 3.1 Generation of *dnrx* Mutants

(A) Genomic structure of *dnrx* with the intron-exon organization. Exons are indicated by black boxes and introns by open boxes. The adjacent genes and directions of their transcription are denoted by gray boxes and arrows, respectively. The P element *XPd08766* is inserted ~200 bp upstream of *dnrx*. *dnrx* alleles *($dnrx^{273}$ and $dnrx^{241}$)* with their deleted regions indicated by hatched boxes were generated by imprecise excisions of *XPd08766*. The primers used for PCR and sequencing to define the break points of deletions are indicated by black arrows.

(B) Western blot of adult head membrane extracts of wild-type and $dnrx^{273}/Df$ flies probed with anti-DNRX (upper panel) and re-probed with anti-NRX IV (lower panel). The ~200 kDa DNRX band is absent in $dnrx^{273}/Df$ mutants. NRX IV is the loading control.

(C-C') Double-staining of $dnrx^{273}/Df$ mutant NMJ with anti-BRP (red) and anti-DNRX (green) showing that DNRX immunoreactivity is absent from synaptic boutons (C') where only BRP immunoreactivity is detected (C). Scale bar, 15 μm.

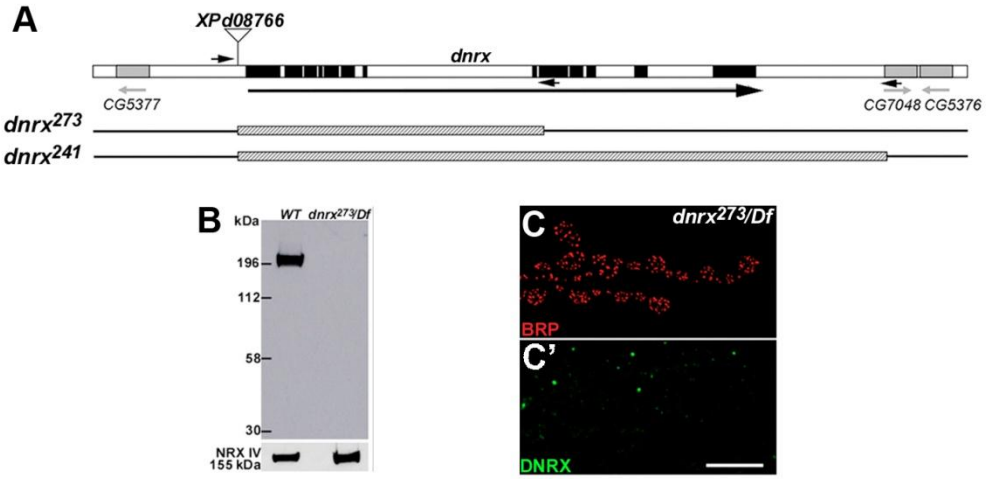
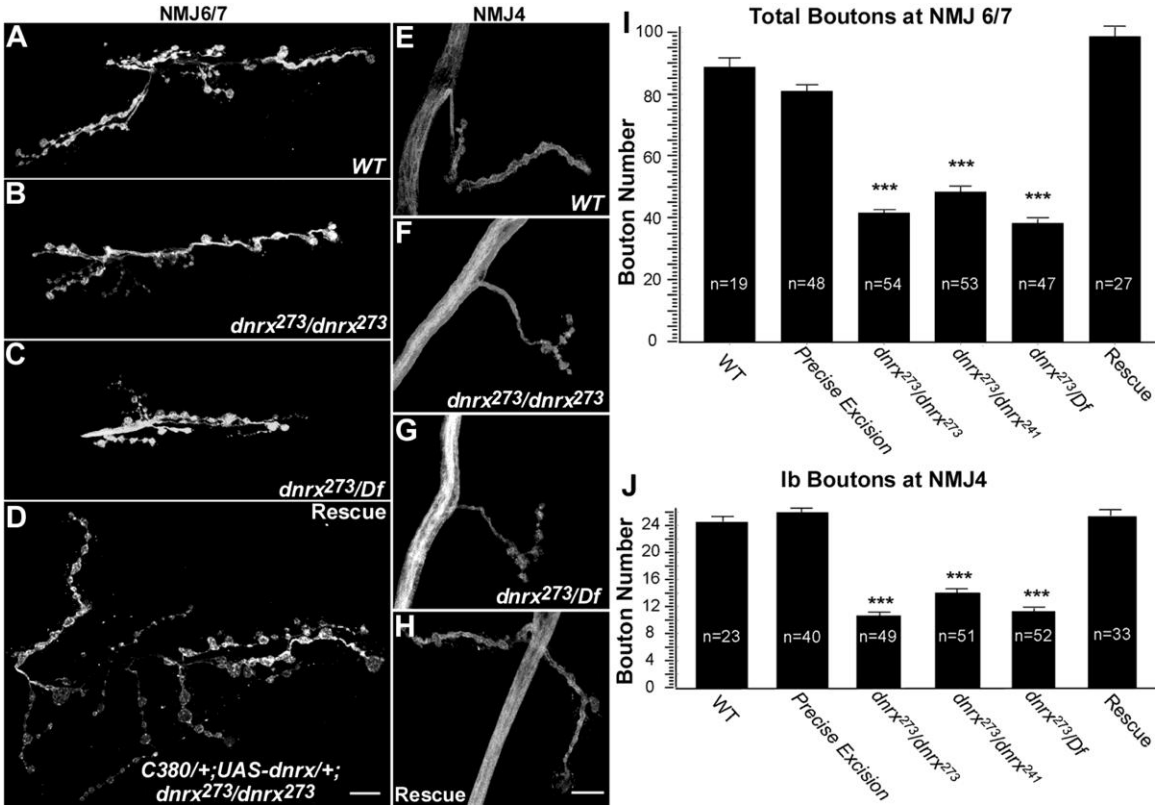


Figure 3.2 DNRX **Loss-of-function Leads to Reduced Synaptic Bouton Number at Larval NMJs**

(A-H) NMJ morphology at muscle 6/7(A-D) and muscle 4 (E-H) of larval abdominal segment 3 labeled with anti-HRP. Compared to wild-type (A and E), *dnrx* null mutants $dnrx^{273/}dnrx^{273}$ (B and F) and $dnrx^{273}/Df$ (C and G) have less NMJ expansion, shorter axonal branches, and fewer boutons. A rescue line with neuronal expression of *UAS-dnrx* cDNA in $dnrx^{273/}dnrx^{273}$ background appears to restore NMJ morphology (D and H). Scale bars, 15 μm.

(I and J) Quantification of total bouton number at NMJ 6/7 (I) and type Ib bouton number at NMJ4 (J). Both quantifications show that *dnrx* mutants $dnrx^{273/}dnrx^{273}$, $dnrx^{273}/dnrx^{241}$ and $dnrx^{273/}Df3R(5C1)$ have a significant decrease in average bouton number when compared to wild-type and precise excision homozygotes. The reduced bouton number is completely rescued by restoring DNRX expression in neurons (rescue: *C380/+; UAS-dnrx/+;* $dnrx^{273}/dnrx^{273}$). ***P<0.001, data are mean±SEM.


NMJ6/7
NMJ4
A
WT
B
dnrx273/dnrx273
C
dnrx273/Df
D
Rescue
C380/+;UAS-dnrx/+;
dnrx273/dnrx273
E
WT
F
dnrx273/dnrx273
G
dnrx273/Df
H
Rescue
I
Total Boutons at NMJ 6/7
Bouton Number
100
80
60
40
20
0
n=19
n=48
n=54
n=53
n=47
n=27
***
***
***
WT
Precise Excision
dnrx273/dnrx273
dnrx273/dnrx241
dnrx273/Df
Rescue
J
Ib Boutons at NMJ4
Bouton Number
24
20
16
12
8
4
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n=49
n=51
n=52
n=33
***
***
***
WT
Precise Excision
dnrx273/dnrx273
dnrx273/dnrx241
dnrx273/Df
Rescue

Figure 3.3 Neuronal but Not Muscular Overexpression of DNRX **Promotes Proliferation of Synaptic Boutons**

(A-C) NMJ morphology at muscle 6/7 in animals with DNRX overexpression in neurons. Compared with control animals **(A)** that lack *dnrx* transgene (*C380-Gal4*) or **(B)** that overexpress an unrelated gene (*C380-Gal4; UAS-lacZ*) in neurons, **(C)** animals with DNRX overexpression in neurons (*C380-Gal4; UAS-dnrx*) have more branching and boutons. Scale bar, 15 μm.

(D) Quantification of bouton number shows that animals overexpressing either one- (*Gal4; UAS-dnrx*) or two-copy (*Gal4; UAS-dnrx; UAS-dnrx) dnrx* transgene driven by either neuron (*C-380*) or panneuron (*elav*) Gal4 divers have a significant increase in average bouton number, when compared to control animals that lack a *dnrx* transgene (*Gal4*) or that express an unrelated gene (*Gal4; UAS-LacZ*). ***P<0.001, Data are mean±SEM.

(E) Quantification of bouton number shows that there is no significant change in average bouton number between animals that express *dnrx* transgene in muscles driven by C57 Gal4 and control animals. Data are mean±SEM.

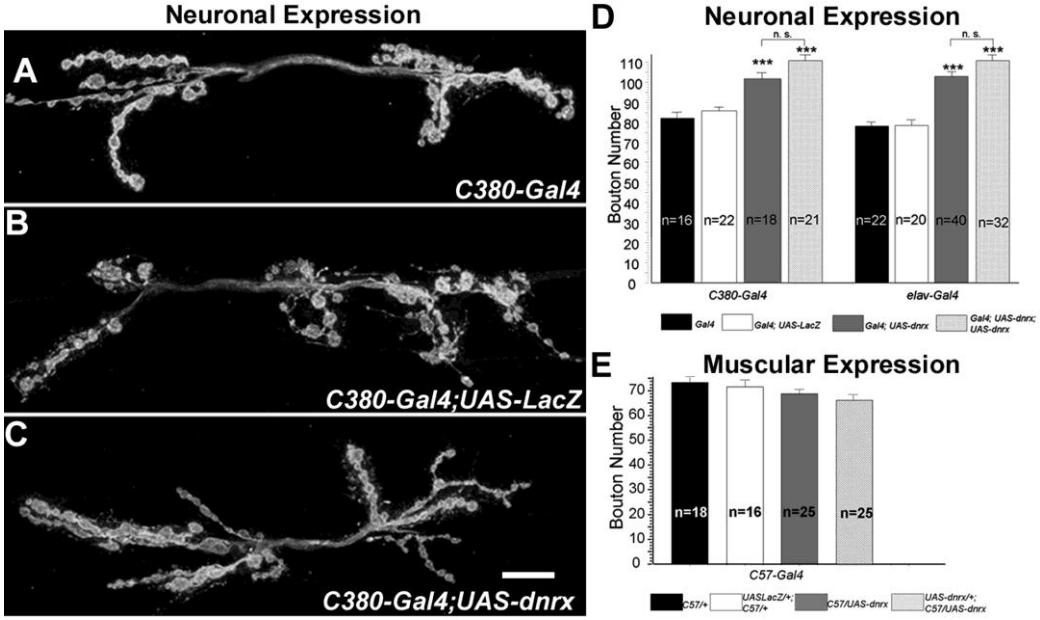

Neuronal Expression
A
C380-Gal4
B
C380-Gal4;UAS-LacZ
C
C380-Gal4;UAS-dnrx
D
Neuronal Expression
n. s.
***
Bouton Number
n=16
n=22
n=18
n=21
n=22
n=20
n=40
n=32
C380-Gal4
elav-Gal4
Gal4
Gal4; UAS-LacZ
Gal4; UAS-dnrx
Gal4; UAS-dnrx; UAS-dnrx
E
Muscular Expression
Bouton Number
n=18
n=16
n=25
n=25
C57-Gal4
C57/+
UASLacZ/+; C57/+
C57/UAS-dnrx
UAS-dnrx/+; C57/UAS-dnrx

Figure 3.4 Synaptic Component Proteins Are Mislocalized to Axons in *dnrx* Mutants

(A-B') NMJ 4 stained with anti-Syt (red) and anti-HRP (green) in wild type (A and A') and *dnrx* mutants (B and B'). *dnrx* mutants display accumulation of Syt staining puncta in motor axons (B and B', arrowheads).

(C-C") NMJ 4 of *dnrx* mutant stained with anti-Syt (green), anti-BRP(red) and anti-HRP (blue), showing co-localization of Syt and BRP staining (arrows) in accumulated puncta along the axon. Scale bar, 15 μm (B' and C").

(D) Quantification of Syt-puncta observed in axons innervating muscle 4 and muscle 12/13 shows that *dnrx* mutants have significantly increased Syt accumulation per unit axon length. n>76; ***P<0.001. Data are mean±SEM.

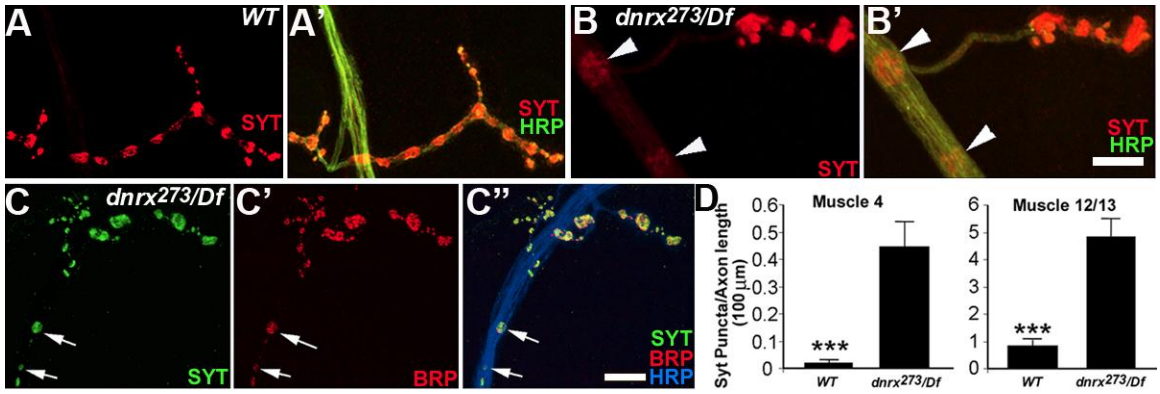


Figure 3.5 Distribution of PSD Proteins Is Enlarged in *dnrx* Mutant Boutons

(A-B'') Synaptic boutons of wild-type (A-A'') and *dnrx* mutant (B-B'') double-stained for BRP (red) and DPAK (green), which labels the presynaptic active zone and PSD, respectively. The pre- and postsynaptic alignment appears grossly unaffected in *dnrx* mutants. However, the distribution of DPAK within synaptic boutons is increased in *dnrx* mutant.

(C-D'') Synaptic boutons of wild-type (C-C'') and *dnrx* mutants (D-D'') double-stained for BRP (green) and GluRIII (red). The apposition of GluRIII and BRP staining appears normal in *dnrx* mutants. However, the distribution of GluRIII cluster is enlarged in *dnrx* mutant. Scale bar: 5 μm (D").

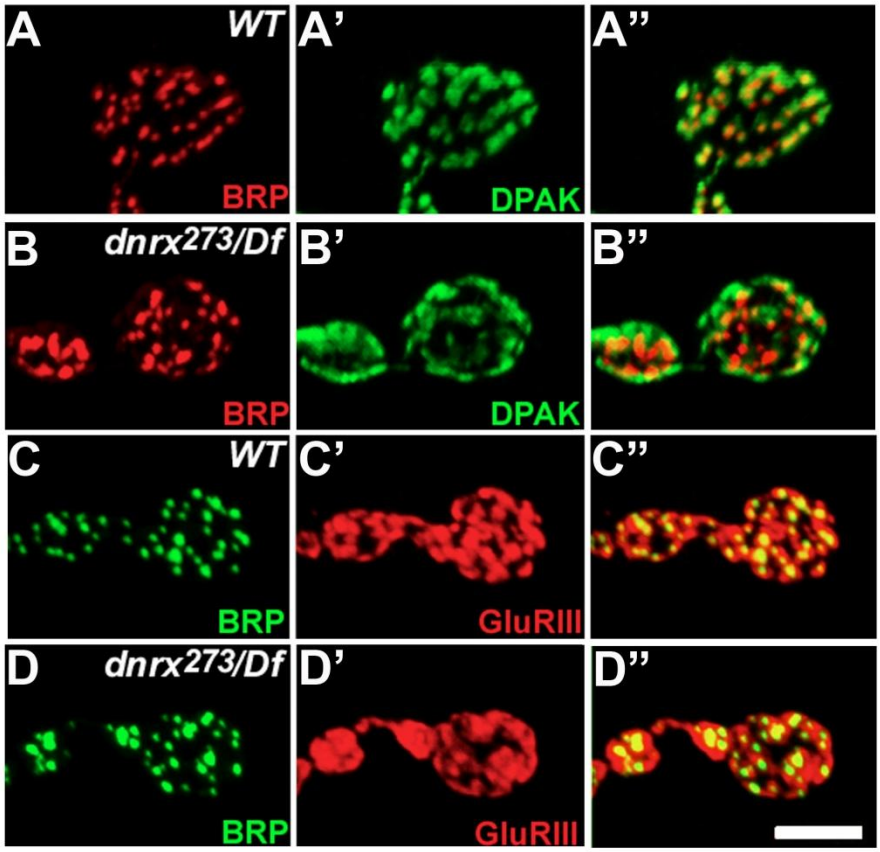


Figure 3.6 Ultrastructural Analysis of Type I Synaptic Bouton in *dnrx* Mutants.

(A-F) TEM micrographs of (A, C, E) wild type, and (B, D, F) $dnrx^{273}$/Df mutants.

(A, B) are low magnification view of synaptic bouton midlines (b), showing the presynaptic active zones (arrows) and post synaptic SSR. Note that in $dnrx^{273}/Df$ mutants the number of active zones is increased.

(C, D) Higher magnification view of the PSD (between arrowheads) and its juxtaposed PRD containing a T-bar. In $dnrx^{273}/Df$ mutants both PSDs and PRDs are much longer than wild type. In addition, the presynaptic membrane shows signs of detachment from the PSD (curved arrows).

(E, F) High magnification view of a region of the PRD and apposed PSD. While in wild type the synaptic cleft between the PSD and PRD has a constant size, in $dnrx^{273}/Df$ mutants this size is variable, due to the presence of detachments of the presynaptic membrane (curved arrows). Also note that at sites of detachments the PRD no longer displays its typical electron density.

(G) Morphometric analysis of synaptic boutons, showing bouton midline area, number of T-bars, synapse length, number of ruffles/synapse, and vesicle density. Numbers in parenthesis below genotypes correspond to the number of samples. ***P<0.001; **P<0.01; and *P<0.05. Data are mean±SEM.

Scale bars, 1 μm (A and B); 0.2 μm (C and D); 0.1 μm (E and F).

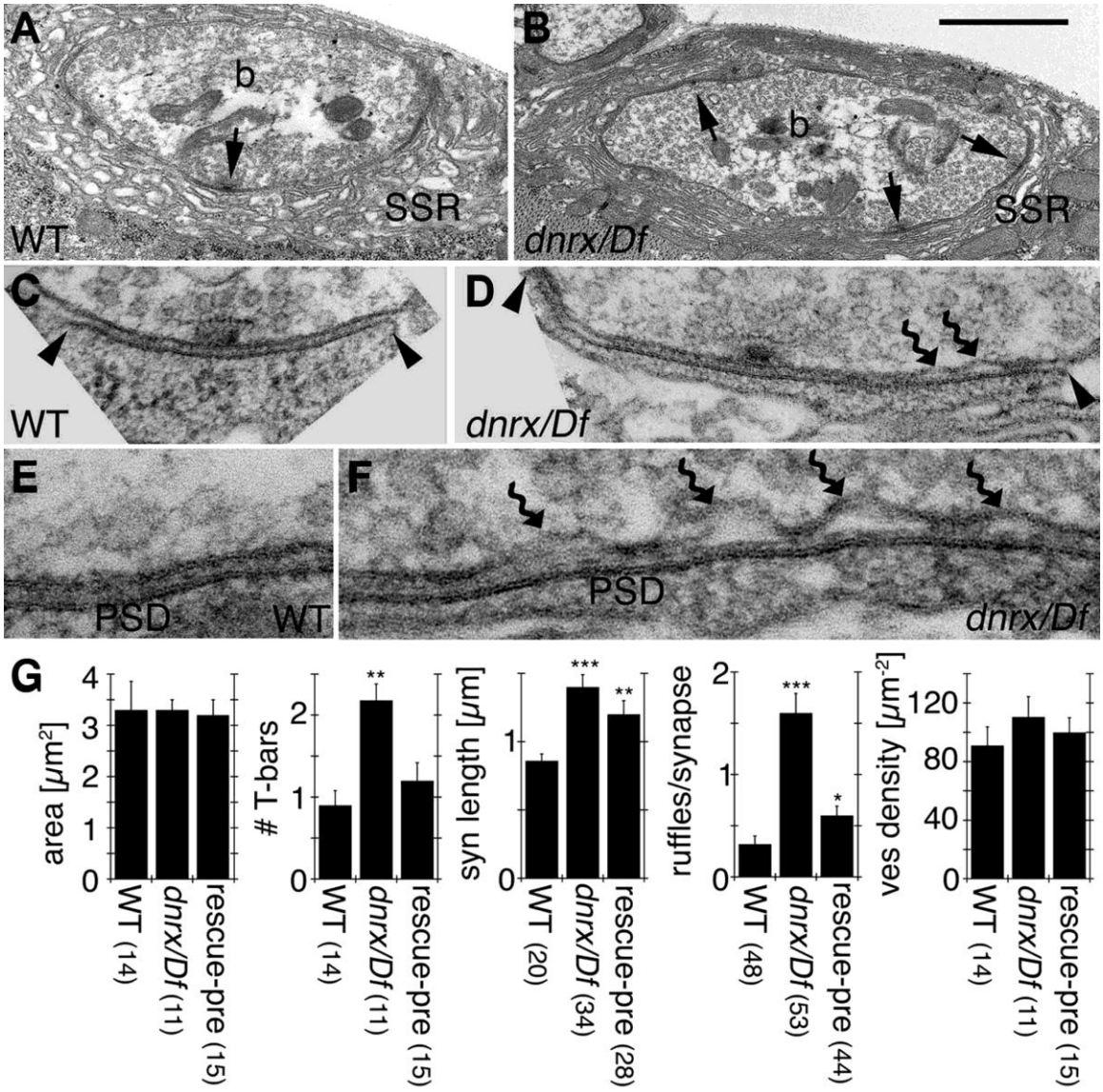

A
b
SSR
WT
B
b
SSR
dnrx/Df
C
WT
D
dnrx/Df
E
PSD
WT
F
PSD
dnrx/Df
G
area [μm²]
WT (14)
dnrx/Df (11)
rescue-pre (15)
T-bars
WT (14)
dnrx/Df (11)
rescue-pre (15)
syn length [μm]
WT (20)
dnrx/Df (34)
rescue-pre (28)
ruffles/synapse
WT (48)
dnrx/Df (53)
rescue-pre (44)
ves density [μm⁻²]
WT (14)
dnrx/Df (11)
rescue-pre (15)

Figure 3.7 Electrophysiological Analysis of NMJs.

(A) Representative traces of mEJPs in the genotypes indicated. Note that both the frequency and amplitude of mEJPs is enhanced in *dnrx/Df* mutants.

(B) Representative traces of evoked responses, showing a decrease in peak amplitude in *dnrx/Df* mutants. Calibration scale: 3 mV and 230 msec (A); 6 mV and 10 msec (B).

(C) Quantification of EJP and mEJP amplitude, mEJP frequency, and quantal content (EJP/mEJP amplitude). ***P<0.001; **P<0.01; and *P<0.05. Data are mean±SEM.

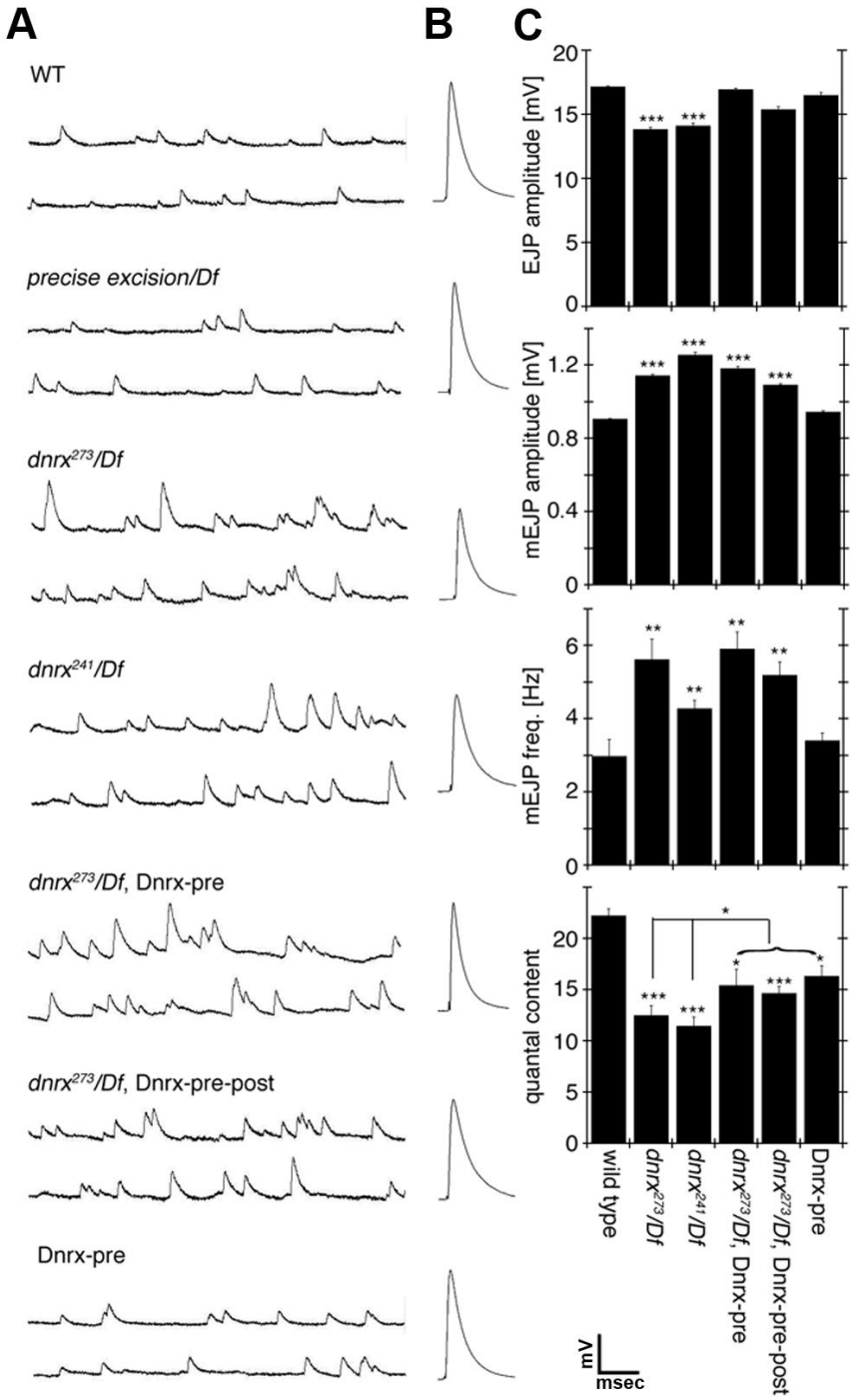

A
B
C
WT
precise excision/Df
dnrx273/Df
dnrx241/Df
dnrx273/Df, Dnrx-pre
dnrx273/Df, Dnrx-pre-post
Dnrx-pre
EJP amplitude [mV]
mEJP amplitude [mV]
mEJP freq. [Hz]
quantal content
wild type
dnrx273/Df
dnrx241/Df
dnrx273/Df, Dnrx-pre
dnrx273/Df, Dnrx-pre-post
Dnrx-pre
mV
msec

Figure 3.8 Mutations in *dnrx* Have Abnormal Calcium Sensitivity without Altering the Distribution of Presynaptic Calcium Channels

(A) EJP amplitude as a function of external Ca^{2+} concentration in wild type and *dnrx* mutants.

(B-C') Confocal images of NMJ 6/7 in preparations expressing Cac-GFP in neurons and imaged live in wild type (B and B'), and *dnrx* mutants (C and C'). No significant changes in the intensity of the signal between the two genotypes were found (WT: 100±1.4, n=9; *dnrx273/Df*: 97.9±1.2, n=6). Scale bars, 17 μm (B and C); 4 μm (B'and C').

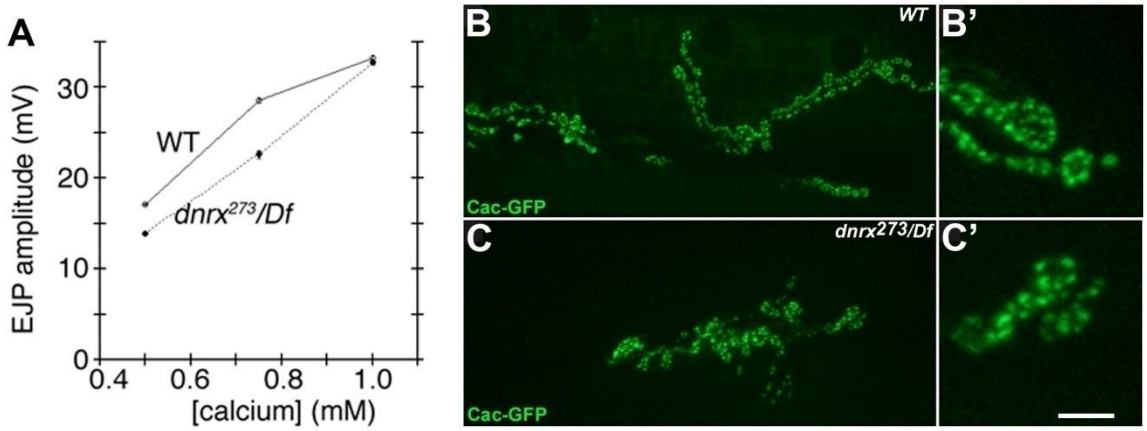
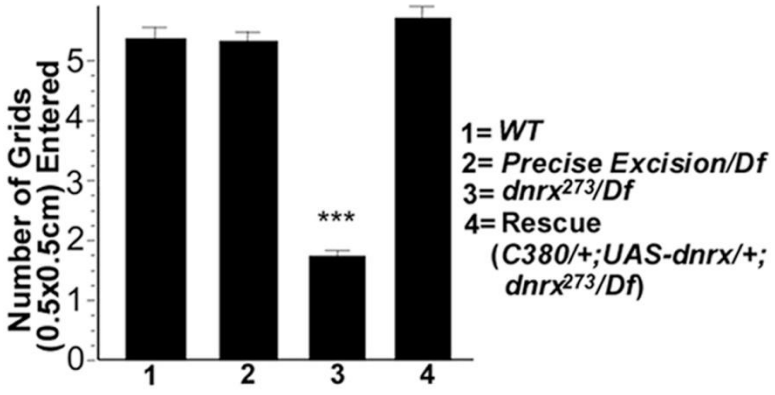


Figure 3.9 *dnrx* Mutants Display Decreased Locomotor Activity.

Quantification of locomotor activity of wall-wandering 3rd instar larvae within a 30 sec time window over a test period of 180 sec. The *precise excision/Df* performed as well as wild-type, whereas *dnrx*^{273}*/Df* displayed significantly reduced locomotor activity (***P<0.0001). This phenotype was completely rescued with neuronal expression of a *dnrx* full-length cDNA (*C380/+; UAS-dnrx/+; dnrx*^{273}*/Df*). For all genotypes, n=20. Data are mean±SEM.



Supplemental Figures

Figure S3.1 Genetic Excision Screen to Generate *dnrx* Deletion Mutants

(A) The genetic scheme of P-element (XP d08766) excision screen mediated by Δ2-3 transposase in *mus309* background (see Materials and Methods).

(B) Location of XP d08766 in *dnrx* region and a PCR strategy to isolate *dnrx* deletion mutants. Primers for PCR screening of deletions are indicated by arrows.

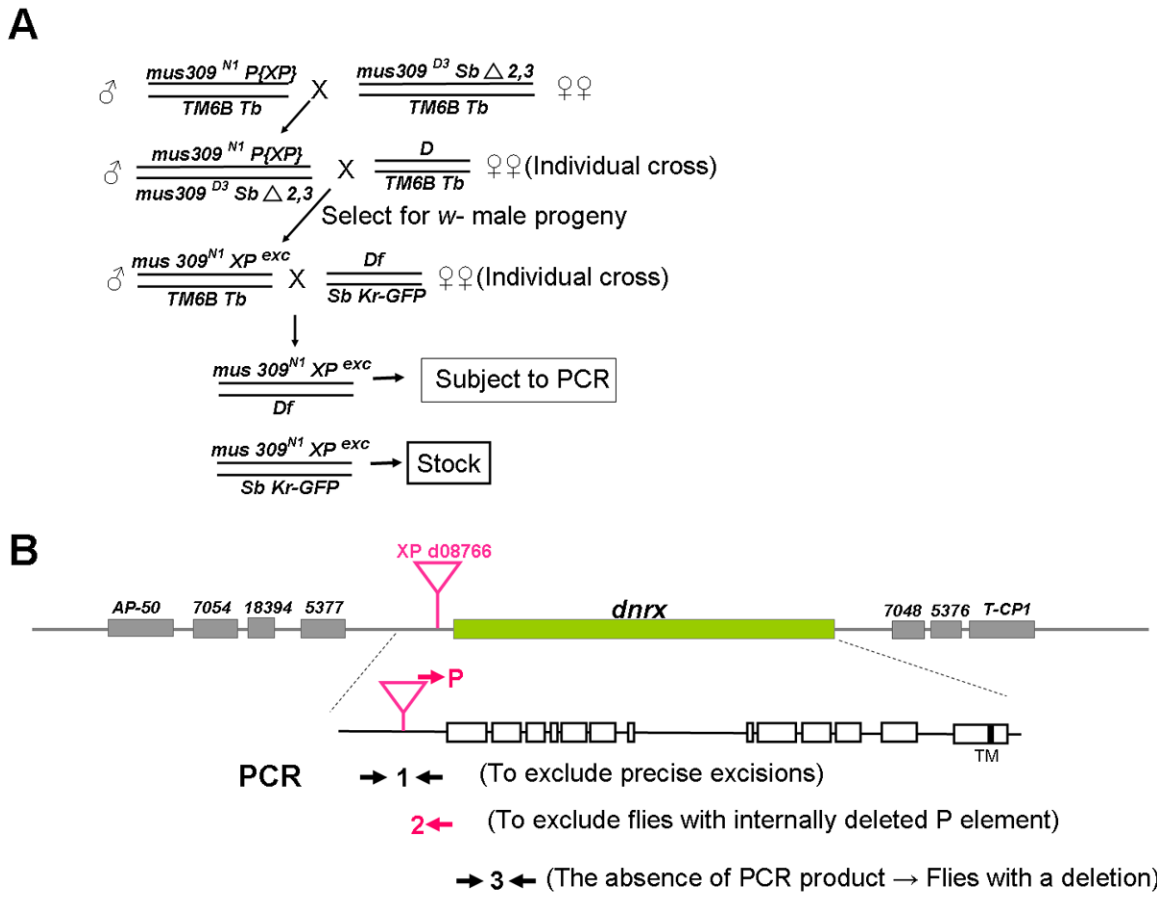


Figure S3.2 Distribution of Active and Periactive Zone Proteins in *dnrx* Mutants

Synaptic boutons of wild-type (A-A'', C-C'' and E-E'') and *dnrx* mutants (B-B'', D-D'' and F-F'') double-stained for active zone (DPAK and BRP, red) and periactive zone markers (FASII, NWK and Dap 160, green). Mutant boutons have increased distribution of DPAK (B). The cell adhesion molecule FASII, the cytoskeleton adaptor protein NWK and the endocytotic protein Dap 160 surrounding the active zone proteins have a similar distribution in *dnrx* mutants (B'-B'', D'-D'', and F'-F'') as in the wild-type (A'-A'', C'-C'' and E'-E''). Scale bar: 5µm

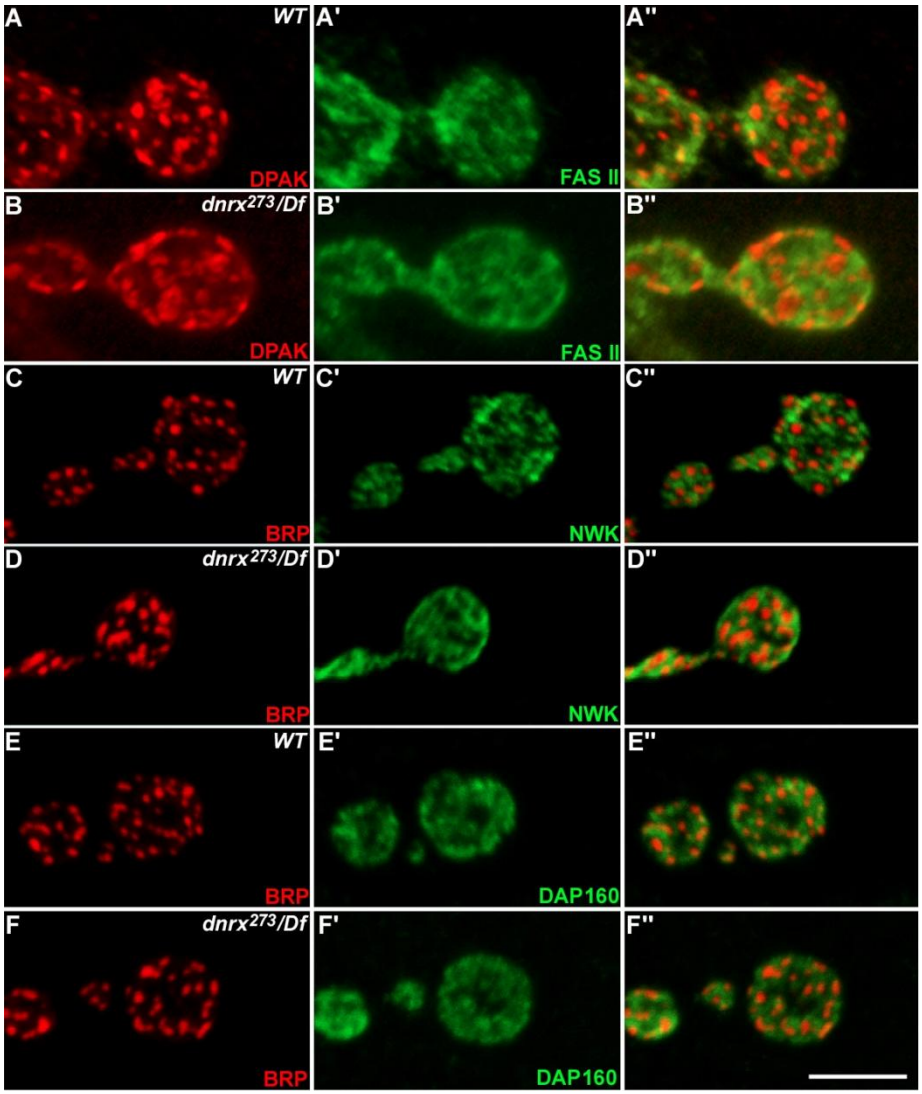
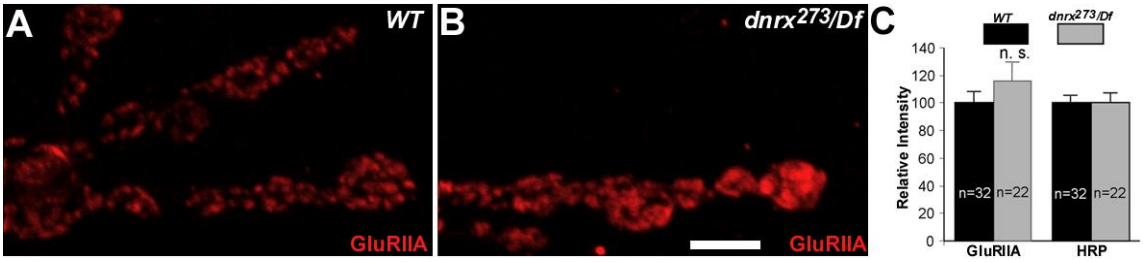


Figure S3.3 Distribution and Intensity of Postsynaptic GluRIIA in *dnrx* Mutants.

NMJ staining for GluRIIA in wild-type (A) and *dnrx* mutants (B). Note that the distribution of GluRIIA clusters is increased in *dnrx* mutants.

(C) Quantification of GluRIIA fluorescence intensity in synaptic boutons. HRP is the internal control. Relative intensity is expressed as percent of wild type level. No significant difference is observed between mutant and wild-type boutons. Data are mean±SEM. Scale bar: 5 μm



CHAPTER FOUR

CONCLUSIONS

4.1 Summary of Findings

The studies presented here characterized the single *Drosophila* neurexin gene *dnrx* and identified its in vivo function in synapse development and function.

We found that during *Drosophila* development, *dnrx* expression starts before the differentiation of presynaptic terminals, and is highly enriched in CNS neurons. DNRX localizes to CNS synaptic regions, axons, and glutamatergic NMJs. At larval NMJ, DNRX is concentrated at active zones, but also extends into periactive zones within synaptic boutons.

dnrx null mutants are viable, but display reduced locomotor behavior. Using *Drosophila* NMJs, we demonstrate that *dnrx* loss of function prevents the normal proliferation of synaptic boutons, while *dnrx* gain of function in neurons has the opposite effect, suggesting an essential role of DNRX in control of synaptic growth. In *dnrx* mutants, synaptic vesicle and active zone component markers are mislocalized along axons, suggesting that DNRX is required for the proper recruitment and localization of key synaptic components during presynaptic differentiation. Conspicuously, *dnrx* null mutants display striking defects in synaptic ultrastructure with the presence of detachments between pre- and postsynaptic membranes, abnormally long active zones, and increased number of T-bars. These abnormalities result in corresponding alterations in synaptic transmission with reduced neurotransmitter release.

Our results provide compelling evidence for an *in vivo* role of DNRX in synaptic growth, the proper formation and organization of active zones, the adhesive interactions between pre- and postsynaptic compartments, and regulation of synaptic transmission. These findings provide novel insights into understanding the function of neurexins *in*

vivo, and offer a strong basis for the interpretation of observations at mammalian central synapses.

4.2 Future Directions

Dissecting the underlying mechanisms by which DNRX functions at synapses

Our findings suggest that DNRX have multiple distinct but interrelated roles during synapse development, from recruiting synaptic building block proteins and promoting synapse growth to mediating the physical link across synaptic cleft and modulating synaptic transmission. The particular aspects of DNRX function might be specified by particular domains of DNRX, their specific interacting proteins, and thereby specific signaling pathways.

We found that the proteins that have been shown to interact with vertebrate neurexins have homologs in *Drosophila*, further supporting the idea that the function of neurexins and underlying signaling mechanisms might be evolutionarily conserved across species. Our initial studies on the expression pattern of these *Drosophila* homologs and protein-protein interactions have identified the potential binding partners of DNRX (Li, J. et al., unpublished data). Among these, *Drosophila* neuroligin and/or dystroglycan (Dg) (Deng et al., 2003) might be potential DNRX ligands. In addition, Camguk, the *Drosophila* homolog of PDZ scaffolding protein CASK (Martin and Ollo, 1996), might be an intracellular binding partner of DNRX.

Drosophila neuroligin mRNA appears at embryonic stage 14 and almost exclusively in CNS neurons with no detectable expression in muscles, which is almost identical to *dnrx* expression pattern in embryos (Li, J. et al., unpublished data). Dg is

highly expressed in the somatic musculature of embryos (BDGP gene expression report), and present at NMJ (Li, J. et al., unpublished data). Dg mutants are embryonic lethal (Deng et al., 2003), and perturbation of Dg function by RNAi and genetic interaction studies suggest a role of Dg in muscle maintenance and axon pathfinding (Shcherbata et al., 2007). Future studies on characterization of *Drosophila* neuroligin and Dg, their functions at NMJ and interactions with DNRX are required to understand the functional significance of neurexin-mediated transynaptic adhesion during synapse development.

Camguk is expressed in neurons (Martin and Ollo, 1996; Lopes et al., 2001) and localized to type I NMJs (Li, J. et al., unpublished data). *Caki*, a null mutant of Camguk, is viable but exhibits reduced locomotor behavior, and defects in learning and memory formation in adult flies (Martin and Ollo, 1996; Lu et al., 2003). The major phenotype of CASK knockout mice and *caki* mutant at synapses are similar, including an increase in the frequency of glutamatergic spontaneous neurotransmitter release, suggesting their primary role in regulation of synaptic function (Atasoy et al., 2007; Zordan et al., 2005). The phenotype of *caki* at larval NMJ has not been reported. Our initial characterization revealed that the NMJ morphology appeared normal and there was no defect in synaptic growth in *caki* mutant. However, similar to the *dnrx* mutant, synaptic component proteins were accumulated in the motor axons of *caki* mutant (Li, J. et al., unpublished data). Thus, the preliminary data indicate that there might be unidentified DNRX intracellular binding partner(s) that mediate the signaling pathway controlling synaptic growth. Further studies are needed to confirm the association of Camguk with DNRX in vivo, their functional relevance and signaling complexes they are involved in. Identification of additional DNRX interacting proteins is also required to dissect the intracellular signaling

pathways that mediate synaptic assembly and growth. In addition, an in vivo structure and function approach should also provide insights into the mechanisms underlying the function of DNRX. For instance, if a particular phenotype of *dnrx* mutant is selectively rescued by a *dnrx* transgene containing a particular domain but not rescued by the transgene lacking this domain; it would suggest that a particular aspect of DNRX function is specified by this domain and associated signal complexes.

Together, future studies on DNRX structure-function relationship, identification and characterization of DNRX binding proteins, their functional relevance to DNRX should shed light on the cellular and molecular mechanisms by which neurexins function in synapse development and function.

Identifying the role of DNRX in the molecular mechanism of retrograde synaptic signaling

Synaptic homeostasis requires two-way communication between pre- and postsynaptic cells to adjust coordinated synaptic performance. For example, during *Drosophila* larval development, the number of synaptic boutons and number of active zones per bouton increase substantially as the larval muscle expands dramatically in size to achieve proper muscle depolarization (Keshishian and Kim, 2004). Recent studies have suggested that retrograde signaling derived from postsynaptic cells is essential for synaptic homeostasis (Haghighi et al., 2003; Sanyal et al., 2004). The retrograde control of synaptic homeostasis is more evident when presynaptic terminals respond to experimental manipulations, e.g. inhibition of postsynaptic transmitter receptors results in a compensatory enhancement of transmitter release (Haghighi et al., 2003).

A variety of diffusible and membrane-bound factors have been proposed to convey the retrograde signaling across synaptic cleft. Among these, TGFβ/BMP signaling pathway has been demonstrated to be involved in the retrograde control of synaptic homeostasis at *Drosophila* NMJ during development and experimental manipulations (Haghighi et al., 2003; Marques and Zhang, 2006). Strikingly, the phenotypes in *dnrx* mutants resemble those arising by mutations in BMP signaling pathway. In this pathway, the BMP homolog Glass bottom boat (Gbb) is released from muscles, and activate its presynaptic receptors on motor neurons including type II receptor Wishful thinking (Wit), type I receptors Thick veins (Tkv) or Saxophone (Sax), leading to the phosphorylation of receptor-regulated Smad (R-Smad). Phosphorylated R-Smad forms a complex with common Smad (Co-Smad) and translocates into the motor neuron nucleus, where it acts as a transcriptional regulator to direct the expression of TGFβ/BMP responsive genes (McCabe et al., 2003). Loss-of-function mutations in the components of BMP signaling pathway such as Wit and Gbb result in a decrease in synaptic bouton number, a reduction in neurotransmitter release and alterations in synaptic ultrastructure that are remarkably similar to the defects observed in *dnrx* mutants (Marques et al., 2002; McCabe et al., 2003; Aberle et al., 2002). This suggests a link between *dnrx* and TGFβ/BMP signaling pathway. Indeed, target-derived BMP4 has been shown to induce upregulation of neurexin expression in mouse sympathetic neurons (Patzke et al., 2001). Therefore, it is likely that neurexins might be one of target genes regulated by TGFβ/BMP retrograde signaling to induce coordinated presynaptic response such as the enhancement of synaptic growth and regulation of neurotransmitter release.

In addition to acting as a presynaptic effecter of TGFβ/BMP-dependent retrograde signaling, it is also plausible that neurexins might be a direct presynaptic mediator of membrane-bound retrograde signaling, given its trans-synaptic location with neuroligins. A recent study demonstrated that postsynaptic PSD-95-neuroligin complex modulates presynaptic neurotransmitter release probability in a retrograde way by increasing sensitivity to extracellular Ca^{2+}. And this retrograde effect might be mediated by presynaptic neurexins (Futai et al., 2007).

Taken together, several lines of emerging evidence indicate a role of neurexins in retrograde synaptic signaling. However, the key evidence for this role is still lacking. Future studies to test whether the retrograde effects trigged by genomic and /or pharmacological manipulations will be abolished in *dnrx* mutant background, and whether DNRX is regulated by TGFβ/BMP signaling pathway should provide the direct evidence identifying the role of neurexins in retrograde signaling and synaptic homeostasis.

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