SCIENCE CHINA
Life Sciences

SPECIAL TOPIC
• REVIEW •

January 2012 Vol.55 No.1: 20–26
doi: 10.1007/s11427-012-4273-3

Cell adhesion molecules in *Drosophila* synapse development and function

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Received July 7, 2011; accepted September 15, 2011

Synapse is a highly specialized inter-cellular structure between neurons or between a neuron and its target cell that mediates cell-cell communications. Ample results indicate that synaptic adhesion molecules are critically important in modulating the complexity and specificity of the synapse. And disruption of adhesive properties of synapses may lead to neurodevelopmental or neurodegenerative diseases. In this review, we will use the *Drosophila* NMJ as a model system for glutamatergic synapses to discuss the structure and function of homophilic and heterophilic synaptic adhesion molecules with special focus on recent findings in neurexins and neuroligins in *Drosophila*.

***Drosophila*, cell adhesion molecules, synapse, neuromuscular junction, neurexin, neuroligin**

Citation: Sun M K, Xie W. Cell adhesion molecules in *Drosophila* synapse development and function. Sci China Life Sci, 2012, 55: 20−26, doi: 10.1007/s11427-012-4273-3

Synapse is a specialized cell adhesion structure between a neuron and its target cell or another neuron. It is involved in neural information processing and storage. Synapse formation is a multi-step process that includes target recognition that requires cell adhesion molecules to initiate cell-cell contact, and subsequent recruitment of proteins and organelles to form the presynaptic and postsynaptic compartments. Many cell adhesion molecules (CAMs) are expressed at the synapse. These are membrane-anchored molecules, consisting of three domains: an intracellular domain that can directly interact with intracellular proteins to mediate signaling processes, a transmembrane domain, and an extracellular domain that interacts either with another molecule of the same CAM protein (homophilic binding) or with different CAM or extracellular matrix proteins (heterophilic binding). Disruption of adhesive properties of synapses may lead to neurodevelopmental or neurodegenerative diseases, such as autism and schizophrenia [1].

Drosophila melanogaster has been widely used as a model system to address fundamental questions related to neuroscience not only because it has a relatively simpler genome and nervous system, but also because many molecular mechanisms underlying neuronal development that operate in mammals, including those involved in synaptogenesis, are highly conserved in this organism. In addition, the fruit fly has a well-characterized repertoire of molecular genetic tools and a relatively short life span [2], thus providing an invaluable model system for genetic dissections of the nervous system on a large scale. In particular, the *Drosophila* neuromuscular junction (NMJ), an asymmetric chemical synapse formed between motor neurons and muscle cells, is an excellent model system for investigating the fundamental mechanisms governing synaptic development and function. The advantageous features of the *Drosophila* NMJ include its structural accessibility, stereotypic feature and amenability to genetic manipulations, electrophysiological and microscopic analyses [3–7].

The *Drosophila* NMJ is a glutamatergic synapse. Many

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of its properties, including the molecular composition, are similar to those of excitatory synapses of mammalian CNS, therefore, the *Drosophila* NMJ is considered as a convenient and useful model for elucidating the mechanisms underlying glutamatergic synapses [8,9]. Indeed, the NMJ has been a site of extensive investigations in the context of synapse formation, synaptic transmission and plasticity, and synaptic degeneration.

Here, we summarize recent findings on the roles of selected synaptic adhesion molecules in synapse development, with special emphasis on the *Drosophila* neurexin-neuroligin complex. Our key focus is to highlight the *Drosophila* Neurexin-neuroligin complex principles that govern the molecular basis of synapse formation and function. We conclude by providing a hypothetical model for neurexin-neuroligin signaling at the NMJ.

1 Homophilic CAMs

The identified homophilic CAMs at the *Drosophila* NMJ include Capricious, Connectin, Fasciclin II, Fasciclin III, Neuroglian, N-Cadherin and Dscam.

1.1 Capricious

Capricious (Caps) is a transmembrane protein with 14 leucine-rich repeats (LRRs). It is expressed presynaptically in the anterior corner cell (aCC), RP2, U, and RP5 motor neurons, and postsynaptically in muscles 1, 2, 9, 10, and 12 [10]. The level of Caps is regulated by the transcription factor Kruppel. Caps is necessary for proper defasciculation of SNb axons [11]. Loss-of-function or ectopic expression of the *caps* gene alters the target specificity of muscle 12 motorneurons [10,12] (Table 1). In muscle 12 cells, Caps is localized at the tips of myopodia before the arrival of motoneuronal growth cones. In *caps* mutants, there are fewer contacts between myopodia of M12 and the presynaptic growth cones during the initial neuromuscular interaction. In addition, the nascent synaptic sites of M12 are also reduced in the *caps* mutants. These results indicate that Caps is required for target recognition at the tips of myopodia [13].

1.2 Connectin

Connectin (Con) is a cell surface protein with 10 LRRs, which may mediate homophilic interaction *in vitro* [14]. The expression of Con is first observed in one to three myoblasts on the lateral side of the body wall at late stage 11 to early stage 12. Con is expressed on the surface of eight muscles, their innervating motoneurons and several surrounding glial cells. The *connectin* mutants or transgenic flies with ectopic expression do not show any significant neuromuscular defects, therefore, the role of this CAM remains elusive [15] (Table 1).

1.3 Fasciclin II

Fasciclin II (Fas II) is a homophilic interaction protein [16,17] that has multiple functions in the *Drosophila* nervous system. The fact that the overall structural features of Fas II are remarkably similar to the vertebrate neuronal cell adhesion molecule (NCAM) suggests that it may represent

Table 1 Cell adhesion molecules at the *Drosophila* NMJ

Genes	Expression patterns	Functions	References
Capricious	Motor neurons aCC, RP2 U, RP5	Defasciculation of SNb axons	[11]
	muscle 1,2,9 10, 12,14 15,16,17 and 30	Target recognition	[12]
Connectin	Muscle 5,8,18, 21-24 and innervate their motor neurons	Muscle pattern formation	[14]
	PG1, PG3 and PG4 Glial cells	Synaptic formation	[15]
FasciclinII	All motor neurons	Presynaptic cell pattern formation	[26]
	All muscles	Postsynaptic accumulation	
FasciclinIII	RP3 motor neuron muscle 6/7	Target recognition	[31]
Neuroglian	All motor neurons	Target recognition	[35]
	All muscles		
N-Cadherin	All motor neurons	Axonal pattern formation	[40]
	All muscles		
Dscam	All motor neurons	Presynaptic cell pattern formation	[41]
	All muscles	Dendritic development	
Neurexin IV	Midline neurons	Epithelial and axo-glial SJs	[47]
	Glia cells		
	Epithelial Cells	Axon guidance	
Neurexin-1	CNS neurons	Synaptic assembly	[50]
	Motor neurons	Synaptic differentiation	[51]
	Body Wall muscles	Learning and memory	[52]
Neuroligin1	Body Wall muscles	Synaptic growth	[54]
		Postsynaptic differentiation	
Neuroligin2	CNS neurons	Synaptic growth	[56]
	Motor neurons	DGluRs recruiment	
	Body Wall muscles		

the fly ortholog of the mammalian NCAM. Fas II has been shown to be important for the development, maintenance, and plasticity of the NMJ.

Fas II is expressed by differentiating neuroblasts during early neurogenesis in the *Drosophila* embryo [18,19], where it is involved in the induction of downstream proneural genes, including *achaete* (*ac*) and *atonal* (*ato*) [20]. Fas II is expressed in all motoneuron axon pathways, including growth cones from early axonal outgrowth to the time of synapse formation [21]. It is also expressed at low levels in all muscle cells [22].

Overexpression of Fas II in presynaptic neurons results in fusion of motoneuron axons, whereas decreased expression of Fas II leads to a complete or partial defasciculation of motor axon pathways [23,24]. In aCC and RP2 pioneer axons, Fas II is necessary and sufficient for guiding follower axons and the establishment of presynaptic cell patterns [25]. At the NMJ, FasII is expressed at both presynaptic and postsynaptic site and is required for the accumulation of scaffolding protein Discs large (Dlg) and glutamate receptor subunits (GluRII A and GluRII B) [26]. In some muscle cells, transient expression of Fas II results in the formation of new ectopic functional synapses [22]. These results suggest that Fas II plays essential roles in pattern formation and postsynaptic specialization (Table 1).

Recent work has also shown that Fas II and Dlg function together to modulate activity-dependent synaptic development and that this role is regulated by activation of CaMKII [27,28]. In addition, axonal Fas II can interact homophilically with a glial isoform of Fas II and this interaction is critical for the glial cell migration and is regulated by Fzr/Cdh1[29].

1.4 Fasciclin III

Fasciclin III (Fas III) is a single transmembrane, homophilic immunoglobulin superfamily (Ig CAM) protein [30]. It plays an important role in cell adhesion, axon pathfinding and fasciculation [31,32].

Fas III is expressed in muscle 6 and 7, in the axons of RP motoneurons, including RP3. *Fas III* mutants displayed defects of RP3 axons guidance, which axons incorrectly innervate their targets. RP3 mistarget the neighboring muscles misexpressing Fas III [31]. Cell-specific expression pattern of Fas III may provide the molecular basis for its target recognition function (Table 1).

1.5 Neuroglian

Neuroglian (Nrg) is a homophilic interaction protein that contains six Ig-like domains and 5 FN type III domains. It is related to a number of vertebrate CAMs although most closely to the mouse L1. Alternative splicing of the *Nrg* gene generates 2 isoforms; the long form of Nrg that is expressed on the surface of specific CNS and PNS neurons, as well as in some PNS support cells, and the short form of Nrg that is expressed in glia and a variety of other non-neuronal tissues, including trachea, hindgut, salivary gland and muscle [33]. Together with Ank, Nrg mediates the neuron-glia interaction to contribute to the axonal and dendritic morphogenesis [34]. Loss of function of Nrg results in motoneuron axon misprojections and stalling close to the target postsynaptic muscle cell [35]. Nrg in sensory neurons is also necessary for the maintenance of sensory axon advance [36] (Table 1).

1.6 N-Cadherin

The *Drosophila* N-cadherin (N-Cad) is an evolutionarily conserved, classic type cadherin with a large, complex extracellular domain and a catenin-binding cytoplasmic domain. There are 12 isoforms of N-Cad that share the same molecular architecture but have different sequences in their transmembrane and extracellular domains, which mediate homophilic interactions [37–39]. N-Cad regulates axonal pattern formation, presumably by regulating axonal fasciculation in the developing embryo [40].

1.7 Dscam

The *Drosophila* Dscam, a homologue of human Down syndrome cell adhesion molecule (DSCAM), is an immunoglobulin (Ig) superfamily protein. It participates in the presynaptic motor neuron pattern formation at the NMJ and is important for precise neuronal connections in the fly brain [41]. Dscam is also required for dendritic self-avoidance in all four classes of *Drosophila* dendritic arborization (da) neurons [42]. The dendrites of these neurons distinguish self and non-self through Dscam1 homophilic interactions [41]. *Drosophila* Dscam1 could generate as many as 19008 different ectodomains by alternative splicing of three exon clusters, with each encoding half or a complete variable immunoglobulin domain. This isoform diversity provides the molecular basis for establishing specific self-avoidance in neurons with complex dendritic arborization [43].

2 Heterophilic CAMs

2.1 Neurexin IV

Neurexin IV (NRX IV) is a transmembrane protein with a cytoplasmic domain homologous to glycophorin C and is shown to be more similar to the vertebrate Caspr protein [44]. NRX IV is localized to septate junctions (SJs) of epithelial and glial cells, and is required for the formation and function of septate junction and blood-nerve barrier [45], and the establishment of epithelial cell polarity [46]. NRX IV is also expressed in the medline neurons, where it interacts with Roundabout and plays a role in repulsive midline

axon guidance and is found a novel interacting component of the Robo/Slit signaling pathway [47–49] (Table 1).

2.2 Neurexin-1

The *Drosophila* Neurexin-1 (DNRX) is a single transmembrane protein with six LamininA/ Neurexin/sex hormone-binding protein domains and three interspersed epithelium growth factor like sequences. The DNRX protein has an identical domain structural organization to mammalian a-neurexins [50–52]. DNRX is expressed throughout the development of the nervous system. During embryonic stages, strong expression is observed along the longitudinal tracts of the VNC and brain. In the adult brain, DNRX is expressed at high levels within the medulla, lobula, lobula plate, mushroom body and antenal lobe [52]. Within synaptic boutons, DNRX mostly localizes to the active zone (AZ, also called T-bar) of presynaptic terminals. However, DNRX is present both pre- and post-synaptically in embryo and third instar larvae stages [50]. *dnrx* loss of function causes reduced proliferation of synaptic boutons at glutamatergic neuromuscular junctions whereas overexpression of DNRX in neurons leads to synaptic overgrowth [51].

DNRX promotes presynaptic AZ specialization, neurotransmitter release, and postsynaptic glutamate receptor clusters [50]. Thus, *dnrx* null mutants display striking defects in synaptic ultrastructure, including the presence of detachment–between pre- and postsynaptic membranes, abnormally long AZs, increased number of T bars, and deficits in synaptic transmission [52]. DNRX is also required for synapse formation in the adult CNS and is important for associative learning [52]. Therefore, DNRX is critically involved in both synapse development and function [50–52].

2.3 Neuroligins

There are four *neuroligin* genes identified in the *Drosophila melanogaster* genome. Their encoded proteins (CG13772, CG34127, CG34139, and CG31146) share significant similarity in amino acid sequence and predicted structure with the vertebrate Neuroligins: a type I membrane protein with an extracellular domain consisting of mostly of a region homologous to acetylcholinesterases but lacking the esterase activity, a transmembrane domain, and a cytoplasmic domains with a PDZ binding motif. Phylogenetic analysis based on protein sequences indicates that the fly neuroligins and mammalian neuroligins share a common ancestor [53].

Of the four *neuroligin* genes, only the transcript of *Drosophila neuroligin 1* (*dnl1*) is specifically expressed in muscle tissues as judged from *in situ* hybridizations. DNL1 protein (CG31146) is also specifically found in postsynaptic muscle cells and accumulates at NMJs, in a site adjacent to PSDs. Moreover, DNL1 forms discrete clusters at the edge of postsynaptic receptor fields [54]. *dnl2* (CG13772) mRNA is primarily detected in the brain and VNC in embryonic stage 14, which is consistent with the expression pattern of DNL2. High levels of DNL2 are also observed in the brain and VNC of third-instar larvae, where it colocalizes with DNRX. DNL2 expression in the CNS appears to be widespread and uniform with no preferential colocalization with any specific neurotransmitter or neuro-hormone. DNL2 is also detected in the abdominal muscles of third-instar larvae, where it is enriched at the NMJs [53]. The expression of the other two *dnls* (CG34127 and CG34139) is also prominent in the CNS and third instar larval NMJs (unpublished).

Ample results indicate that DNL1 is required for effective addition of synaptic boutons at developing NMJ terminals, and postsynaptic differentiation, including the accumulation of postsynaptic glutamate receptors, scaffold proteins, and subsynaptic membrane components. Because mutant animals of *dnl1* showed overgrown glutamate receptor fields, at the same time, boutons that are positive for presynaptic markers frequently lacked postsynaptic receptor fields [54]. Meanwhile ectopic DNL1 expression triggers ectopic postsynaptic differentiation via its cytoplasmic domain.

DNL1 plays an important role in the regulation of synaptic function. In *dnl1* mutant flies, eEJC amplitudes are reduced to approximately 50% of control levels at both low and high extracellular [Ca^{2+}], possibly due to less AZs formed. Moreover, eEJC decay kinetics is also prolonged in the *dnl1* mutants, which might be in agreement with enlarged postsynaptic receptor fields. Overall amplitudes and frequencies of mEJCs are, however, not altered significantly from controls [54].

Null mutants of *dnl2* display reduced axonal branching and fewer synaptic boutons with an increase in the number of AZs per bouton, but a decrease in the thickness of subsynaptic reticulum (SSR) and in the length of postsynaptic densities [53]. *dnl2* mutants also exhibit a decrease in the total glutamate receptor density and a shift in the subunit composition of glutamate receptors in favor of GluR IIA complexes. *dnl2* mutants also show increased neurotransmitter release and altered kinetics of stimulus-evoked transmitter release [53].

3 Trans-synaptic neurexin-neuroligin signaling at *Drosophila* NMJs

Although CAMs have been shown to regulate synapse development and function, the exact underlying molecular mechanisms and *in vivo* significance remain unclear. It is known, however, that mutations in genes encoding several CAMs, such as *neurexin* and *neuroligin*, are linked to human neurological and mental disorders, including autism and schizophrenia [1]. *In vitro* studies suggest that the vertebrate neurexins and neuroligins play critical roles in the initial establishment of the synapse (reviewed in [1,55]), but *in vivo* results obtained from knockout animals suggest that

these molecules are only important for the maturation or validation of the synapse. However, this conclusion is complicated by the existence of multiple genes and splice variants for both neurexins and neuroligins in mammals. In contrast, the *Drosophila* has relatively fewer members/splice variants of *neurexin* and *neuroligins,* providing some advantages to analyze the *in vivo* function and underlying mechanism [50–54].

DNRX localizes at the presynaptic membrane, where its intracellular domain interacts with CAKI/CMG, the *Drosophila* homolog of the vertebrate CASK, a member of the MAGUK scaffolding protein family. This interaction is important for synaptic vesicle trafficking [56]. *dnrx* mutants are reduced in the expression of Bruchpilot (BRP), a presynaptic protein known to be important for the structural integrity and function of synaptic AZs in *Drosophila*. BRP mutants exhibit deficits in T-bars, calcium channel clustering, and synaptic short-term plasticity [57,58]. Some of these deficits are also observed in *dnrx* mutants [57,58]. These results suggest that DNRX may regulate presynaptic assembly and functional maturation through interacting with BRP [59].

Interestingly, *Drosophila* Syd-1 (DSyd-1) has been identified to interact with BRP, and *dsyd-1* mutants display smaller terminals with fewer release sites and reduced neurotransmitter release. In addition, the remaining AZs of the mutants are often large, ectopic, and abnormal in shape with electron-dense accumulations of BRP in boutons and axons [60]. These alterations are similar to those observed at *dnrx* deficient NMJs [51]. Therefore, it is possible that DNRX functions in AZ assembly by forming BRP-DSyd-1-DNRX complexes (Figure 1). It has been proposed that DSyd-1 regulates effective nucleation of newly forming AZs via interacting with DLiprin-α [60].

In addition to presynaptic deficits, both *dnrx and dsyd-1mutants* are also altered in postsynaptic specialization. Although DNRX localizes predominantly close to AZs, *dnrx* mutants show unexpected changes in the levels of glutamate receptor and DNL1 at the NMJs [50,51,54]. Similarly, d*syd-1* mutants also exhibit alterations in glutamate receptors at NMJs. These results suggest that DNRX and DSyd-1 are also important in the regulation of postsynaptic glutamate receptors, likely through trans-synaptic interaction with postsynaptic DNLs.

Interestingly, the postsynaptic deficits associated with *dnrx* and *dsyd-1* mutants are remarkably similar to those found in *dnl1*and *dnl2* mutants. For example, loss of DSyd-1 or DNL2 leads to a dramatic increase in the amount

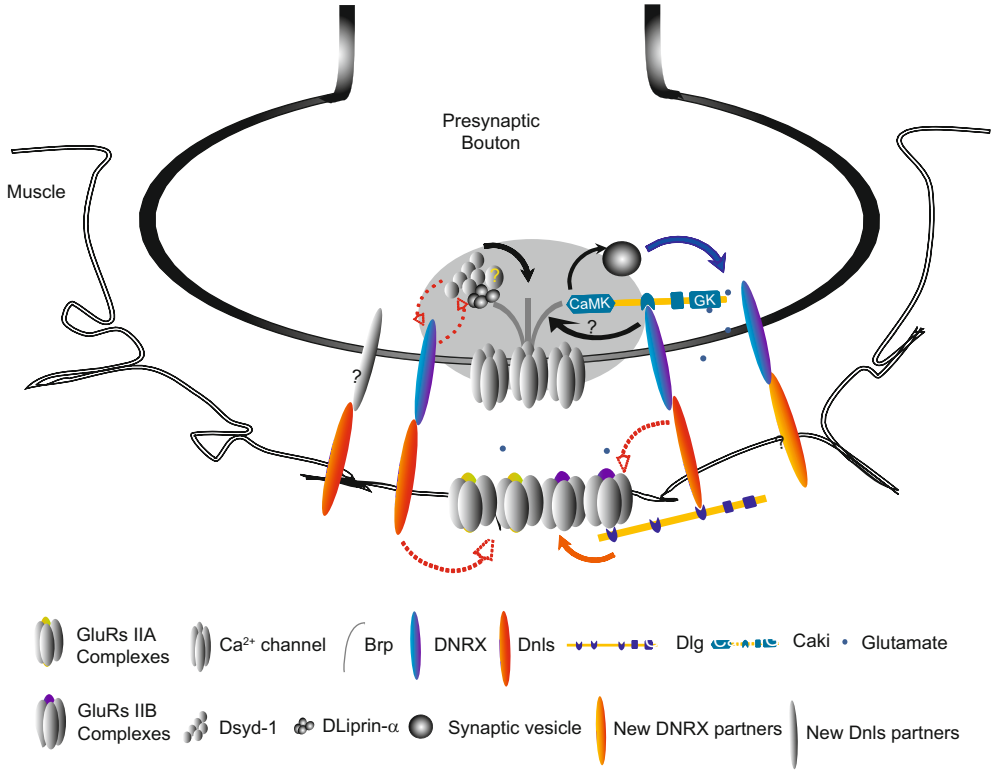

Figure 1 Model of neurexin-neuroligin mediated intercellular signaling. DNRX and DNL bind to each other to bridge the presynaptic and postsynaptic sites and strengthen signaling across the synapse. DNRX interacts with CASKI at the presynaptic site to regulate synaptic vesicle trafficking; DNRX also forms a protein complex with BRP and Dsyd-1 and this complex is important for the formation of active zone; DSyd-1 directly interacts with BRP at the AZ to regulate the localization and motility of Liprin-α; DNRX is proposed as a direct substrate for DSyd-1. DNL can be regulated by DSyd-1 or other presynaptic proteins via trans-synaptic DNRX-DNL interaction, which in turn regulates postsynaptic receptor fields. Other potential DNRX and DNL interacting partners may also participate in this trans-synaptic signaling. The broken lines indicate links (direct or indirect) of unknown nature.

of DGluRIIA and a decrease in DGluRIIB, resulting in a shift in the relative ratios of these receptors. These results are consistent with the idea that DNRX and Dsyd-1 regulate postsynaptic properties through DNRX-DNL trans-synaptic interaction. Both genetic and biochemical evidence supports a direct interaction of DNRX and DNL2 in *Drosophila* [53]. Exactly how DNL2 regulates GluRs remains unknown, but direct protein interactions with DLG may play an important role in this process [51,53,60] (Figure 1).

Considering the overall phenotypes in mutant *dnrx* [50–52] and *dnl* [53,54], it is reasonable to conclude that DNRX and DNL regulate synaptic development and function at the *Drosophila* NMJ through DNRX-DNL trans-synaptic interaction. However, the fact that the *dnrx* phenotype is weaker than the *dnl1* phenotype suggests that not all DNRX function is mediated by DLN1 [53]; indeed, *dnl2* null flies also display a significant reduction in the number of synaptic boutons. In addition, *dnrx*/*dnl2* double mutants exhibit more severe phenotypes than those observed in *dnrx* or *dnl2* single mutants [53]. These results suggest that additional postsynaptic proteins may interact with and mediate the effect of DNRX. LRRTM2 has been recently identified as a synaptic cell-adhesion molecule that could act as a NRX receptor in mammals [61,62] and this may also occur in *Drosophila*. It is also possible that other partners than NRX exist for DNL. To identify these novel molecules and their specific roles in the regulation of synaptic development and function will be one of most important research topics and the fruit flies will continue to provide a useful system to investigate them.

We thank Drs. Jia ZhengPing and Zhou ZiKai for reading the manuscript and comments. This work was supported by the National Natural Science Foundation of China (Grant Nos. 31171041 and 31000486) and National Basic Research Program of China (Grant No. 2012CB517903).

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