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The roles of dystrophin and dystrobrevin : in synaptic signaling in drosophila

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Citation

Potikanond, S. (2012, January 19). *The roles of dystrophin and dystrobrevin : in synaptic signaling in drosophila*. Retrieved from <https://hdl.handle.net/1887/18388>

Version: Corrected Publisher's Version

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CHAPTER 1

Introduction

Chapter 1 (Part 1): The Roles of the Dystrophin-Associated Glycoprotein Complex at the Synapse

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Published in *Molecular Neurobiology*. February 2010; 41(1): 1–21.

Abstract

Duchenne muscular dystrophy is caused by mutations in the *dystrophin* gene and is characterized by progressive muscle wasting. A number of Duchenne patients also present with mental retardation. The dystrophin protein is part of the highly conserved dystrophin-associated glycoprotein complex (DGC) which accumulates at the neuromuscular junction (NMJ) and at a variety of synapses in the peripheral and central nervous systems. Many years of research into the roles of the DGC in muscle have revealed its structural function in stabilizing the sarcolemma. In addition, the DGC also acts as a scaffold for various signaling pathways. Here, we discuss recent advances in understanding DGC roles in the nervous system, gained from studies in both vertebrate and invertebrate model systems. From these studies, it has become clear that the DGC is important for the maturation of neurotransmitter receptor complexes and for the regulation of neurotransmitter release at the NMJ and central synapses. Furthermore, roles for the DGC have been established in consolidation of long-term spatial and recognition memory. The challenges ahead include the integration of the behavioral and mechanistic studies and the use of this information to identify therapeutic targets.

Keywords: Dystrophin, DGC, NMJ, CNS, PNS, Retina, Duchenne muscular dystrophy, Mammals, *Drosophila*, *C. elegans*

Introduction

Duchenne muscular dystrophy (DMD) is a common X-linked, fatal genetic disorder characterized by progressive muscle wasting [1]. The disease was named after the French neurologist G.B.A. Duchenne who, in the mid-1800s, described patients that suffered not only from muscular dystrophy but also from mental retardation [2]. A major advancement in understanding the underlying cause of DMD came in 1987, when dystrophin was identified as the protein that is absent in DMD patients [3, 4]. Mental retardation occurs in approximately one third of DMD patients and is apparently dependent on the specific location of the mutation within the dystrophin gene (in mammals called the DMD gene) [5]. Despite more than two decades of intense clinical and basic research into the DMD disease process and the interactions and roles of the dystrophin protein, a cure for the disease remains elusive and treatments palliative. Thus, much remains to be learned about the basic biological roles of the dystrophin protein in the musculature and in the brain.

The DMD gene is one of the largest genes in the human genome, spanning 2.3 Mb [6]. It has three upstream promoters that control expression of full-length dystrophin Dp427 and four internal promoters which regulate expression of the short dystrophin isoforms, Dp260, Dp140, Dp116, and Dp71 (reviewed at www.dmd.nl; **Table 1**; **Textbox 1** in “Appendix”). The complexity of DMD gene expression, which results in multiple transcripts and protein isoforms, has made understanding the functions of individual dystrophin protein isoforms difficult. Dp427 has an amino-terminal actin-binding domain, and Dp427, Dp260, Dp140, and Dp116 have a variable number of spectrin-like repeats that are predicted to form triple-helical rod-like structures. Dystrophin proteins invariably bear a number of protein–protein interaction domains, some of which are bound by other dystrophin-associated glycoprotein complex (DGC) members: a WW domain, a cysteine-rich region containing a ZZ domain, and a highly conserved carboxy-terminal region including a coiled-coil domain.

Dystrophin is predominantly expressed in the musculature and the nervous system (**Fig. 1**, **Table 2**, and references therein). More specifically, the three distinct Dp427 variants that are transcribed from different promoters and have distinct first exons are expressed in skeletal muscle (Dp427M), throughout the brain (Dp427B) and in Purkinje cells (Dp427P), respectively. Dp260 is expressed in the retina, Dp140 in brain and kidney, Dp116 in the peripheral nervous system, and Dp71 is uniformly expressed, but most prominently present throughout the mammalian brain (reviewed in [7]; **Fig. 1**; **Table 2**). In mammals, there are two proteins which are significantly homologous to dystrophin, utrophin [8], and the much shorter dystrophin-related protein 2 (DRP2) [9]. Utrophin is more ubiquitously expressed compared to dystrophin, hence its name. It is found in developing skeletal and smooth muscle, the nervous system, the lung, kidney, liver, spleen, and stomach [10, 11].

Many components of the DGC have been identified, including the dystrobrevins (DB- α and DB- β), the syntrophins (α 1, β 1, β 2, γ 1, and γ 2), dystroglycan (DG- α/β), the sarcoglycans (SG- α , SG- β , SG- γ , SG- δ , SG- ϵ , and SG- ζ), and sarcospan [10, 12]. Together with dystrophin, these proteins form a large, molecularly heterogeneous cluster [13], known as the DGC, that differs in composition depending on the tissue or organs where the components are expressed (reviewed in [10, 14–16]; **Fig. 1**; **Table 2**).

Table 1 Compilation of the Known DGC components in Human/Mice, Zebrafish, *C. elegans* and *Drosophila*

DGC member	Species			
	Human/mice (www.dmd.nl) Proteins (gene)	Zebrafish [180] (www.zfin.org)	<i>C. elegans</i> [33] (www.wormbase.org)	<i>Drosophila</i> [32] (www.flybase.bio.indiana.edu/)
Dystrophin	Long isoforms: Dp427 (M, B, P) Short isoforms: Dp260, Dp140, Dp116, Dp71 (DMD)	Long isoform: a 400 kD dystrophin ortholog Short isoform: Dp71 (dmd)	Dystrophin-like protein (<i>dys-1</i>)	Long isoforms: DLP(1-3) Short isoforms: Dp205, Dp186, Dp117 (<i>Dys</i>)
Utrophin	a-, b-, g-utrophin (UTRN)	utrophin (utrn) [181]		
DRP2	Dystrophin-related protein (DRP2)	drp2 (drp2) [181]	-	-
Dystrobrevin	α -dystrobrevin (DTNA) β -dystrobrevin (DTNB)	α -dystrobrevin(dtna) β - dystrobrevin (dtnb) γ - dystrobrevin (dtng) [181]	Dystrobrevin (<i>dyb-1</i>)	Dystrobrevin-like (<i>Dyb</i>)
Dystroglycan	α/β -dystroglycan (DAG1)	α/β -dystroglycan (dag1)	Dystroglycan (<i>dgn-1</i>)	Dystroglycan (<i>Dg</i>)
Sarcoglycan	α -, ϵ -, β -, γ -, δ -, ζ -sarcoglycan (SCGA-Z)	α -, ϵ -, β -, γ -, δ -, ζ - sarcoglycan	α -, β -, δ/γ -Sarcoglycan (α -, β -, δ/γ - <i>sgn</i>)	Sarcoglycan- α , Sarcoglycan- β , Sarcoglycan- δ (<i>scg</i> - α , <i>scg</i> - β , <i>scg</i> - δ)
Sarcospan	sarcospan (SSPN), (Microspan in mice)	Not determined	-	-
Syntrophin	α -, β 1-, β 2 syntrophin γ 1-, γ 2-syntrophin	Not determined	β 1-Syntrophin (<i>stm-1</i>) γ -Syntrophin	Syntrophin-like 1 and Syntrophin- like 2 (<i>syn-1</i> , <i>syn-2</i>)

Table 2: Compilation of the known expression domains of DGC components of Human/Mice and *Drosophila*¹

<i>Species</i>	<i>Human/mice</i>	Expression domain	<i>Drosophila</i>	Expression domain (embryo)
DGC member				
Dystrophin	Dp427	Muscle (M), Brain (B), Purkinje cells (P), Retina	DLP1	Visceral Mesoderm
	Dp260	Retina	DLP2	Visceral Mesoderm, Musculature, Mesectoderm
	Dp140	Fetal tissues, Brain, Retina, Kidney	DLP3	Adult
	Dp116	Schwann cells (brain and spinal cord)	Dp205	Pericardial cells, VNC, Visceral Mesoderm
	Dp71	Brain (microvascular glial and gyrus dentatus cells), retina (ILM, perivascular astrocytes), other non-muscular tissues, Liver	Dp186	CNS, Midgut
	a-Utrophin	Fetal Muscle, muscle, Pia mater, Choroid plexus, Astrocytes	Dp117	Musculature, VNC
	b-Utrophin	Vascular endothelial cells	-----	-----
	g-Utrophin (homologue of Dp116) DRP2	Sensory ganglia, Brain CNS, Schwann cells		
Dystrobrevin	α -DB-1/-2	Skeletal Muscle, Brain	Dystrobrevin-like	Musculature, VNC, Brain
	α -DB-3	Skeletal and cardiac muscle, the inner layer of plexiform plexus		
	β -DB	Outer layer of the plexiform plexus of retina		
Dystroglycan	α -, β -DG	most tissues	Dystroglycan	Musculature, Brain, VNC
Sarcoglycan	α -, β -, γ -, and δ -sarcoglycan	Skeletal and cardiac muscle β - ϵ sarcoglycan in mouse retina	Sarcoglycan- α -, β -, δ	CNS, Gut, musculature
	ϵ -sarcoglycan	Brain, Smooth muscle, Schwann cells		
	ζ -sarcoglycan	Brain, Muscle, Kidney, Spleen, Pancreas		
Sarcospan	Long, short isoforms	Skeletal and Cardiac Muscle (short isoforms also in other tissues)	---	---
Syntrophin	α 1-, β 1-, β 2-syntrophin	Skeletal and Cardiac Muscle, Brain, Retina, Lung, Kidney, Liver, Pancreas, Testes	Syntrophin-like-1	Brain, VNC
	γ 1-syntrophin	Brain, General expression	Syntrophin-like-2	Mesoderm

¹ The data reviewed in this Table is taken from references [1, 7, 10, 14-16, 25, 32, 39, 182].

The proteins of the DGC are highly conserved between species, although there are fewer homologs and isoforms in the lower vertebrates, such as zebrafish, and in the invertebrates, *Caenorhabditis elegans* and *Drosophila* (**Table 1**).

Due to the complex and variable composition of the DGC, it is difficult to assign a general function to this transmembrane complex. However, in all organisms studied thus far, the different DGCs localize to the plasma membrane (reviewed in [12]). In muscle, the DGC has long been thought to be required for the stabilization of the plasma membrane by linking the actin cytoskeleton to the extracellular matrix [17]. Disruption of the DGC caused by the absence of dystrophin consequently renders the sarcolemma susceptible to mechanical damage during contraction and ultimately results in muscle degeneration. More recently, it has been established that the DGC also functions as a scaffold for proteins involved in signaling, including neuronal nitric oxide synthase (nNOS), phosphoinositol triphosphate 2, calmodulin, and growth factor receptor-bound protein 2 (grb2; reviewed in [18]). Additional evidence supports the involvement of the DGC in the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) signaling cascade [19], epidermal growth factor receptor-mediated signaling [20], and insulin signaling [21]. Moreover, it has been shown that the DGC is required for neurotransmitter receptor and ion channel clustering and in maintaining calcium (Ca^{2+}) homeostasis [22, 23].

Since the 1990s, the role of the DGC in brain function has also become the topic of extensive clinical and basic research; however, the mechanisms of action of the different dystrophin isoforms in brain are still largely unknown [5]. Interestingly, a patient was recently described carrying a mutation in the carboxy-terminal region of the DMD gene, who suffered from a mental disorder but showed no signs of muscle weakness [24]. This finding raises the possibility that there may be more patients with cognitive impairments caused by alterations in dystrophin expression or function and demonstrates the important role for dystrophin and its associated proteins in the brain (reviewed by [25]). Moreover, mutations in the gene encoding an enzyme that glycosylates α -dystroglycan, protein O-mannosyltransferase (POMT) 1, have been associated with a range of muscular dystrophies that are often accompanied by severe mental retardation and ocular abnormalities [26–28]. In recent years, novel gene therapy strategies, such as antisense oligonucleotide-mediated exon-skipping, have been directed toward restoring dystrophin expression in muscle fibers of DMD patients [29]. Promising results have been obtained in a phase I clinical trial [30]. While the application of such approaches in the brain will likely be more challenging, these reports validate the need for preclinical studies to establish the agents and means of delivery required for treatment of DMD-associated nervous system deficits.

Mammalian DMD models, such as the mdx mouse, which lacks the three full-length dystrophin Dp427 isoforms, have been studied most extensively. However, it is now becoming clear that invertebrates such as *C. elegans* and *Drosophila* can also serve as models to study the fundamental and likely conserved functions of the DGC [31–33]. The reduced functional redundancy of the DGC members [31], the genetic tools available, and the ease of transgenic manipulation render these invertebrate animal models useful. Both the worm and the fly have only a single dystrophin/utrophin ortholog, and their functions may therefore reflect roles played by both dystrophin and utrophin in mammals. *Drosophila* exhibits muscle degeneration and a reduced lifespan when the expression of certain DGC members are reduced or absent [21, 34–37]. *C. elegans* lacking DGC components also display severe dystrophy of the musculature, but only in a genetically sensitized background where muscle differentiation is impaired [38]. These findings illustrate the relevance of these models for DMD.

This review focuses on recent insights into the specific roles of the DGC at the synapse gained from studies using mouse and invertebrate animal models. First, we summarize what is known about the function of the DGC at the neuromuscular synapse with emphasis on results obtained from mouse, *C. elegans* and *Drosophila*. We then describe the roles of DGC members in central synapses in the brain and the retina and how disruption of the complex may cause cognitive impairment. Our focus is on the possible signaling pathways interacting with the DGC that may be important for the development or maintenance of peripheral and central synapses.

1. Roles of the DGC at the Neuromuscular Junction

In this section the involvement of the DGC at the mammalian neuromuscular junction (NMJ) is discussed in receptor clustering, synaptic transmission, and its participation in a number of important signaling cascades acting at the NMJ, such as the agrin/muscle-specific kinase (MuSK)/rapsyn, nNOS, and Ca²⁺/calmodulin-dependent protein kinase II (CAMKII)-mediated signaling pathways. In the following Sections, insights obtained in DGC NMJ function using the *C. elegans* and *Drosophila* invertebrate model systems which reveal novel roles for dystrophin in neurotransmitter clearance and in the regulation of neurotransmitter release, respectively, are reviewed.

1.1 The Involvement of the DGC in AChR Clustering, Synaptic Transmission, and Signaling at the Mammalian NMJ

1.1.1 AChR Density, Junctional Folds, and Transmission Are Reduced at DGC-Deficient NMJs

The six families of proteins encompassing the DGC can be divided into three subcomplexes: (1) a cytoplasmic complex comprised of dystrophin (or utrophin), dystrobrevin and syntrophin, (2) the transmembrane dystroglycan complex, and (3) the sarcoglycan–sarcospan complex (reviewed in [1, 18, 25, 39, and 40]). Dystrophin is expressed at the sarcolemma of mammalian skeletal muscle and is also found to accumulate at the troughs of the postsynaptic membrane where it colocalizes with voltage-gated sodium channels [41]. Dystrophin is only consistently detected at the mammalian NMJ after postnatal day 7 [42]. Utrophin, however, accumulates at the postsynaptic side of the neuromuscular junction during early postnatal development as part of a protein complex, the utrophin glycoprotein complex (UGC; **Fig. 1b**), which includes the syntrophins, dystrobrevins, sarcoglycans, sarcospan, and dystroglycan [43]. Dystroglycan interacts with the more general structural and signaling components of the NMJ, including agrin, laminin, and rapsyn and also associates with clusters of acetylcholine receptors (AChRs) at the crests of the junctional folds [41, 42]. In addition, the adaptor protein ankyrin B is required for localizing the DGC at the mammalian postnatal NMJ [44].

Much has been learned about the mechanisms that underlie the development and function of the vertebrate NMJ (reviewed in [45–47]). A synapse is formed with a highly specialized presynaptic membrane, the active zone, opposing a postsynaptic density enriched in AChRs (in vertebrates) in the crests of the junctional folds and voltage-gated sodium channels in the troughs. Several signaling pathways involved in organizing the initial stages of NMJ formation have been, at least in part, elucidated. One such pathway includes agrin, a heparin sulfate proteoglycan that is released from the motor neuron to activate the postsynaptic tyrosine kinase receptor, MuSK. Engagement of MuSK leads to a second messenger cascade resulting in the clustering of the AChRs, the major neurotransmitter receptors present at the NMJ. Recently, it was reported that postsynaptic Lrp4 and

MuSK act as coreceptors of agrin [48, 49]. Furthermore, Tid1 was identified as a downstream effector of agrin/Lrp4/MuSK-induced AChRs clustering and maintenance, possibly by modulating the organization of the subsynaptic cytoskeleton [50]. The pre- and postsynaptic specializations required for establishment and maintenance of NMJ synapse function are spatially aligned, at least in part, by these interactions.

Rapsyn, an effector protein of MuSK signaling, is also required for AChR clustering. Rapsyn physically links the AChRs to the UGC, and this interaction seems to be required for AChR stabilization and maturation of the NMJ (reviewed in [51]). Specifically, when agrin binds to the extracellular region of dystroglycan, it localizes to the NMJ, where it participates with other proteins to cluster the AChR via its intracellular association with rapsyn. While the UGC complex as a whole seems to be required for stabilizing AChR clusters, rather than being responsible for their initial formation, several components of the UGC, i.e., α -dystrobrevin and α -syntrophin, are also involved in other signaling events at the NMJ (discussed below).

A clear indication for an involvement of the UGC in NMJ function came from the observation that knockout mice of dystroglycan, utrophin, α 1-syntrophin, or α -dystrobrevin display reduced AChR densities at the NMJ [52–56]. These mice also have a reduction in the number of the junctional folds, a morphological defect that might possibly underlie the altered receptor density. The reduction of postsynaptic folds and reduced AChR density is further exacerbated in the double knockout *mdx;utrn* mouse which lacks both utrophin and dystrophin, indicating that dystrophin also plays an important role at the NMJ [40, 57, 58]. Most of these studies were done in mice older than 7 days of age at which stage both dystrophin and utrophin can be detected at the NMJ. Mice deficient in α -dystrobrevin show a 70% reduction in AChR density, and the remaining receptors are irregularly distributed in aggregates as the distinction between crests and troughs of junctional folds becomes less apparent [59, 60]. Importantly, the initial formation of the NMJs is normal in these mice, but their postnatal maturation is compromised, resulting in the described abnormalities.

Few electrophysiological studies of the DGC/UGC-deficient NMJs have been reported. However, *utrn* knockout mice exhibit decreased miniature endplate current amplitudes [54], consistent with the reduction in postsynaptic acetylcholine (ACh)-dependent neurotransmitter receptor clusters observed. Interestingly, miniature endplate potentials were also found to be decreased in *mdx* mice, while these mice display only subtle changes in junctional fold morphology and AChR density [61, 62]. The structural roles of the UGC and DGC in stabilizing NMJ receptor clusters and junctional folds may therefore be independent of their roles in regulating synaptic transmission.

1.1.2 DGC Participation in Signal Transduction Pathways at the Mammalian NMJ

While the role of the DGC in stabilizing the sarcolemma during repeated cycles of contraction and relaxation is well studied, much less is known about the involvement of the complex as a scaffold for signaling pathways. α -Dystrobrevin knockout mice were found to exhibit muscle degeneration in the absence of apparent structural deficits of the sarcolemma [59], suggesting that dystrobrevin may play roles in signaling. Since these mice also show a displacement of nNOS from the membrane, it was hypothesized that impaired nitric oxide (NO) signaling likely contributed to the observed dystrophy [59].

A second clear indication for possible roles of the DGC in signaling was suggested by the observed physical association of the DGC with numerous signaling molecules such as calmodulin, CaMKII,

protein kinase A (PKA), phosphoinositide 3-kinases (PI3K), grb2, son of sevenless, and Ras in addition to nNOS. Thirdly, since several DGC pathway members contain potential phosphorylation sites for serine/threonine or tyrosine kinases, DGC function and activation might be regulated by phosphorylation. Indeed, dystrophin is a target of CaMKII-, MAPK-, p34-cdc2 kinase-, and casein kinase-dependent phosphorylation in vivo (reviewed in [18]). In cell culture, phosphorylation of dystrophin has been shown to alter its affinity for actin and syntrophin [18].

Direct evidence for a functional role of tyrosine phosphorylation of DGC proteins has only been established for dystrobrevin. α -Dystrobrevin mutated at a presumptive tyrosine phosphorylation site failed to fully rescue the NMJ defects in the dystrobrevin knockout mouse [63]. Dystrobrevin is also a target of the serine/threonine kinase PKA [64], a protein implicated in synaptic plasticity, learning, and memory acquisition [65]. Thus, phosphorylation of dystrobrevin at several distinct sites likely modulates its activity during synaptic remodeling.

The DGC actively participates in signaling pathways that are associated with cell survival, cellular defense, and cell growth in a variety of tissues mediated by not only the aforementioned molecules calmodulin, PI3K, and nNOS but also by MAPK, protein kinase B (Akt), and the insulin receptor pathway (reviewed in [18]). Here, two examples of pathways relevant for DGC function in the nervous system will be discussed, i.e., DGC participation in nNOS signaling and in Ca^{2+} mediated homeostasis.

nNOS

NOS, which generates NO, plays roles in a number of cellular signaling pathways which, among others, include those involved in apoptosis, in the protection against ischemia, and in regulating vascular tone [66]. While these more general cellular functions are relevant for NOS involvement in the progression of DMD [67], there is also evidence for a more specialized role of NOS in modulating UGC function at the NMJ. NO also acts as both an antero- and retrograde modulator of synaptic transmission at central synapses [68]. While the role of NO in the cerebellum and hippocampus has been studied in great detail, little is known about its possible interactions with the DGC at these synapses. Indications that nNOS and the UGC/DGC play interdependent roles at the NMJ are supported by the following observations, (1) nNOS is physically associated with the UGC [69], (2) nNOS levels are substantially reduced and nNOS is dislocated from the postsynaptic membrane of DMD patients and in mdx and α -syntrophin mutant mice [67, 70] and (3) NMJ abnormalities in the mdx mouse (and to a lesser extent in α -syntrophin null mutant mice) can be fully rescued upon expression of a nNOS transgene in muscle [71]. However, no such rescue was observed in the α -syntrophin/ β 1-syntrophin double knockout mouse, indicating that syntrophins may be responsible for recruiting nNOS to the NMJ [71]. It has been recently established that dystrophin itself can also serve as a scaffold for nNOS sarcolemmal targeting [72]. How do alterations in NO levels result in changes in UGC function? Increased NO levels lead to the activation of guanylate cyclase and elevated production of cyclic guanosine monophosphate (cGMP) [73], which subsequently affects contractile function, glucose metabolism [74], and Ca^{2+} mobilization in muscle fibers [75]. nNOS also acts as an effector of agrin-induced postsynaptic differentiation at the NMJ (reviewed in [76, 77]). In brief, changes in NO and cGMP levels are sufficient to modulate the activity of a number of protein kinases that in turn modify the interaction of the UGC with actin, thereby affecting postsynaptic differentiation. The rod-like region of the dystrophin and utrophin proteins contain potential protein kinase G (PKG) phosphorylation sites,

while potential protein kinase C (PKC) phosphorylation sites are located near the actin-binding sites [78]. Depending on which site is phosphorylated, F-actin binding to dystrophin and utrophin is promoted (PKG) or inhibited (PKC). According to one proposed model [77], agrin-induced increases in NO/cGMP stimulate PKG and inhibit PKC, resulting in increased interaction of dystrophin and utrophin with actin which results in stabilization of AChR aggregates at the NMJ. Another target of NO, the Src family kinases, then phosphorylates the β -subunit of AChRs, which promotes their binding to rapsyn and association with other AChRs [51]. Can muscular degeneration in DMD patients be explained solely by a reduction in nNOS levels? This is unlikely, since NOS knockout mice do not display overt dystrophy [79]. However, since increased NO production results in enhanced utrophin expression [80] and treatment of mdx mice with a nNOS substrate, l-arginine, also increases utrophin levels and its membrane localization [81], clinical manipulation of nNOS levels in the muscle is considered a potential starting point for therapeutic intervention [82].

Ca²⁺ signaling and homeostasis

Ca²⁺ levels in dystrophic muscle are thought to be increased due to disruption of the sarcolemma and abnormal activation of Ca²⁺ leak channels [83]. It is not clear whether the changes in intracellular Ca²⁺ levels and disrupted Ca²⁺ homeostasis observed in dystrophin-deficient muscle are a cause or a result of the pathogenic processes, such as apoptosis and oxidative stress, that occur in dystrophic muscle [83, 84]. It is generally thought, however, that the elevated Ca²⁺ levels will eventually elicit Ca²⁺-dependent proteolysis via the activation of calpains and other Ca²⁺-dependent proteases. In this section, Ca²⁺ involvement in apoptosis and necrosis of dystrophic DMD fibers is not further discussed. Instead, potential roles for the DGC in the regulation of Ca²⁺ homeostasis in muscle and at the NMJ under normal physiological conditions are reviewed. A first insight into a role of the DGC in Ca²⁺-mediated signaling came from the findings that two of the DGC proteins, i.e., dystrophin and syntrophin, physically interact with calmodulin [85] (reviewed in [18]) and that calmodulin activity is reduced in dystrophin-deficient muscle fibers [86]. Calmodulin is a Ca²⁺-binding protein that regulates the activity of many Ca²⁺-sensitive enzymes and acts as a sensor of Ca²⁺ levels in the cell. Calmodulin is likely to regulate binding of dystrophin/utrophin to actin [87], while the Ca²⁺/calmodulin dependent kinase, CaMKII, actively phosphorylates both dystrophin and syntrophin [88]. Dystrophin's interaction with syntrophin is inhibited upon phosphorylation [88], further indicating the regulatory function that CaMKII exerts on DGC activity. In addition, it has been shown that both CaMKII and dystrophin are required for maintaining appropriate levels of neurotransmitter release at the NMJ in *Drosophila* [89, 90], although at present it is not clear whether they act in the same or parallel pathways. The DGC also regulates the activity of several Ca²⁺ channels. Changes in mechanosensitive Ca²⁺ channel function in mdx muscle fibers, measured by cell-attached patch-clamp recordings, revealed that the probability of these channels being open was increased, presumably leading to increased Ca²⁺ influx [91]. Changes in ACh neurotransmitter receptor aggregation caused by the disorganization of the membrane-associated cytoskeleton have been proposed to account for the disturbed Ca²⁺ homeostasis and increased Ca²⁺ leakage into dystrophin-deficient muscle [22]. Evidence supporting this hypothesis includes the observations that AChR and voltage-gated l-type Ca²⁺ channel aggregates display unusual physical interactions in dystrophic muscles, which may result in the abnormal Ca²⁺ influx observed at these sites [22, 92]. Since channel activity measurements vary significantly between different reports, possibly depending on the tissues used, i.e., cultured myotubes versus muscle fibers, or the type of channels studied, a defined role for the DGC in regulating Ca²⁺ channel activity is far from clear.

1.2 Dystrophin Regulates Cholinergic Transmission at the *C. elegans* NMJ

The highly conserved *C. elegans* dystrophin ortholog, *dys-1*, was first identified in 1998 [93]. In contrast to mammals, the worm usually has one ortholog for each of the six DGC gene families [33]. For example, it has a dystrophin gene, but no utrophin, DRP2, or sarcospan genes, single dystrobrevin and dystroglycan genes, and three sarcoglycan and two syntrophin orthologs (Table 1). Furthermore, the *C. elegans* dystrophin gene encodes only one protein isoform [94]. The reduced potential for functional redundancy has made *C. elegans* and other genetically tractable invertebrates, such as *Drosophila*, attractive models to study the individual roles of each DGC member.

The *dys-1* loss-of-function mutants display a characteristic behavioral phenotype: They are hyperactive, bend their head inappropriately when moving forward, and tend to hypercontract [93]. Muscle degeneration is observed only when the *dys-1* mutation is placed into sensitized backgrounds with reduced expression levels of the worm ortholog of the myogenic factor, MyoD, which is required for muscle differentiation, or reduced *egl-19* Ca²⁺ channel function [38, 95]. *dys-1* mutants, however, display hypersensitivity to ACh and exhibit reduced ACh-esterase activity, indicating a role for dystrophin in cholinergic synaptic transmission at the worm NMJ [93, 96]. The loss of other worm DGC members, i.e., dystrobrevin or syntrophin, also gives rise to increased cholinergic activity [97].

A subsequent report revealed that inactivation of *snf-6*, a gene encoding a ACh transporter in *C. elegans*, results in an almost identical hyperactivity phenotype to that seen in *dys-1* mutants [98]. Moreover, *snf-6* was found to bind to *stn-1*, the β 1-syntrophin ortholog expressed in the muscle, and *snf-6* expression is lost from the NMJ of both *dys-1* and *stn-1* mutants [98]. As observed for *dys-1*, the *snf-6* mutation in combination with a mutation of MyoD results in muscle degeneration. Thus, inefficient clearing of ACh from the synaptic cleft, due to both a reduction of esterase activity and the loss of the ACh transporter at the DGC mutant NMJ, likely accounts for the behavioral phenotypes observed. Furthermore, it may also contribute to the muscle degeneration observed in DGC sensitized mutant backgrounds. Whether altered cholinergic transmission underlies the dystrophic phenotype in DMD patients remains to be investigated, although increased sensitivity to ACh has been reported for human cultured myotubes derived from DMD patients [99].

1.3 Dystrophin Regulates Glutamatergic Transmission at the *Drosophila* NMJ

1.3.1 *Drosophila* Dystrophin Is Required to Maintain Muscle Integrity

Genomic analyses and expression studies indicate that the *Drosophila* dystrophin gene encodes three full-length (DLP1-3) and three short dystrophin isoforms (Dp186, Dp205, and Dp117) [32, 94, 100, 101]. DLP2, which is expressed in the musculature throughout development, is most closely related to the mammalian Dp427 isoform. Similarly to *C. elegans* *dys-1* mutants, but unlike mutations in the human Dp427 isoform, *Drosophila* DysDLP2 E6 mutants do not display obvious muscle degeneration. However, electrophysiological analyses revealed that neurotransmitter release is increased at the larval NMJ in these mutants [90].

The short *Drosophila* dystrophin isoform Dp117 is also expressed in the musculature [37]. Interestingly, muscle degeneration was observed in larvae, when all dystrophin isoform expression levels, or Dp117 specifically, were reduced in muscle by transgenic RNA interference [37]. In adult flies, progressive muscle degeneration and impaired climbing ability were observed when

dystrophin or dystroglycan expression was reduced [21]. The dystrophin isoform-specific mutant studies suggest that the *Drosophila* DLP2 protein, which accumulates at the postsynaptic side of the larval NMJ [90], is likely functionally analogous to utrophin at the mammalian NMJ and required for regulating synaptic transmission. The mechanisms by which Dp117 maintains muscle integrity are not yet clear as this isoform lacks an apparent actin-binding domain. Generation of classical Dp117 mutants and identification of Dp117 protein expression domains should shed further light on its roles.

Further evidence for a role for *Drosophila* dystrophin in muscle function comes from a study that described an age-dependent disruption of the myofibrillar organization of the myocardium in dystrophin mutants lacking the large isoforms, accompanied by reduced cardiac performance and lifespan [36]. Progressive impairment of locomotive ability, disrupted flight muscles, and reduced cardiac function and lifespan were also observed in flies lacking δ -sarcoglycan [34]. Furthermore, in larvae that express reduced levels of dystroglycan or lack POMT1 or POMT2, the protein O-mannosyltransferases required for dystroglycan glycosylation in vivo, abnormalities in muscle attachment, muscle contraction, and muscle membrane resistance are reported [35, 102]. In summary, the requirements for dystrophin, dystroglycan, POMT, and sarcoglycan in maintaining muscle integrity in flies illustrate the usefulness of this invertebrate as a model system for the study of muscular dystrophies.

1.3.2 Does a DGC Exist in *Drosophila*?

Although in general the orthologs of the mammalian DGC complex are highly conserved in *Drosophila* (Table 1), little is known about whether they form DGC-like complexes. No biochemical analyses of dystrophin-containing protein complexes derived from *Drosophila* tissue preparations or transfected cells have been reported to date. Perhaps the strongest evidence for the existence of a DGC-like protein complex comes from antibody colabeling experiments for multiple members of the DGC at the larval NMJ, the central nervous system (CNS), and the muscle. Dystroglycan and laminin colocalize in repeating stripes at sarcomeres [35], suggesting the existence of a T-tubule DGC-like complex, similar to that which is present in vertebrate cardiac muscle. Similarly, dystrophin and dystrobrevin are expressed in overlapping domains at the larval NMJ and in the CNS (S.P., L.G.F., J.N.N, unpublished), while dystrophin and dystroglycan have also been shown to colocalize at the postsynaptic side of the NMJ [103]. Genetic experiments have further indicated that at the NMJ, dystroglycan controls the synaptic localization of dystrophin and laminin [102, 103]. While direct biochemical data are currently lacking, together these data are consistent with the existence of DGC-like complexes at the *Drosophila* NMJ.

1.3.3 The Dystrophin Isoform DLP2 Modulates Neurotransmitter Release at the NMJ

Drosophila larvae that lack the large DLP2 dystrophin isoform exhibit elevated levels of evoked neurotransmitter release at the NMJ [90]. Electrophysiological analyses of DLP2 mutants showed that the resting muscle membrane potential in DysE6 DLP2 larvae and the depolarization of the muscle caused by spontaneous vesicle release from nerve terminals (miniature amplitude) are unaffected [90]. This suggests that the postsynaptic receptor field is not functionally altered in the mutant. An insight into dystrophin function at the NMJ was revealed by analysis of the effects of evoked stimulation. DLP2 mutants display significantly higher levels of muscle depolarization as

compared to control animals indicative of an increased release of presynaptic glutamate. As DLP2 is expressed and required in the muscle [90], this finding indicates that the absence of a postsynaptic dystrophin isoform results in changes in presynaptic function.

Possible explanations for the observed increased neurotransmitter release at the DLP2 mutant NMJ are (a) an increase in probability of release of vesicles or (b) an increase in the available vesicle pool. The vesicle pool was shown to be unaltered in DLP2 mutants, but the probability of release, as deduced from paired-pulse facilitation (PPF; **Textbox 2** in “Appendix”), was elevated. Increased numbers of presynaptic active zones with T-bars, specialized structures involved in vesicle docking and release, were also observed thus providing a likely structural correlate to the observed altered synaptic physiology [90]. Postsynaptic dystrophin may act as a scaffold for signaling molecules required for the retrograde control of neurotransmitter release from the presynaptic apparatus. The signaling pathways involved in the dystrophin-dependent increase in synaptic vesicle release remain, however, to be elucidated.

In the last decade, it has become clear that both anterograde, nerve to muscle, and retrograde, muscle to nerve, signals are required to achieve proper synaptic homeostasis (reviewed in [104]). Little is known about the molecular mechanisms required for retrograde control at the NMJ, but one pathway identified as playing roles in this process is the bone morphogenetic protein (BMP) pathway (reviewed in [105]). The secreted BMP pathway member, glass bottom boat, is believed to mediate a signal emanating from the muscle by binding to the presynaptic Wishful Thinking receptor. Interestingly, a genetic interaction was observed with DLP2 and Wishful Thinking at the NMJ, suggesting that dystrophin functions in a BMP-dependent pathway to control homeostatic plasticity [90]. Dystrophin also interacts with BMP signaling pathways in the *Drosophila* wing where their interplay is required for proper vein formation [106].

What roles do other members of the putative *Drosophila* DGC play at the synapse? Thus far, only dystroglycan has been examined. Intriguingly, the lack of dystroglycan or the enzyme required for its glycosylation, dPOMT1, both cause a phenotype that is opposite to the DLP2 dystrophin phenotype. Loss of postsynaptic dystroglycan or POMT leads to a decrease in the number of transmitter quanta released from the presynaptic terminal and a concomitant decrease in the efficacy of synaptic transmission [102, 103]. The probability of release is decreased in these mutants, while the number of release sites is not altered. In addition, while the overall morphology of the NMJ is intact, the ratio of glutamate receptor subunits is altered in dystroglycan and POMT mutants because the levels of the DGluRIIB subunit are reduced.

In *Drosophila*, the best studied postsynaptic glutamate receptors are GluRIIA and GluRIIB (reviewed in [107]). The subunit composition of the postsynaptic glutamate receptors determines their function and trafficking. Furthermore, the regulation of glutamate receptor subunit ratios may underlie aspects of synaptic homeostasis and plasticity. How these changes would result in alterations in presynaptic release is not known. Nevertheless, changes in postsynaptic subunit composition are also important for the maturation of mouse cholinergic endplates and glutamatergic central synapses [108].

How might the seemingly differential roles of dystrophin and dystroglycan at the *Drosophila* NMJ be explained? The single mutant analyses have clearly indicated that each of the two proteins function at the neuromuscular synapse, but, surprisingly, in distinct and even opposite ways. Since the phenotypes of dystrophin/dystroglycan double mutant flies have not yet been reported, the epistatic relationship between dystroglycan and dystrophin at the NMJ remains unclear. Dystrophin

likely modulates a retrograde signal that limits transmitter release by, at least in part, inhibiting the formation of T-bars. Dystroglycan likely acts via a different mechanism since no alterations in T-bar numbers were observed in the dystroglycan mutant [103]. It is also not yet clear whether the change in glutamate receptor composition observed in dystroglycan mutants reflects a direct role of dystroglycan in receptor clustering, as has been shown for the vertebrate dystroglycan protein in AChR clustering [109]. Further studies are required in order to understand these dramatically different roles of dystrophin and dystroglycan at the NMJ.

The increased synaptic efficacy at the *Drosophila* DLP2-deficient NMJ is reminiscent of the elevated levels of cholinergic neurotransmission observed in *C. elegans* dys-1 mutants. However, as discussed above, the increased depolarization observed in mutant worm muscle is most likely due to the delocalization of the Snf-6 ACh transporter which results in the inappropriate persistence of ACh in the synaptic cleft. A similar role of a *Drosophila* dystrophin-interacting glutamate transporter at the NMJ cannot be ruled out.

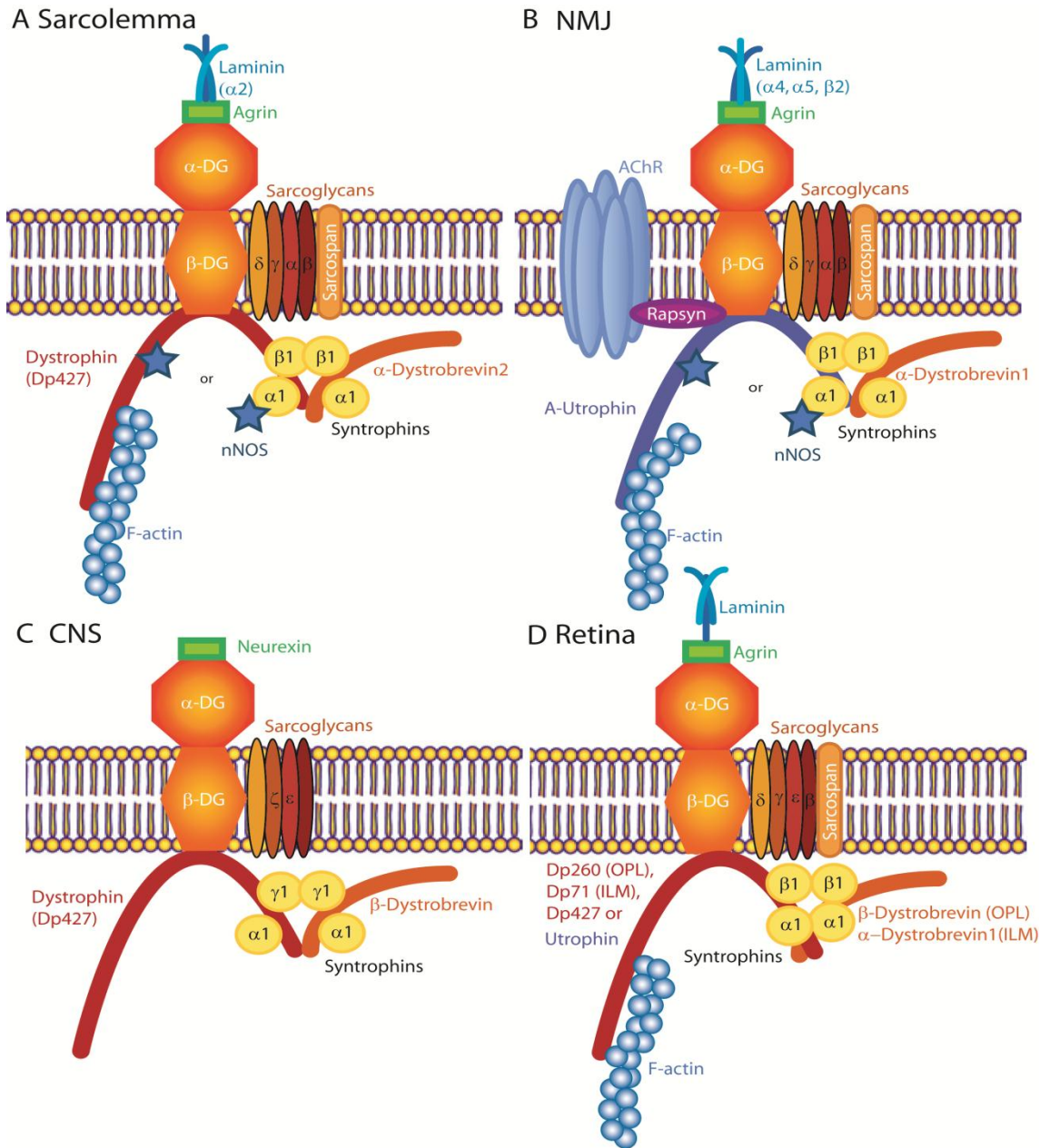


Figure. 1 Differential DGC complex composition at mammalian muscle, NMJ, CNS, and retina. The compositions of the different DGC/UGC complexes that have been reported [1, 7, 10, 14–16, 25, 32, 39, 182] to be present in vertebrate muscle (a), at the NMJ (b), in brain (c), and at the retina (d) are shown. a In skeletal muscle, the N-terminal actin-binding domain and specific spectrin repeats in the central rod of dystrophin bind to costameric F-actin. The cysteine-rich region and the C terminus of dystrophin establish the link with the DGC through binding to β -dystroglycan [183] and α -dystrobrevin2, respectively [184, 185]. β -Dystroglycan is linked to the extracellular α -dystroglycan, which, depending on the tissue, links laminin α 2 along the sarcolemma. In addition, β -dystroglycan associates with δ -sarcoglycan by which the sarcoglycan–sarcospan complex is stabilized at the sarcolemma [186]. The DGC can bind four syntrophins (α 1 and/or β 1), which in turn recruit sodium channels and signaling molecules, such as nNOS, via their PDZ domains. nNOS can also associate with dystrophin directly via its spectrin-like repeats [72]. b At the NMJ, a similar complex, the UGC, is formed, in which dystrophin can be replaced by A-utrophin and α -dystrobrevin1 replaces α -dystrobrevin2 [185, 187]. After postnatal day 7, dystrophin can be detected at the rat NMJ, and therefore, a DGC can also be formed. These proteins bind α -syntrophin, which is present at crests and troughs of the junctional folds. β 2-Syntrophin is mainly localized to the troughs. α -Dystroglycan not only binds the laminins β 2, α 4,

and $\alpha 5$ but also the proteoglycans agrin and perlecan. Perlecan is necessary for synaptic localization of acetylcholine esterase, which is involved in termination of cholinergic neurotransmission [188]. The synaptic localization of β -dystroglycan is dependent on the presence of rapsyn [189, 190], which is important for stabilization of AChR clusters and linkage of these clusters to the UGC at the crests of the junctional folds (reviewed in [51]). β -Dystroglycan binds to utrophin. c In the brain, the DGC consists of at least four main components, dystrophin or utrophin, dystroglycan, dystrobrevin, and syntrophin, with variations in the isoforms present depending on the cell type. The composition of the sarcoglycan–sarcospan complex is not known for this tissue, but δ - and ϵ -sarcoglycan are detected in brain samples. The neuronal DGC consists of a full-length dystrophin isoform (Dp427) bound to β -dystrobrevin and dystroglycan, which in turn associates with presynaptic neuexin- α . The complex also contains $\alpha 1$ - and $\gamma 1$ -syntrophin. d In the retina (reviewed in [155]), different dystrophin isoforms are expressed; Dp427, Dp260, Dp140, and Dp 71. Dp260 is highly specific in the photoreceptor terminals of retina [119]. Utrophin is present in the end-feet fraction of Müller glial cells [166]. Dp71 and/or utrophin and α -dystroglycan from Müller cells bind actin and laminin, respectively [191]. The sarcoglycan complex which is composed of δ -, γ - [191], β -, and ϵ -sarcoglycan, and sarcospan [182] is bound to β -dystroglycan and dystrobrevin. β -Dystrobrevin is expressed at the outer plexiform layer (OPL) of the retina, while α -dystrobrevin1 is located at the inner limiting membrane (ILM) [134]. Syntrophins, $\alpha 1$ and $\beta 1$, interact with the carboxy-terminus of dystrophin [192].

2. Roles of the DGC at Central Synapses

We now focus on the roles of dystrophin in the CNS. The first section describes what has been learned about dystrophin function in cognitive studies and behavioral assays which reveal DGC roles in long-term spatial and nonspatial memory consolidation and emotion, respectively. The following section discusses new mechanistic insights into requirement for dystrophin in receptor clustering and synaptic plasticity in the mammalian hippocampus and cerebellum gained, in part, through electrophysiological analyses. The subject of the last section is the recently revealed role of dystrophin in regulating neurotransmitter release at a defined cholinergic central synapse in *Drosophila*.

Reports that describe dystrophins' involvement in metabolic pathways in the nervous system, such as the altered glucose metabolism in the brains of DMD patients and *mdx* mice, and those that examine the defects in the establishment of the blood–brain barrier in these mice fall outside the scope of this review. These subjects are reviewed in [5, 25].

2.1 Roles for Dystrophin in Behavior

2.1.1 DMD Is Associated with Mental Retardation

Cognitive impairments occur in approximately one third of Duchenne patients, highlighting a role for dystrophin in the nervous system (reviewed in [5, 7, 25]). The average IQ of boys diagnosed with DMD is 85, while 30% have an IQ less than 70 (reviewed in [5]). These mental deficits appear to be nonprogressive and unrelated to the severity of the muscular dystrophy. Their causes remain unknown. Moreover, morphological abnormalities in the brains of postmortem DMD patient are highly variable, ranging from no abnormalities [110–112] to slight and severe abnormalities, such as partial cerebral atrophy, neuronal loss and gliosis, and abnormal arborization and dendritic branching of cortical pyramidal neurons [111, 113–115].

There are various neuropsychiatric complications associated with DMD, such as autism, attention deficit hyperactivity disorder, obsessive–compulsive disorder, epilepsy, and febrile convulsion [116]. Mutations in the human fukutin-related protein gene and POMT1/2 genes, which affect the glycosylation of α -dystroglycan, also result in syndromes associated with severe mental retardation

(Fukuyama congenital muscular dystrophy and Walker–Warburg syndrome), further indicating roles for the DGC in the development of circuitry required for cognition [26]. Lastly, mutations in ϵ -sarcoglycan are implicated in myoclonus–dystonia syndrome, a disease characterized by dystonia and psychiatric complications, including anxiety and obsessive–compulsive disorder [117].

The brain-specific DGC(s) consists predominantly of four components, dystrophin (or utrophin), dystroglycan, dystrobrevin, and syntrophin (**Fig. 1**), with variations in the isoforms present, depending on the specific cell type being examined. Some neuronal cell types also express the ϵ - and ζ -sarcoglycans, but sarcoglycans are more predominantly expressed in muscle. Dystrophin isoforms are differentially expressed in subsets of neurons and glia: Dp427 is present in the cerebral cortex, the limbic system, including the areas CA1-CA3 of the hippocampus, the basolateral nucleus, and the lateral nucleus of amygdala, and cerebellar Purkinje cells [118]. Dp260 is present in the retina [119], Dp140 in the microvasculature and throughout the brain [120], Dp116 in Schwann cells of the peripheral nervous system [121], while Dp71 is highly abundant in most, if not all, brain areas examined [122]. It is not evident that the lack of one particular dystrophin isoform is solely responsible for the neurological disorders associated with DMD, but mutations in the 3' end of the gene affecting all isoforms are most frequently linked to lower IQ scores [123]. Furthermore, most DMD patients, who exhibit neurological complications, have a genetic deficiency or duplication distal to exon 44 which most likely alters expression of Dp140, Dp116, and/or Dp71 [116]. Expression of the Dp140 isoform, whose promoter and first exon lie in the large intron between exons 44 and 45 [124], is likely to be affected by these deletions [125, 126].

2.1.2 Requirements for the DGC in Memory Acquisition and in Defense and Motor Behavior

Conflicting reports exist concerning possible roles for dystrophin in learning and memory that have been established using mouse models. The discrepancies are likely the result of the use of different behavioral paradigms that test distinct learning tasks of dystrophin-deficient mice [127–129]. In addition, it has become apparent that results obtained from tests employing the mdx mouse (which only lacks full-length dystrophin isoform Dp427) likely differ from those using the mdx3cv mouse (which lacks all dystrophin isoforms). Together, the results suggest that the full-length isoform is essential for the function of dystrophin in cognition and memory involving hippocampal and forebrain function (reviewed in [25]). The specific roles for the smaller isoforms in cognition are not yet clearly defined. Since mdx mice do not exhibit any significant signs of muscular dystrophy during the first 6 months of postnatal life, possibly due to sufficient regeneration of muscle, behavior of mdx mice can be studied in the absence of extensive muscle degeneration.

Recently, Vaillend and colleagues addressed memory impairment in mdx mice using spatially and nonspatially defined tasks that can be rapidly learned [130]. The use of these behavioral paradigms reduces the possibility that extended training procedures would inadvertently improve memory function and mask memory deficits [131, 132]. The tests were based on (1) object recognition and (2) spatial learning in a water maze. Naturally occurring novelty-seeking behavior was not altered in the mdx mouse confronted with new objects. Both mdx and control mice spent longer times exploring a novel object when it was copresented with a familiar object to which they had been exposed 10 min prior. However, when the time between exposure and the test was prolonged to 24 h, the mdx group would explore both objects equally, whereas the control group would still prefer a novel object. This observation is consistent with the mdx mice exhibiting long-term recognition memory impairments. Similar results were obtained when mice were trained to find a submerged

platform in a water bath. Mice of the control group would still be able to efficiently locate the platform 24 h after training, as measured by the time spent in the target area, whereas the mdx mice would not show a preference for the area where the platform was located. Thus, apparently, consolidation or expression of long-term memory is impaired in mdx mice [130].

The lack of the large dystrophin isoforms in mdx mice also impacts on emotional behavior and fear memory, which reflects amygdala transmission. Mdx mice have an increased defensive freezing response to restraint compared to control mice [133]. This behavior can be partially rescued by oligonucleotide-mediated exon skipping that facilitates expression of a truncated dystrophin in the brain, not in the muscle [133]. These data support the hypothesis that dystrophin is required for wild-type emotional behavior. Behavioral tests have also been performed on double knockout α - and β -dystrobrevin mice [134]. These mice exhibit abnormal sensorimotor behaviors that reflect cerebellar dysfunction, while the single dystrobrevin knockout mice apparently behave normally.

In the next two sections, electrophysiological studies that examine the roles of the different DGC members at mammalian central synapses are discussed. Furthermore, studies are reviewed that indicate that a number of the DGC members (dystrophin, dystrobrevin, syntrophin, and dystroglycan) are present at the postsynaptic side of mammalian inhibitory GABAergic synapses in the hippocampus and in cerebellar Purkinje cells [135], where they are required for synapse function and plasticity. Lack of expression of dystrophin at these sites might contribute to the behavioral defects observed in mdx mice and the mental retardation displayed by DMD patients.

2.2 Dystrophin Function in Receptor Clustering and Synaptic Plasticity of Mammalian Hippocampal and Cerebellar GABAergic Synapses

In the mammalian brain, full-length dystrophin, Dp427 is expressed at the postsynaptic membranes of hippocampal pyramidal neurons, neocortical pyramidal neurons [136], amygdala, and cerebellar Purkinje cells [133] where it colocalizes with inhibitory GABA_A receptor clusters [133, 137]. No gross changes in morphology or increased apoptosis were apparent in the mdx mouse hippocampal CA1 area [138]. However, in other areas of the mdx brain, for example in the spinal trigeminal nucleus, the number of neurons is almost 50% decreased compared to wild-type mice [139]. Interestingly, in these same mice, α 1- and α 2-GABA_A clusters are significantly reduced in number [133, 137, 140]. Dystrophin colocalizes with other DGC proteins such as dystrobrevin, the syntrophins, and β -dystroglycan to inhibitory GABAergic synapses [25, 141]. Furthermore, in dystroglycan conditional knockout mice, these synapses no longer contain dystrophin [142]. α - and β -Dystrobrevin double knockout mice have lost dystrophin, and mdx mice have lost dystrobrevin localization from the cerebellar GABA_A clusters [134] and exhibit a decrease in the size and numbers of α 1 subunit GABA_A receptor clusters. Together, these data suggest that the DGC proteins are, at least partially, interdependent for their targeting to GABAergic synapses and that the DGC likely stabilizes GABA_A receptor clusters at these synapses.

There is ample evidence that the DGC members also modulate synapse function at inhibitory GABAergic synapses in the cerebellum [140], the amygdala [133], and the hippocampus (reviewed in [25]). In the mdx cerebellar Purkinje cells, there are significant reductions in both the frequency and amplitude of spontaneous inhibitory postsynaptic currents (mIPSP; Textbox 2 in “Appendix”), consistent with the reduction in the number and size of GABA_A receptor clusters at postsynaptic densities [140]. A reduction in inhibitory input in these cells was also recorded when GABAergic signaling was blocked resulting in a smaller increase in the amplitude of evoked postsynaptic

potentials (EPSPs; Textbox 2 in “Appendix”), compared to wild-type controls [143]. Furthermore, decreased long-term depression (LTD; Textbox 2 in “Appendix”) was observed in mdx Purkinje cells, which possibly leads to a disruption of cerebellar long-term plasticity [144]. These results may, at least in part, explain some of the behavioral problems and cognitive impairments reported in mdx mice and DMD patients [140].

In the hippocampus, loss of Dp427 in mdx mice does not affect inhibitory postsynaptic currents (IPSCs; Textbox 2 in “Appendix”) evoked in the pyramidal cell layer [145]. However, a lack of facilitation was shown by PPF analysis, which suggests that the probability of inhibitory synaptic release is higher in mdx mice compared to control mice at this particular synapse. This effect correlates with an observed increased mIPSC frequency, contrary to what is observed at the cerebellar Purkinje cell synapses discussed above [145]. Several studies by Vaillend and colleagues [129, 130, 146] demonstrated, again in mdx mice, an increase in neuronal facilitation, a sustained effect leading to enhancement of the maintenance phase of hippocampal long-term potentiation (LTP; Textbox 2 in “Appendix”), as well as an enhancement of short-term potentiation (STP; Textbox 2 in “Appendix”) and depression (Textbox 2 in “Appendix”). The enhanced STP and STD were prevented when the GABA_A receptor antagonist, bicuculline, was applied [130, 146]. Collectively, these data point toward the dystrophin Dp427 deficiency being associated with a sustained increase in synaptic efficacy and excitability of CA1 hippocampal neurons. Moreover, dystroglycan conditional knockout mice that lack dystroglycan in brain also exhibit deficits in hippocampal LTP [147].

The above-mentioned studies, while showing different electrical responses to dystrophin deficiency in the cerebellum versus the hippocampus, suggest that the alteration of inhibitory synaptic transmission may contribute to memory deficits evident in mdx mice. Similar memory impairments correlating with enhanced synaptic efficacy and LTP have been observed in other mouse models, including a model for Alzheimer’s disease, and mice with disrupted genes encoding PSD-95 or a subunit of the ionotropic glutamate receptor [148–151].

Finally, the effects of the loss of the Dp71 isoform on mouse brain function employing electrophysiology and behavioral studies were recently reported [152]. Interestingly, glutamatergic transmission is enhanced and synaptic plasticity reduced in Dp71-deficient CA1 hippocampal neurons. Furthermore, Dp71 knockout mice display reduced exploratory and novelty seeking behavior, mild retention deficits in inhibitory avoidance, and impairments in spatial learning and memory acquisition [152]. Together, the studies described above highlight the different roles that distinct dystrophin isoforms play at central synapses.

2.3 *Drosophila* Dystrophin Modulates Neurotransmitter Release of Cholinergic Central Synapses

Similarly to its mammalian counterpart, the *Drosophila* dystrophin gene also encodes several short isoforms. One of these, Dp186, is expressed predominantly, if not exclusively, in the CNS [32]. Dp186 has been localized, in particular, to cholinergic synapses between interneurons and motoneurons. Whole-cell voltage clamp recordings from identified motoneurons, in the larval CNS, show that excitatory synaptic currents are significantly increased in the absence of Dp186 compared to those measured in controls [153]. Recordings were also performed in the presence of tetrodotoxin, which blocks evoked transmitter release, allowing measurement of only spontaneous miniature synaptic currents. The amplitudes of these miniature synaptic currents were not altered in

the mutants, indicating that the postsynaptic AChR field was not affected by the absence of Dp186. However, although amplitude was normal, the frequency of the miniature synaptic currents was significantly increased in the mutants compared to wild types. Together, these data indicate that lack of Dp186 in the CNS results in an increased probability of presynaptic (ACh) neurotransmitter release. This mutant phenotype could be fully rescued only when a wild-type Dp186 transgene was expressed postsynaptically, but not presynaptically, demonstrating that Dp186 exerts a role consistent with retrograde signaling at interneuronal CNS synapses. Based on similarity of effect, this role likely is similar to that of the large *Drosophila* DLP2 isoform at the NMJ [90].

3 Roles of the DGC in the Retina

One of the earliest described nervous system-related clinical features of DMD patients affects the visual system [154, 155]. While DMD patients have generally normal vision, they do exhibit altered responses to certain light/dark stimuli as measured by electroretinography. Here, recent findings are reviewed that shed light onto DGC function at retinal synapses gained from the use of electrophysiological approaches in animal models.

3.1 The DGC Is Required for Correct Electrical Activity and Formation of the Mammalian Retina

Several dystrophin isoforms are differentially expressed in the murine retina [6, 156]. Based on a number of studies, in which different dystrophin isoforms have been localized to specific regions within the retina, it is proposed that each isoform likely contributes a unique function [157]. For example, Dp427 and Dp260 are located in the outer plexiform layer (OPL), where photoreceptors form synapses with horizontal and bipolar cells. Dp71 is detected in the inner limiting membrane, the Müller glia, and the perivascular astrocytes, while Dp140 is also present at perivascular astrocytes [158, 159]. A special type of synapse at the OPL, called the ribbon synapse, connects axons of photoreceptor cells to bipolar cell dendrites. Visual information is transmitted from the photoreceptor cell to the ganglia cell via the bipolar cells. At the ribbon synapse, Dp260, Dp140, and Dp71, as well as β -dystroglycan, are expressed presynaptically, in contrast to the postsynaptic expression of DGC members in other parts of the brain and NMJ [51, 160–163].

As indicated above, general eye exams of DMD patients indicate normal visual abilities and no gross abnormalities nor evidence of night blindness. However, DMD patients present with abnormal electroretinograms (ERG; [155]; Textbox 3 in “Appendix”). When measured in a dark-adapted (scotopic) retina, the ERG shows a reduced amplitude of the b-wave response in the majority of DMD patients. Further analyses of these ERG waveforms suggest that aberrant dystrophin expression impairs synaptic transmission specifically between the rod and cone photoreceptor cells and their postsynaptic targets, the bipolar ON cells in the OPL [164]. Consistent with these findings, dystrophin expression is observed at these synapses.

Although *mdx* mice, lacking Dp427, display normal ERG [154], *mdx*^{CV3} mice, lacking all dystrophin isoforms, show a decreased b-wave response [165]. Mice that only lack Dp71 [166] have normal ERGs with no significant changes of the b-wave amplitude and kinetics [167]. Based on these results and the above-mentioned expression analyses, the Dp260 isoform is thought to be the isoform likely required for proper b-wave formation and timing. Collaborating evidence for this hypothesis has been obtained from clinical studies of DMD patients. A subset of DMD patients with deletions downstream of exon 30, affecting the splicing and transcription of Dp260, exhibit a red–

green color vision defect, while DMD patients, who have dystrophin mutations upstream of exon 30 (solely affecting Dp427) have seemingly normal color vision. Thus, this color vision defect may be caused by a loss of the dystrophin isoform Dp260 [159, 168].

Little is known about the involvement of other DGC members in retinal function. While β -dystrobrevin colocalizes with dystrophin and β -dystroglycan at photoreceptor cell termini in the OPL, dystrobrevin null mutant mice display normal ERGs [134]. The localization of dystrophin in the retina is, moreover, not dependent on dystrobrevin expression, unlike their interdependence at inhibitory cerebellar Purkinje synapses. Interestingly, a novel dystroglycan ligand, called pikachurin, has been recently identified which presynaptically colocalizes with dystrophin and dystroglycan at the photoreceptor ribbon synapse [169]. Pikachurin null mice have improper apposition of bipolar dendritic tips to the photoreceptor ribbon synapse, leading to prolonged retinal synaptic transmission from photoreceptors to bipolars. Pikachurin is therefore important for both the development and function of the ribbon synapse; synapses are formed in Pikachurin null mutants, but the subsequent invagination of the photoreceptor axon surrounding the dendrites of bipolar cells is abnormal, affecting the physiology of visual perception [169]. These animal studies might further our understanding of the exact nature of the defects in vision of DMD patients.

3.2 *Drosophila* DGC Is Required for Axon Guidance of Photoreceptor Neurons

Studies of the *Drosophila* optic system recently demonstrated that dystroglycan and dystrophin are required during axon pathfinding of both photoreceptor neurons and their supporting glial cells present in the lamina plexus of the larval brain [21]. Lack of either dystroglycan or dystrophin results in similarly aberrant axon projection patterns: clumping photoreceptor axons at the lamina that are irregularly distributed at the termination zone of the lamina plexus. These abnormalities were also observed when expression of dystroglycan or different isoforms of dystrophin (all isoforms, DLP1-3, or the smaller isoforms only) were reduced by RNA interference either in the photoreceptor axons or the glia, suggesting that both cell types are required for wild-type axon patterning. The observed axon pathfinding phenotypes are reminiscent of phenotypes observed in flies deficient for genes encoding the adaptor protein dock [170] and the insulin receptor (InR) [171]. Genetic interaction studies showed that dystroglycan indeed strongly interacts with dock and InR, whereas dystrophin does not. From these observations, a model was proposed in which dystroglycan may selectively interact with either dystrophin or InR and dock, thereby modulating the cytoskeletal rearrangements in the photoreceptor neurons required for appropriate axonal projections in the retina [21].

Concluding Remarks and Future Perspectives

Major advances have been made in the clinical assessment of the cognitive and neuropsychiatric impairments of DMD patients and toward the development of animal models to investigate DGC function. However, despite these advances, our understanding of the roles of the complex remains incomplete. A primary challenge ahead for elucidating DGC function in the nervous system is to comprehend how the absence of the complex leads to the observed defects in synaptic plasticity, behavioral abnormalities, and visual impairments. At the mechanistic level, it is clear that both at the NMJ and at central synapses, the DGC is required for clustering of major neurotransmitter receptors, such as the AChRs at the NMJ and inhibitory GABAA receptor clusters in the

hippocampus, the cerebellum, and the amygdala. For a number of these synapses, it has been established that the absence of dystrophin results in aberrant synapse maturation and neurotransmission. Interestingly, DGC/UGC function is apparently not neurotransmitter specific, since the complex is required for appropriate regulation of glutamatergic (*Drosophila* NMJ and mammalian brain), GABAergic (mammalian brain), and cholinergic (the mammalian and *C. elegans* NMJ and the *Drosophila* central synapse) transmission. Furthermore, it functions at inhibitory (GABAergic) and excitatory (glutamatergic and cholinergic) synapses.

The definition of the precise synaptic roles of the DGC is complicated by the findings that the effects of DGC deficiency clearly differ depending on the particular synapse studied. In the DGC mutant hippocampus, no gross structural changes in neuronal fate or axon outgrowth were observed. Clear abnormalities in synaptic plasticity and LTP, correlating with alterations in receptor clustering and enhanced synaptic transmission, were, however, evident. In mdx cerebellar Purkinje cells, a reduction in the number of postsynaptically localized GABAA clusters has been associated with a decreased amplitude and frequency of spontaneous inhibitory postsynaptic currents and reduction in LTD. These defects may underlie the cognitive impairments these mice exhibit in learning and memory behavioral paradigms. In the coming years, it will hopefully be feasible to directly correlate the mechanistic defects in mdx mice GABAA receptor function in the hippocampus and the cerebellum with the observed abnormalities in synaptic plasticity and behavior.

While studies on DGC function in the brain and at the NMJ point toward a mainly postsynaptic role, the complex accumulates at the presynaptic side of the ribbon synapse in the visual system. Here, it is required not only for correct (light-induced) synaptic transmission but also for the structural development of this synapse. In contrast, a role in the stabilization and maturation of the postsynaptically localized neurotransmitter receptor clusters and synaptic folds is evident at the mammalian NMJ, but early morphological development of the NMJ seems to be unaffected in DGC mutants.

Invertebrate organisms, particularly the worm and the fruit fly, have also proven useful for investigating DGC function at the synapse. The fruit fly larval NMJ and central synapses are highly amenable to electrophysiological analysis and specific roles for individual dystrophin isoforms have been identified. Thus, it has been shown that the large dystrophin isoform DLP2 at the glutamatergic NMJ and the smaller Dp186 isoform at cholinergic central synapses, both postsynaptically localized, are required for wild-type levels of presynaptic neurotransmitter release. At the NMJ, at least, this function likely requires BMP signal transduction. Future studies using these invertebrate models, where electrophysiology can be combined with genetic manipulation of single identified cells, should continue to reveal the molecules with which dystrophin interacts at the synapse. Invertebrate animals might also be employed for the large scale screening of potentially therapeutically useful compounds that target DGC function.

Mammalian dystrophin isoforms may also play roles in the regulation of presynaptic neurotransmitter release as has been observed in *Drosophila*. First, the lack of the Dp71 isoform in the mouse hippocampus leads to alterations in PPF suggesting an increase in presynaptic glutamate release. Secondly, increased mIPSC frequency and reduced PPF of the eIPSCs are observed in the Dp427-deficient hippocampus also indicating increased probability of release. However, the mechanisms underlying these effects on transmitter release remain unknown.

While *Drosophila* models of dystrophin dysfunction are being increasingly employed, it is currently difficult to precisely determine which of the various fly and mammalian isoforms are functional

orthologs. This is for two main reasons: (1) the primary amino acid sequences of the isoform-specific amino-termini differ significantly between *Drosophila* and mammals (Textbox 1 in “Appendix”) and there are no conserved motifs evident and (b) mutants of many of the small isoforms in both species have yet to be examined; thus, their functions are unknown. It remains possible, however, that apparently divergent dystrophin amino-termini play analogous roles in different species. Even if certain aspects of dystrophin function prove, however, to be different between species, studying its roles at various types of synapses in multiple species should help to reveal the biological strategies that have evolved to wire a complex nervous system. This information will aid in the development of novel therapeutic interventions for neurological disorders.

Acknowledgments

This work was supported by grants from the “Nederlandse Organisatie voor Wetenschappelijk Onderzoek, N. W. O.” (J. N. N. and L. G. F.) and by the Wellcome Trust and BBSRC (R. A. B.). We acknowledge Drs. J. T. den Dunnen, J. J. Plomp, A. M. Aartsma-Rus, and J. J. G. M. Verschuuren for providing insightful suggestions on this manuscript.

Appendix

Textbox 1: Dystrophin Isoforms in Mammals, *Drosophila*, and Worms and Their Mutants

Mammals

The human/mouse *DMD* gene has seven promoters driving the expression of mRNAs encoding the isoforms, Dp427 (B), Dp427 (M), Dp427 (P), Dp260, Dp140, Dp116, and Dp71. Three promoters drive expression of the brain- (B), muscle- (M), and Purkinje cell (P)-specific large Dp427 isoforms which differ in their 5' untranslated and first codon exons which are spliced to a common exon 2. All three large isoforms bear an actin-binding domain, four hinge domains interrupted by 24 spectrin-like repeats that form triple-helical coiled-coils (CC) and a C-terminal region that contains domains involved in protein-protein interactions, including a WW domain, a ZZ domain within a cysteine-rich region and a CC domain near the carboxy-terminus. The smaller isoforms have the common carboxy-terminal region, no apparent actin-binding domains, and a variable number of spectrin-like repeats (Dp260 has 15, Dp140 has 5, Dp116 has 2, and Dp71 has none). Unlike *Drosophila* and *C. elegans*, mammals also have an *utrophin* gene whose encoded protein is highly similar to dystrophin with respect to sequence and domain structure, particularly in the carboxy-terminal domain.

Drosophila

Drosophila's *dystrophin* gene contains six identified promoters driving the expression of the following isoforms: DLP1, DLP2, DLP3, Dp186, Dp205, and Dp117. Similar to the large mammalian isoforms, *Drosophila* DLP1, 2, 3 differ only in their untranslated and first coding exons, but all contain the other conserved protein domain domains described for the mammalian Dp427 isoforms, including 22 spectrin-like repeats [101]. The smaller fly isoforms do not contain conserved actin-binding domains, but Dp186, Dp205, and Dp117 contain unique amino-terminal domains conserved with other *Drosophila* species and the carboxy-terminal domains including WW, ZZ, and CC domains that are present in the mammalian dystrophin protein isoforms. Dp186, Dp205, and Dp117 contain 4, 2 and no spectrin-like repeats, respectively [101].

C. elegans

C. elegans' dystrophin gene, *dys1*, encodes a single large isoform which bears an actin-binding domain, seven spectrin-like repeats, and the conserved carboxy-terminal domain containing the WW, ZZ, and CC domains.

Dystrophin Isoforms Affected in Human DMD Patients and Model Organisms

Approximately two thirds of all DMD patients have large deletions in the *dystrophin* gene, mostly located at two hot spots that result in the truncation of all protein isoforms and the loss of their common C-terminal domains. The remainder of DMD patients has small deletions or point mutations that introduce stop codons and other mutations that lead to (partial or full) loss of dystrophin protein isoform production or decreased protein stability. Mouse studies discussed in this review employ three different dystrophin-deficient mouse strains, *mdx* (lacking only the large Dp427 isoforms), *mdx*^{3Cv} (lacking all isoforms), and *Dp71*-specific knockout mice. An additional mouse model, *mdx*⁵², lacking the Dp427, Dp260, and Dp140 isoforms has been generated, but no nervous system phenotypes have been reported. The various mammalian models of DMD have been recently reviewed [172].

The *Drosophila* mutants described here include mutants lacking only the large DLP2 or CNS-specific Dp186 isoforms or with Dp117 or all dystrophin isoform expression levels reduced by transgenic RNA interference. The *C. elegans dys1* mutant lacks the sole dystrophin isoform. Dystrophin isoform expression domains are presented in Table 2.

Textbox 2: Electrophysiological Terminology

EPSP is a transient depolarization of the postsynaptic membrane caused by the flow of positively charged ions (usually Na^+) into the postsynaptic cell as a result of the opening of ligand-gated channels. Depolarization increases the chance of the occurrence of an action potential as the membrane is pushed toward a threshold for the opening of voltage-gated cation channels (Na^+ and/or Ca^{2+}). In contrast, IPSPs (or IPSCs) are mediated by ligand-gated anion channels (Cl^-) and their activation hyperpolarizes the membrane, decreasing the chance of firing action potentials. The spontaneous release of single neurotransmitter quanta from the presynaptic excitatory neuron generates mEPSPs, while the mIPSPs (or mIPSCs) are generated by the release of single quanta at inhibitory synapses [173].

PPF can be described as a short-term form of synaptic plasticity. PPF is the short-lasting (<1 s) increase in postsynaptic potential evoked by a second impulse. It results in an increase in the probability of transmitter release by residual Ca^{2+} only when the probability of synaptic vesicle release is low [174].

Another physiological feature in the hippocampus is LTP, first described by Bliss and Lomo [175]. LTP has been defined as a long-lasting increase in synaptic efficacy, meaning that a presynaptic neuron gains an increased ability to fire an action potential as a result of brief high-frequency stimulation. LTP can be induced in three major interconnected synaptic circuits of the hippocampus from the dentate gyrus via the CA3 to the CA1 pyramidal neurons. LTP is measured as the percentage increase in the slope of the EPSP relative to the baseline EPSP slope before high-frequency stimulation. LTD is the opposite process, a decrease in neuronal synapse efficacy which can be long lasting, from an hour to days. It is thought to result from changes in postsynaptic receptor density, although changes in presynaptic release may also play a role. LTD is involved in learning and memory processing mediated by the hippocampus and may be important for the clearing of old memory traces [176]. In the cerebellum, LTD has been hypothesized to be important for motor learning. The changes in synaptic strength may be either transient (short term) or persistent (long term). STP refers to synaptic enhancement that can be induced rapidly but maintained only for up to 20 min [177], while STD results from an activity-dependent reduction in neurotransmitter release [178].

Textbox 3: Electroretinography

Electroretinography is used to measure the activity of groups of retinal neurons in response to light stimulation, including the photoreceptors (rods and cones) and the ganglion cells. Essentially, the technique records the alterations in the extracellular ion concentration surrounding neurons that changes during action potential firing. If the ERG is performed on a dark-adapted eye (scotopic), it reflects the rod system, and the ERG performed on a light-adapted eye (photopic) shows the response for the cone system. There are two ERG waves: a-waves which are initial negative waves, followed by positive b-waves. The a-wave and b-wave are responsible for a hyperpolarization of rod cells and the summation of the depolarization of ON-bipolar cells, respectively. There are two types of bipolar cells, ON (depolarizing)-bipolar cells which activate the ganglion cells in response

to an increased brightness, or OFF (hyperpolarizing)-bipolar cells that activate after decreased brightness [179].

Chapter 1 (Part 2): General Introduction to the use of *Drosophila*

1. *Drosophila melanogaster* as an animal model

Drosophila, often called fruit flies, are frequently employed as an animal model for studying numerous human disease genes for a number of reasons, chief among them, their short life cycle (Fig.1), the availability of powerful genetic tools such as UAS-GAL4 system and their relatively simple body plan (reviewed in [1]). Moreover, the frequently similar basic cellular defects observed in diseased humans and flies mutant for conserved disease genes make *Drosophila* an attractive model for further studies on clarifying the cellular and molecular roles of these genes. Approximately 80 % of human disease genes can be identified in highly conserved form in the fruit fly. Unlike mammals, there are generally fewer likely redundant gene family members encoded in the fly genome; therefore, it is often less complicated to explore the function of a gene.

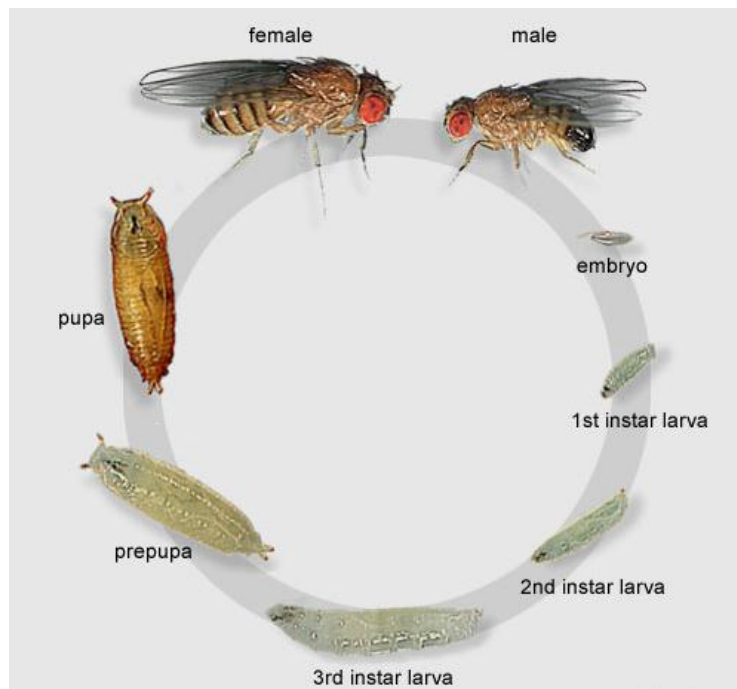


Figure1. The life cycle of *Drosophila* usually takes around 15 days at 25°C. The embryo first transforms into larvae in about 24 hours. The larval form has three phases (first instar, second and third instars larva) before it turns into pre-pupa, pupa and finally ecloses as an adult fly. This picture was downloaded from <http://www.hoxfulmonsters.com/2008/05/drosophila>

2. Formation of *Drosophila* motoneuronal synapses

The *Drosophila* NMJ is a glutamatergic synapse as are vertebrate central synapses. Glutamate receptors at the *Drosophila* NMJ are composed of five different subunits; GluRIIA, GluRIIB, GluRIID, GluRIIE and GluRIII (GluRIIC). The glutamate receptor is formed by four essential glutamate receptor subunits: GluRIIC, GluRIID, and GluRIIE together with either GluRIIA or GluRIIB [2]. The *Drosophila* NMJ forms boutons in the embryo and grows by sprouting new boutons throughout larval life. In *Drosophila* embryo and larva, each hemisegment contains a

stereotyped pattern of 30 muscles [3]. These muscles are innervated by approximately 40 motor axons that make specific connections with particular muscles. *Drosophila* motoneurons are unipolar. Only one primary neurite exits the cell body, which in the neuropile divides into a dendritic tree making contact with its innervating interneurons and one axon that travels out of the CNS into the periphery to find its targets. Its cell bodies are located in the CNS cortex in the ventral ganglion [4]. Motor axons project into the muscle field via two main nerves, the intersegmental (ISN) and the segmental nerve (SN). The transverse nerve (TN), a minor nerve, has few motor axons and runs along the segment border to innervate the external set of muscles. One of the two motoneurons exiting in the TN contacts the lateral bipolar neuron (LBD) which innervates the dorsal muscle (**Fig.2**).

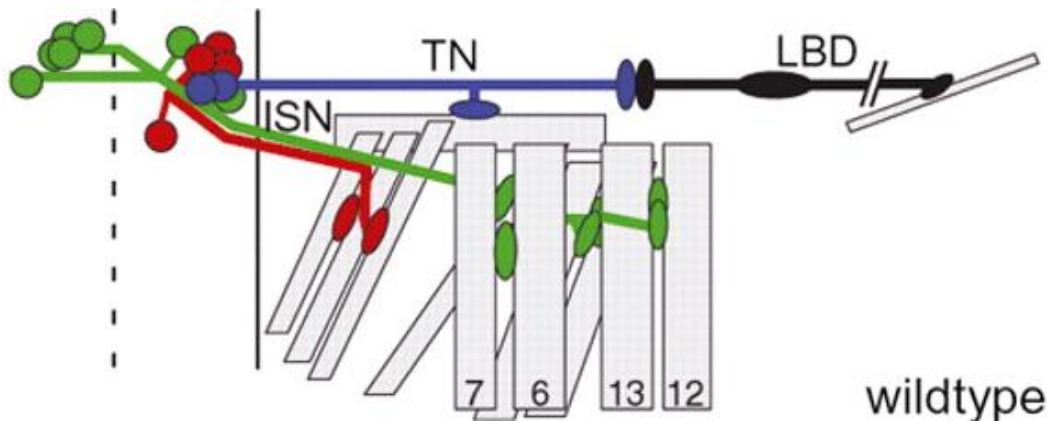


Figure2. Simplified scheme of wild-type motoneuron pattern and their muscle targets in the *Drosophila* embryo. Three main motor axons are the intersegmental (ISN), the segmental nerve (SN in red) and the transverse nerve (TN). TN contacts lateral bipolar neuron (LBD, in black line) which innervates the dorsal muscle. The ventral nerve cord is to the left, the dorsal part is to the right. This picture was taken from the website:

<http://dev.biologists.org/content/131/21/5429/F5.large.jpg>

The muscle of third instar larvae (**Fig 3**) are innervated by synaptic terminals of two major types; type I (large boutons, 2-5 μm diameter) and type II (smaller boutons, 1 μm diameter). Muscle 4 and muscle 6/7 (**Fig. 3B**) have only type I terminals. Boutons containing active zones and T-bars are observed in the late stage 17 embryo, just prior to larval hatching. The growth cone contacts its target muscles, transforms into an initial protosynapse, and subsequently the synapse matures into distinct boutons with active zones and T-bars (**Fig. 3C**). After synapse formation, the hemophilic cell adhesion molecule Fasciclin II (FasII) becomes localized both pre- and postsynaptically where it controls synapse stabilization and synaptic growth. Various FasII mutant alleles display either an increase or decrease in bouton sprouting depending upon the level of FasII [5].

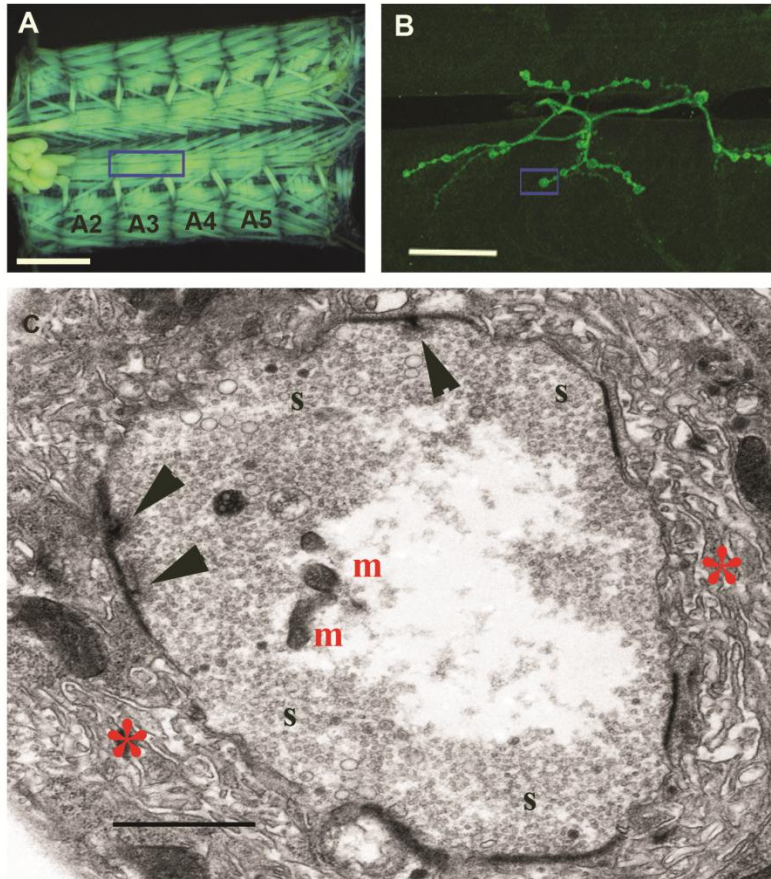


Figure 3. The *Drosophila* NMJ. (A) The third instar larva body wall (anterior to the left, dorsal up) was dissected and fixed with Bouin's fixation for 15 min. Individual muscles and synapses can be easily identified. The third abdominal segment of muscle is shown as A2-A5. Scale bar = 500 μm (B) High magnification view of the synapse on muscles six and seven [frame in (A)]. The synaptic terminals labeled with anti-HRP-FITC. Scale bar = 50 μm . (C) Transmission electron micrograph of a type Ib synaptic bouton [frame in (B)]. Note that the electron dense active zones, some of them contain T-bars (arrowheads). Mitochondria (m) and synaptic vesicles are clearly visible (s). Note that the presence of subsynaptic reticulum (SSR) membrane folds (*) surrounding the bouton. Scale bar = 1 μm .

3. Wing vein formation

The *Drosophila* wing is characterized by a stereotypical pattern of five longitudinal veins (L1–L5) and two crossveins, the anterior crossvein (ACV) and the posterior crossvein (PCV) (**Fig. 4**). The development of veins in the wing imaginal discs has been well characterized (reviewed in [6-8]). Four different signaling pathways have been identified acting during wing morphogenesis: the Epidermal Growth Factor Receptor (EGFR) pathway, the Bone Morphogenetic Protein (BMP) pathway, the Notch pathway, and a pathway involving Rho-GTPases [9]. The Dystrophin DLP2 null mutant (*Dys^{E6}*) exhibits the crossvein defect. In this mutant, the early BMP signaling that initiates crossvein specification is not maintained, particularly in the pro-vein territories adjacent to the longitudinal veins. This is resulted in the incomplete production of a crossvein fragment in the intervein between the two longitudinal veins, L4 and L5 [10]. We used the visual wing vein phenotypes to find Dystrophin interactors that either increase or decrease the wing vein phenotype [11].

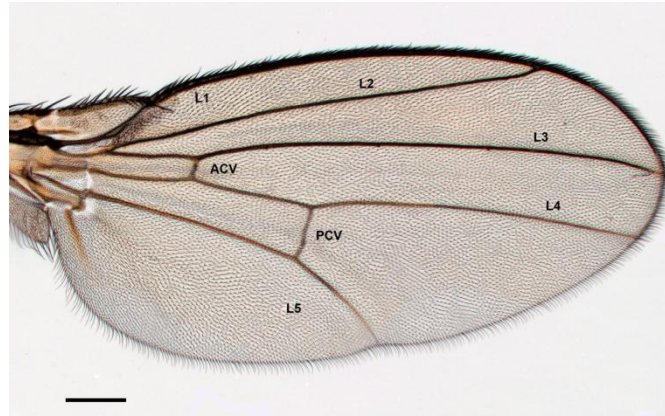


Figure4. The wild type wing has 5 longitudinal veins (L1–L5). The anterior cross vein (ACV) forms between L3 and L4 and the posterior cross vein (PCV) forms between L4 and L5. Scale bar = 200 μm .

4. The Olfactory system of *Drosophila*

For most animals, included *Drosophila*, smell is a primal sense that affords the ability to detect food, predators and mates. The olfactory system of *Drosophila* is an attractive model system to understand mechanism underlying synaptic transmission for many reasons. First, the fly's olfactory system is organizationally similar to that of the human but much simpler (**Fig. 5**). Second, the olfactory sensory genes encoding the odorant receptors have been completely mapped [13-15]. Third, morphological, functional and behavioral studies can be readily performed using the fly's olfactory system.

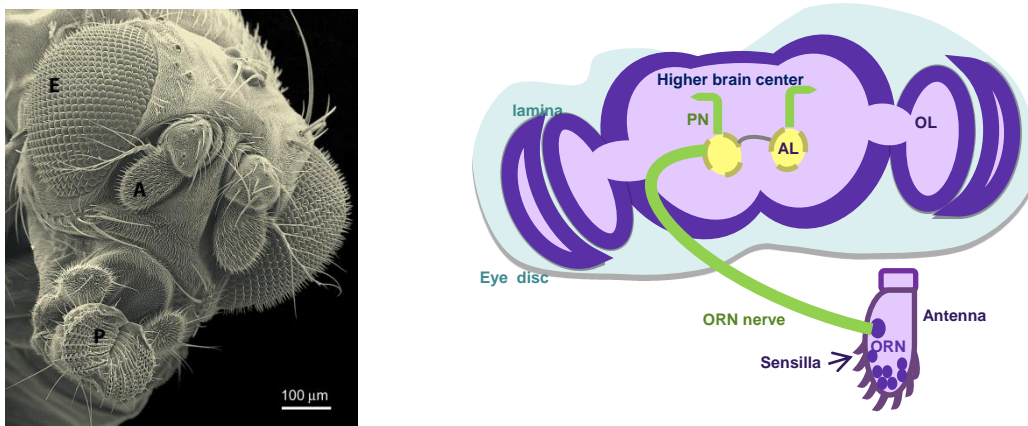


Figure5. *Drosophila* head showing the primary scent organ, the antennae (left) (from http://www.rochester.edu/news/photos/hi_res/Jaenike-Wolbachia/origs/Drosophila_head1.jpg). A cartoon of *Drosophila* olfactory system is shown on the right panel. The olfactory receptor neurons (ORN) are located in sensillae on the antennae. After the odorant binds to the ORNs, the signal will be transfer red via the ORN nerve to the antennal lobe (AL). A= antenna, E= complex eye, AL= antennal lobe, OL=optic lobe, P= proboscis, PN= projection neuron.

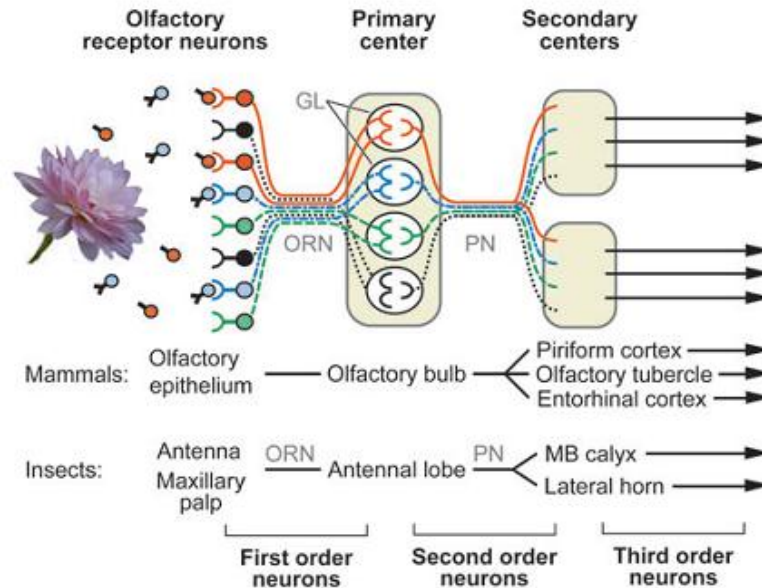


Figure 6. Comparison of the olfactory systems of mammals and insects. This picture was taken from Vosshall, L. and Stocker, R. [16]. ORNs are activated by specific odor and transmit the information through the antennal lobes to the higher brain centra.

Drosophila has two olfactory organs, the antenna and maxillary palp, which contain ~1200 and 120 olfactory receptor neurons (ORNs), respectively [17]. ORNs are compartmentalized into sensillae, which can be subdivided into three major morphological types: basiconic, coeloconic and trichoid. These ORNs can be subdivided into distinct functional classes on the basis of their odor response spectra [18, 19]. Odor information is first received by olfactory receptor neurons (ORNs), located in the sensillae of the antenna (**Fig. 6**). ORNs respond to odors with a sequence of action potentials that reflects the quality, intensity and temporal structure of the odor stimulus. The generation of action potentials by ORNs leads to the activation of second-order neurons in the brain, called glomeruli or antennal lobes. The axons of ORNs expressing the same gene converge onto one or a few glomeruli. Recent studies have essentially completed the OR-to-glomerulus map [20, 21]. The action potentials from the glomeruli are transmitted to projection neurons and then onto higher brain centra, the mushroom body and the lateral horn.

5. Electrophysiology in *Drosophila*

5.1. Electrophysiology at the NMJ

The *Drosophila* NMJ has been extensively used as a model to study the molecular mechanisms underlying synaptic transmission. The *Drosophila* NMJ is relatively simple in structure. The glutamate receptors are located in the postsynaptic membrane called the subsynaptic reticulum (SSR) and opposite the vesicle release sites which are known as active zones. When glutamate binds its receptor, the receptors allow Ca^{2+} to enter the muscle leading to muscle depolarization. The absence of an action potential in the *Drosophila* muscle is one of the reasons why it is a desirable model for NMJ study. In mice, the action potential has to be prevented by blocking Na^+ channels in order to be able to measure the endplate potential, but in *Drosophila*, the muscle

depolarization can be directly measured. Furthermore, the *Drosophila* third instar larval NMJ can be recorded at room temperature and its electrical properties can be maintained for many hours.

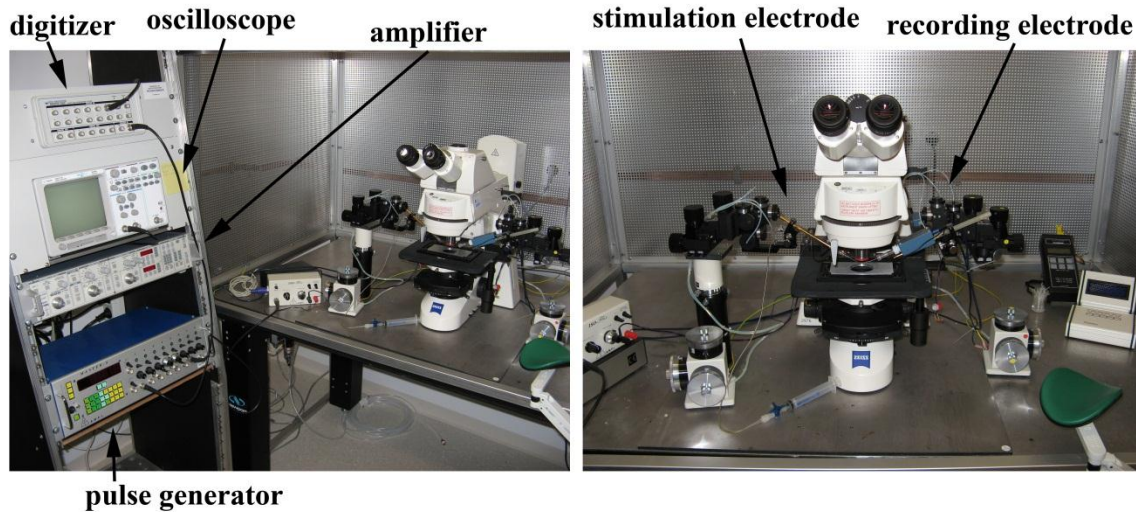


Figure 7. The equipment used in performing electrophysiology measurements. The stimulation electrode is connected to pulse generator and used to stimulate the motoneuron. Muscle depolarization is monitored by a recording electrode. The signal is sent to an amplifier to make the signal visible on the oscilloscope. The digitizer also receives the signal and converts it from an analog signal to a digital signal. The computer records the signal for subsequent analysis.

Electrophysiological recordings are often used to study the function of the *Drosophila* NMJ. In order to record from the muscle, a glass electrode filled with 3M KCl is inserted in the abdominal muscle cell (segments A2-A4) of muscle 6. The motoneuron nerve is cut at the distal end of neuropile and taken up by a suction electrode which connected to a pulse generator (**Fig. 7**). Two types of signals can be recorded from a muscle in this way; the first is those corresponding to spontaneous muscle depolarizations and called miniature excitatory junction potentials (mEJPs). They are thought to represent muscle depolarization from a single vesicle release or quanta from the motoneuron, therefore are also called the quantal size. The second type of signal that can be recorded is the evoked response muscle depolarization also known as the excitatory junction potentials (EJPs). These depolarizations occur when the motoneuron is triggered by stimulus. Therefore, it represents the response to the contents of many vesicles released from the motoneuron. These two measurements can be used to calculate the quantal content, which is the amount of vesicles released upon stimulation of the motoneuron. The quantal content (QC) is calculated by dividing the mean amplitude of the EJPs by the mean amplitude of the mEJPs (amount of vesicles released = amount of depolarization by multiple vesicles/amount of depolarization by one vesicle). To calculate the QC at each NMJ the following formulas are used:

First the amplitudes are adjusted to a standard resting potential of -60 (V_{std}) in *Drosophila*

$$mEJP_n = [mEJP_m * V_{std}] / V_m$$

$$mEJP_n = \text{normalized amplitude}$$

$$mEJP_m = \text{the amplitude as measured}$$

$$V_{std} = \text{standard resting potential (= -60)}$$

$$V_m = \text{the resting membrane potential as measured}$$

Since the measurements are done in a current clamp mode, meaning that the amount of current flowing through the recording electrode is held constant, we have to add a correction to the calculation to compensate for non-linear summation. This is based on the study of McLachlan and Martin, 1981 that the voltage – current relation is not linear for the junction potential above 10 mV [22-23]. The rate of increase of junction potential become progressively smaller as the current increased. In order to correct for this underestimation of the total amount of vesicles released, the following formula was generated for the mouse NMJ and adapted by B.A. Stewart (personal communication) for the *Drosophila* NMJ.

$$\begin{aligned} \text{Formula: } EJP_c &= EJP_n / [1-f*(EJP_n / V_d)] \\ &= EJP_n / [1- 0.4 *(EJP_n / 50)] \end{aligned}$$

EJP_c = the corrected EJP amplitude for nonlinear summation

EJP_n = the normalized EJP amplitude as calculated by $EJP_n = [EJP_m * V_{std}] / V_m$

EJP_m = the average EJP as measured

V_{std} = standard resting potential (= -60)

V_d = the driving force (difference between resting membrane potential and reversal potential; $V_r - V_{std} = -10 - (-60) = 50$)

V_m = the resting membrane potential as measured

V_r = the reversal potential = - 10 mV [24]

f = the membrane capacitance factor (dependent on the duration of transmitter action relative to the membrane time constant ([22-23] and adapted by personal communication with B.A. Stewart). This number is 0.4 in *Drosophila*.

5.2 Electrophysiological recordings in the *Drosophila* antennae Electroantennogram (EAG)

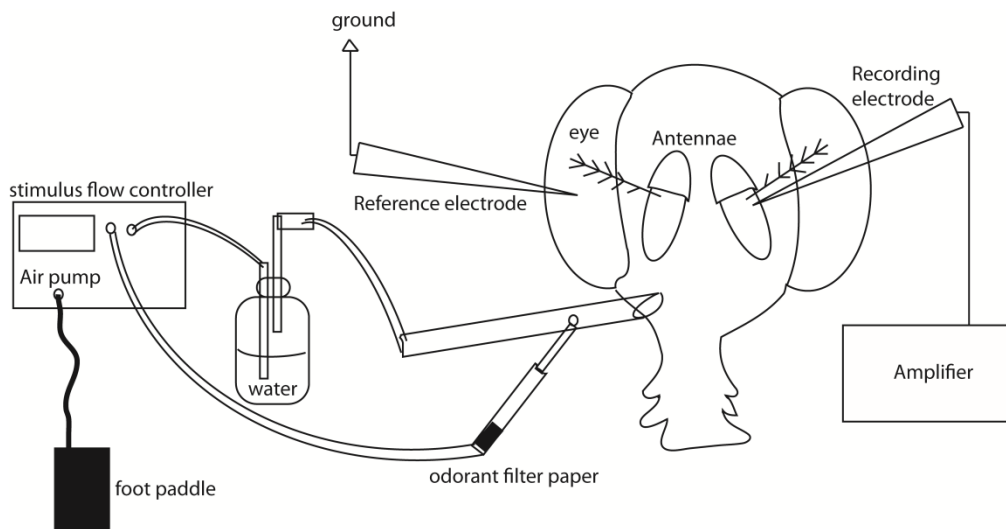


Figure 8. Diagram showing the general settings of EAG measurements. The recording electrode was inserted at the proximal part of the antenna and connected to the amplifier. Another electrode, the reference electrode, was placed at

the eye and was grounded to the table. The stimulation flow controller is connected to the glass tube through the water flask for giving continuous flow and is controlled by a foot paddle. Different odorants were used to stimulate the olfactory receptor neurons in the antenna. The computer records the signal for subsequent analyses.

We performed this technique for studying the electrical function of the overall olfactory receptor neuron field present in the *Drosophila* antennae. A glass electrode, filled with a physiologic buffer solution is inserted in the eyes to serve as a reference electrode (**Fig. 8**). Extracellular recording (recording electrode) at the proximal funiculus has been used to describe the electrical responses of the whole *Drosophila* antenna to different odorants. Qualitative differences in responses to various odorants and quantitative differences in amplitude of the EAG in response to different concentrations have been reported [25].

Chapter 1 (Part 3): Outline of the thesis

In this thesis, my findings on the roles of the DGC proteins in maintaining wild type synaptic function at the neuromuscular junction are presented. I reviewed the literature pertaining to general roles of the DGC in the muscle and discussed recent advances in understanding the DGC roles at various types of synapses in both vertebrate and invertebrate model systems (**Chapter 1**).

Previously, our lab has shown that Dystrophin is required for appropriate retrograde control of neurotransmitter release. We set out to identify signaling molecules that interact with Dystrophin by using a wing vein genetic interaction screen and subsequently evaluated mutant NMJ physiology of candidate dystrophin interacting molecules. We demonstrated that Dystrophin interacts with the Rho-GAP Crossveinless-c in a pathway targeting the Rho GTPase, Cdc42, to regulate presynaptic neurotransmitter release (**Chapter 2**).

To evaluate the role of another DGC member, Dystrobrevin, at the NMJ we first made a knock-out null allele of the Dystrobrevin gene by performing homologous recombination, a relatively new technique in *Drosophila*. I present our finding that Dystrobrevin is delocalized from the synapse in the Dystrophin mutant background and that this delocalization likely accounts for the Dystrophin mutant phenotype. Furthermore, I demonstrated that Dystrobrevin acts through the same Rho-GAP and Rho-GTPase as Dystrophin, thus firmly placing Dystrobrevin in this pathway. Finally, I demonstrate that the Ca^{2+} /calmodulin-dependent kinase II (CaMKII) is a likely postsynaptic target downstream of Cdc42 (**Chapter 3.1**).

Our initial studies on Dystrobrevin indicated that it likely has additional role at the presynaptic side. Indeed, we find that the regulatory subunits of Protein Kinase-A (PKA-R1/R2), the highly conserved mammalian orthologs of which were recently shown to bind Dystrobrevin, also interact genetically and physically with Dystrobrevin in *Drosophila* (**Chapter 3.2**). Further experiments exploring the functions of Dystroglycan, yet another DGC member, at the synapse are also presented (**Chapter 4**).

Chapters 2 to 4 are focused on the functional roles of the DGC at a peripheral synapse, the NMJ. However, possible roles for the DGC at central synapses have been inferred from studies in mammals and clinical evaluations of Duchenne patients who frequently present with mental retardation which is not correlated with decreased muscle strength. Previously, we identified roles for the *Drosophila* Dystrophin brain-specific isoform, Dp186, in regulating homeostasis at an identified interneuronal synapse. Here, I present my findings, employing both electrophysiological and behavioral experiments, describing a role for Dp186 in the olfactory system neural circuit. Its absence leads to decreased output by primary olfactory receptor neurons and also apparently decreases the efficacy of downstream synaptic transmission (**Chapter 5**).

Finally in the last chapter (**Chapter 6**), my studies are summarized and integrated to generate a model for DGC function at the synapse. I also discuss possible future extensions to the studies presented in this thesis.

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