# A Drosophila model for Duchenne muscular dystrophy

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# CHAPTER 1

## Introduction

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# 1. General introduction

## 1.1 Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a severe X-linked progressive degenerative muscle disease affecting approximately 1:3500 boys. Symptoms arise as early as the age of 3, when the patients walk with a 'wobbling' gait. Muscle degeneration further ensues, causing patients to be wheelchair bound by the age of twelve. Due to lung and heart failure, most patients do not live beyond their twenties. In addition to the muscle degeneration, approximately one third of the patients display slight mental retardation, the severity of which is correlated with mutations in the 3' region of the *dystrophin* gene (Moizard *et al.*, 2000). The disease was first described by the French neurologist G.B.A. Duchenne in 1861. In 1987, it was discovered that the disease is caused by mutations in the *dystrophin* gene (Hoffman *et al.*, 1987). Furthermore, mutations causing the complete absence of the Dystrophin protein were found to result in DMD, whereas mutations causing the expression of a truncated, but partially functional, protein cause a much milder form, called Becker's Muscular Dystrophy (BMD). Dystrophin localizes to the muscle plasma membrane where it clusters together with several other glycoproteins, forming a large transmembrane protein complex called the Dystrophin Glycoprotein Complex (DGC, also called the Dystrophin Associated Protein Complex or DAPC).

## 1.2 The mammalian Dystrophin Glycoprotein Complex

The DGC (Figure 1) is composed of multiple proteins. The composition of the DGC can vary between different cell types. It has been detected in virtually all tissue types, including muscle, liver, kidney, retina, nervous system, as well as at the blood brain barrier and the choroid plexus. The DGC is usually located at the plasma membrane facing a basal lamina in tissues that have a secretory function or form a barrier between functional compartments (reviewed in Haenggi & Fritschy, 2006). Although some evidence for tissue-specific DGCs has been provided, in general the DGC contains a Dystrophin-like protein (Dystrophin or Utrophin), two Dystroglycans (α-DG and β-DG), a Dystrobrevin (either α-DB or β-DB), four Sarcoglycans (α-SG or ε-SG, β-SG, γ-SG or ζ-SG, and δ-SG), four Syntrophins (α1-SYN, β1-SYN, β2-SYN, γ1-SYN, or γ2-SYN) and a Sarcospan. In muscle the complex is located along the sarcolemma and at the neuromuscular junction (NMJ).

The complex is hypothesized to serve a mechanical role at the sarcolemma, since it links the Actin cytoskeleton of the muscle cell (bound by Dystrophin) to Laminin in the extracellular matrix (ECM)(bound by α-DG), thereby stabilizing the sarcolemma during contraction. This mechanical function was inferred from the observation of membrane leakage in degenerating muscle fibers in DMD patient muscles. Exercise leads to significantly more contraction-induced injury in the absence of the DGC at the sarcolemma. The DGC is located in costameric structures that correspond to the Z-bands of sarcomeres, where it aids in perpendicular force transmission (Porter *et al.*, 1992; Heydemann & McNally, 2007). However, recently it has become clear, that the DGC also has important signaling roles, as deduced from physical binding studies showing that several signaling molecules are capable of binding the complex. The complex likely serves as a scaffolding complex, where other proteins can bind and interact with their signaling partners (reviewed in Rando, 2001).

Since the DGC has proven to play a vital role at the sarcolemma, it is not surprising that after the discovery of Dystrophin, mutations in a number of other DGC members-encoding genes have been linked to a variety of muscle degenerative diseases. For instance, mutations in *sarcoglycan* genes lead to several types of limb girdle muscular dystrophies (Laval & Bushby, 2004), and hypoglycosylation of α-DG or mutations in the laminin α2 chain result in congenital muscular dystrophy (Muntoni, 2002). The different disease types often have common pathophysiological characteristics, but are nonetheless also very different, making it

difficult to determine the underlying common mechanisms of disease. Therefore, there is still little known about the disease mechanisms involved in the onset and the progression of muscular dystrophy. Several possible functions, related to the mechanical and signaling roles mentioned above, have been proposed for the DGC (for review see Petrof *et al.*, 2002; Deconinck & Dan, 2007), which can explain the different disease symptoms, but it is as yet unclear, whether these symptoms are primary causes or simply consequences of the degeneration of DMD muscle cells.

Dystrophin-like proteins

Dystrophin-like proteins are part of the spectrin superfamily (Koenig *et al.*, 1988; Davies & Nowak, 2006). The Dystrophin-like proteins in mammals are Dystrophin, Utrophin, DRP2 and Dystrobrevin. Recently, Dystrotelin has been added to this list (Jin *et al.*, 2007), but this is a very distant member of the family and will not be discussed here.

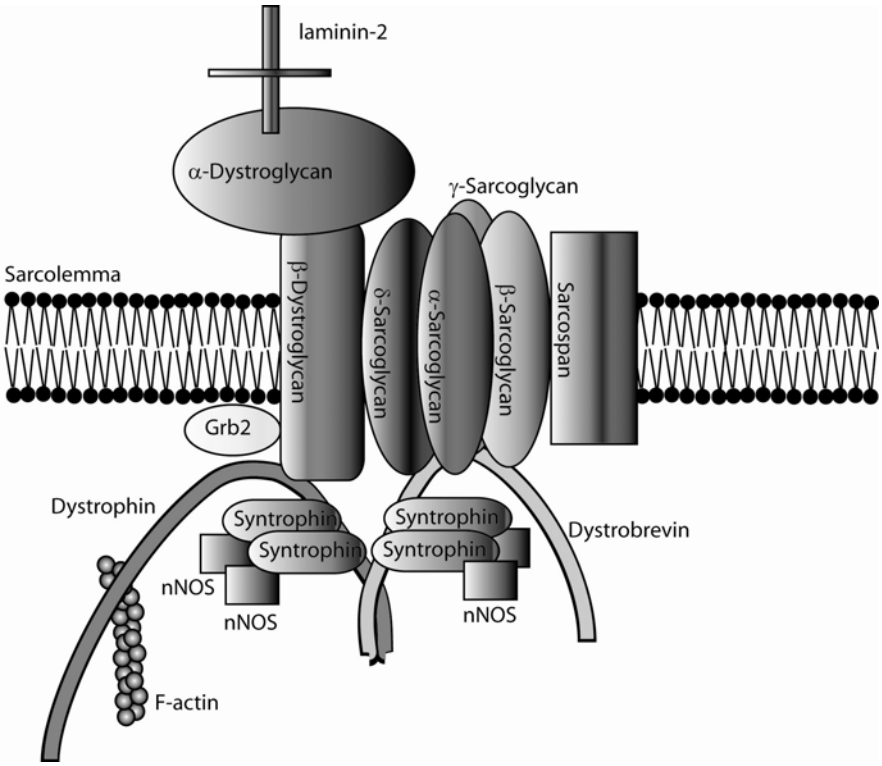


**Figure 1:** A schematic of the Dystrophin Glycoprotein Complex in mammalian skeletal muscle. Also indicated are the DGC interacting proteins laminin-2, Grb2, nNOS, and F-actin.

Dystrophin

The human *dystrophin* gene is located on the X-chromosome and spans approximately 2.5Mb. It is composed of 79 exons and has three independently regulated promoters, which control expression of the large Dystrophin isoform (427 kDa) in muscle, brain and Purkinje cells in the cerebellum (Haenggi & Fritschy, 2006). Several short *dystrophin* isoforms are transcribed from four internal promoters, encoding proteins of 260, 140, 116, and 71 kDa (Dp260, Dp140, Dp116, and Dp71).

One or more Dystrophin isoforms can be found in almost all tissues. The large Dystrophin isoform (Dp427) in skeletal muscle is predominantly located at the sarcolemma within costameres, but is also present at the troughs of the postsynaptic membrane along with voltage-gated sodium channels (Sealock *et al.*, 1991).

The Dystrophin Dp427 protein is composed of four functional domains: 1) an actin binding domain at the N-terminus, 2) a rod domain consisting of spectrin repeats and a second actin-binding domain, 3) a cysteine-rich domain, and 4) a carboxy-terminal domain, unique to Dystrophin, Utrophin and Dystrobrevin (Ervasti, 2007).

Dystrophin binds to cytoplasmic γ-actin filaments of the cytoskeleton and anchors β-Dystroglycan to the sarcolemma. Dystrophin binds to β-Dystroglycan using the WW, EF hand and ZZ protein interaction domains, located in the cysteine-rich region of the protein (Ishikawa-Sakurai *et al.*, 2004). As seen from a missense mutation (C3340Y) in Dystrophin, which causes loss of binding between β-Dystroglycan and Dystrophin, disruption of this binding is sufficient to cause DMD in patients.

Dystrophin deficiency in humans and mice leads to sarcolemmal fragility, muscle weakness, necrosis, and the disorganization of costameres, which are protein lattices surrounding the Z-disk of peripheral myofibrils (Williams & Bloch, 1999; Cohn & Campbell, 2000; Blake *et al.*, 2002).
Both α-Dystrobrevin and α-Syntrophin are lost from the sarcolemma in the absence of Dystrophin in mice. However, expression of Dystrophin transgenes, lacking the α-Dystrobrevin and α-Syntrophin binding domains, in *dystrophin* mutant mice can restore these proteins to the sarcolemma, thereby indicating that their link to Dystrophin via these binding sites is not necessary for their localization. Furthermore, these mice have normal muscle function, suggesting that the link with Dystrophin is also not required for α-Dystrobrevin and α-Syntrophin to function at the sarcolemma. Both these proteins might have a function other than mechanical and can associate with the DGC via other members than Dystrophin; for instance, α-Dystrobrevin may interact with the Sarcoglycans (Yoshida *et al.*, 2000).

Utrophin

*utrophin* is an autosomal gene, which has significant homology with *dystrophin*. Utrophin protein is expressed in almost all tissues with particularly high expression levels in lung, kidney, the nervous system, and vascular endothelial and smooth muscle cells. Two similar full length isoforms have been identified in adult skeletal muscle, A- and B-Utrophin (Burton *et al.*, 1999; Weir *et al.*, 2002). Utrophin protein is located along the sarcolemma in fetal and regenerating muscle, but is restricted to the myotendinous and neuromuscular junctions in adult muscle. A-Utrophin, the most common Utrophin isoform expressed in skeletal muscle, is confined to the neuromuscular junction (NMJ) and is closely associated with acetylcholine receptors at the crests of the postsynaptic membrane.
Utrophin has two actin-binding domains in the amino-terminal CH-domain and the first 10 spectrin repeats for actin binding, whereas Dystrophin has two actin-binding domains consisting of the amino-terminal CH-domain and spectrin repeats 11-17 (Amann *et al.*, 1999; Rybakova *et al.*, 2006). Similarly, Utrophin appears to bind to β-Dystroglycan in a different way than Dystrophin (Ishikawa-Sakurai *et al.*, 2004).

Utrophin deficiency has not been linked to a human disease and *utrophin* null mice do not develop muscular dystrophy (Deconinck *et al.*, 1997a). At the sarcolemma, Dystrophin is normally localized in these mice, but it is upregulated at the neuromuscular junction (NMJ). The NMJ is properly assembled at birth, but develops less postsynaptic folds and acetylcholine receptor clustering is decreased (Deconinck *et al.*, 1997b; Grady *et al.*, 1997a). Utrophin is not required for acetylcholine receptor clustering perse, but has a role in maturation of the synapse (Willmann & Fuhrer, 2002). Utrophin does not localize at the NMJ in the absence of acetylcholine receptors (Slater *et al.*, 1997; Sieb *et al.*, 2000).
Utrophin and Dystrophin bind the same complement of proteins, suggesting that Utrophin may be able to compensate for Dystrophin loss. In support of this theory, continued Utrophin expression in Dystrophin deficient muscle has been shown to partially attenuate the dystrophic phenotype. Furthermore, mice lacking both Dystrophin and Utrophin display a more severe phenotype than both single mutants, and are therefore a better model for the pathophysiology in DMD patient muscles (Deconinck *et al.*, 1997a; Grady *et al.*, 1997b).

DRP2

The third Dystrophin-like protein found in humans is Dystrophin-related protein 2 (DRP2). DRP2 is restricted in its expression to punctuate structures in the CNS (Roberts & Sheng, 2000) and patches between the Cajal bands of Schwann cells in the PNS (Sherman *et al.*, 2001). Interestingly, the N-terminal of DRP2 has a unique elaboration, which is not found in the other Dystrophin-like proteins. DRP2 is closest related to the Dystrophin Dp116 isoform and G-Utrophin (Jin *et al.*, 2007). The recently discovered *Drosophila dystrophin* isoform Dp205 (Neuman *et al.*, 2005) is the closest homologue of DRP2 in *Drosophila* (Jin *et al.*, 2007). Mutation of DRP2 is not associated with human disease, except for the finding that DRP2 levels are reduced in periaxin-deficient myelination disorder (Sherman *et al.*, 2001), likely reflecting the interaction of DRP2 and periaxin in the PNS.

Dystrobrevin

The C-terminus of Dystrobrevin has significant homology to the C-terminus of Dystrophin and Dystrobrevin is therefore a member of the Dystrophin-related protein family (Wagner *et al.*, 1993; Blake *et al.*, 1996). There are two mammalian Dystrobrevin genes, α-Dystrobrevin and β-Dystrobrevin (Peters *et al.*, 1997; Blake *et al.*, 1998; Nawrotzki *et al.*, 1998). Dystrobrevins contain a ZZ domain, two EF hands, two Syntrophin binding sites, a coiled-coil domain and a unique C-terminal tail (Albrecht & Froehner, 2002). The α-Dystrobrevin gene gives rise to at least five splice variants. α-Dystrobrevin1, -2, and -3 are located at the sarcolemma; α-Dystrobrevin1 is restricted to the NMJ, α-Dystrobrevin2 is distributed similarly to Dystrophin along the sarcolemma and at the NMJ (Nawrotzki *et al.*, 1998; Peters *et al.*, 1998; Newey *et al.*, 2001). Both isoforms bind Dystrophin and Utrophin via their coiled-coil regions on their C-terminal side (Blake *et al.*, 1995; Sadoulet-Puccio *et al.*, 1997). β-Dystrobrevin is expressed in many non-muscle tissues, but not in striated muscle. It associates with Dp71 and Utrophin (Blake *et al.*, 1999; Loh *et al.*, 2000). α-Dystrobrevin binds to the Sarcoglycan complex at its N-terminus (Yoshida *et al.*, 2000).

Mutations in human α-Dystrobrevin have so far only been linked to congenital heart disease (Ichida *et al.*, 2001). α-Dystrobrevin null mice have a mild muscular dystrophy in both skeletal and cardiac muscle, characterized by centrally located myonuclei, expression of embryonic and fetal myosin and heterogeneity in muscle fiber size (Grady *et al.*, 1999). Interestingly, unlike DMD muscles, the degenerating muscles remained impermeable for Evans blue dye, a dye used to indicate sarcolemmal damage (Albrecht & Froehner, 2002). Other components of the DGC are normally localized in these mice, however nNOS is displaced from the sarcolemma into the sarcoplasm, likely due to the loss of interaction between Dystrobrevin and Syntrophin (Newey *et al.*, 2000). The NMJ morphology is abnormal in α-Dystrobrevin null mice (Grady *et al.*, 2000), in that the acetylcholine receptor (AChR) clusters are not evenly distributed, but patchy, likely due to destabilization of the acetylcholine receptor clusters. This is similar to the defects seen in Dystrophin/Utrophin double mutants as well as in α-Syntrophin null mice, suggesting a function of the DGC via α-Dystrobrevin and α-Syntrophin in maturation of the synapse.

Dystroglycan

The human *dystroglycan* gene, DAG1 is located on chromosome 3 and consists of two exons and an intron. It encodes a single polypeptide, which is post-translationally cleaved resulting in the α-Dystroglycan and β-Dystroglycan fragments, which remain non-covalently linked (Holt *et al.*, 2000; Esapa *et al.*, 2003; Ibraghimov-Beskrovnaya *et al.*, 1992).
α-Dystroglycan is located extracellularly, where it binds to laminin 2, agrin, perlecan and neurexins sharing LNS domains (Rudenko *et al.*, 2001; Sugita *et al.*, 2001; Ibraghimov-Beskrovnaya *et al.*, 1992). α-Dystroglycan is glycosylated on its mucin-domain. This glycosylation is apparently necessary for its function as a laminin/agrin receptor involved in basal membrane formation and synaptogenesis (Winder, 2001). Binding of α-Dystroglycan to

Perlecan is required for the clustering of acetylcholine esterases at the NMJ (Peng *et al.*, 1999).
β-Dystroglycan is a single transmembrane protein and intracellularly binds either Dystrophin in muscle cells or Utrophin in epithelial cells through its WW domain near the C-terminus. Caveolin-3 binds to the same site, suggesting that these proteins may compete for binding, modulating the membrane anchoring of the complex. β-Dystroglycan binds directly to α-Dystrobrevin (Davies & Nowak, 2006). The Dystroglycan complex is also associated with the Sarcoglycan complex. When mutant Dystrophin isoforms that are unable to bind β-Dystroglycan are expressed, α-Dystroglycan and all Sarcoglycan proteins are delocalized from the membrane, suggesting that β-Dystroglycan is responsible for assembly and anchoring of the Sarcoglycan complex to the DGC (Davies & Nowak, 2006).

No human disease has been linked to mutations in Dystroglycan, possibly due to its important function in embryonic development. Loss of Dystroglycan leads to embryonic lethality in mice (Williamson *et al.*, 1997). Reduction of α-Dystroglycan has also been correlated with increased invasiveness of cancer cells (Muschler *et al.*, 2002). Loss of α-Dystroglycan might be an early event in carcinogenesis, by causing an abnormal cell-ECM interaction and thus contributing to progression to metastatic disease, rather than a consequence of neoplastic transformation (Sgambato & Brancaccio, 2005; Sgambato *et al.*, 2003).
Hypoglycosylation of α-Dystroglycan, caused by mutations in glycosyltransferases, does lead to muscular dystrophy (Barresi & Campbell, 2006). Chimeric mice with reduced Dystroglycan survive with intact basement membranes, but develop muscular dystrophy accompanied by severely disrupted NMJs, possibly due to the loss of its interaction with Rapsyn (Cote *et al.*, 1999; Petrof, 2002).
Dystroglycan has been linked to the transduction and modulation of various signaling pathways, such as FAK signaling via GRB2 and integrin signaling (Henry & Campbell, 1999; Winder, 2001). Dystroglycan is also important for proper epithelial development *in vivo* (Henry & Campbell, 1998) and epithelial morphogenesis *in vitro* (Durbeej *et al.*, 2001).

Sarcoglycans
All six human Sarcoglycans (α-, β-, γ-, δ-, ε-, and ζ-Sarcoglycan) are single pass transmembrane proteins that co-assemble in subsets into a stable tetrameric complex (Ozawa *et al.*, 2005). However, a recent study by Anastasi *et al.* (2007) suggests that there may also be pentameric, or hexameric complexes. Mutations in any *sarcoglycan* gene usually lead to a loss of the entire complex at the sarcolemma. The tetrameric complex usually consists of α-, β-, γ-, and δ-Sarcoglycan in skeletal muscle. However, ε-Sarcoglycan, whose structure is very similar to α-Sarcoglycan, can take its place in the complex. Similarly, ζ-Sarcoglycan is homologous to both γ- and δ-Sarcoglycan and can replace γ-Sarcoglycan in the complex (Ozawa *et al.*, 2005).
The presence of β-and δ-Sarcoglycan is essential in Sarcoglycan complex formation. When either of these subunits is missing, the entire complex is delocalized from the sarcolemma (Ozawa *et al.*, 2005). When γ-Sarcoglycan is missing, the other three subunits remain at trace levels. In the absence of α-Sarcoglycan, only γ-Sarcoglycan can be detected at the sarcolemma. Thus, β- and δ-Sarcoglycan form a core complex with α- and γ-Sarcoglycan binding to this core.
When Dystrophin is absent, the Sarcoglycan complex is destabilized and the sarcolemma becomes leaky. However, none of the Sarcoglycans likely bind directly to Dystrophin (Davies & Nowak, 2006). The complex does bind to the Dystroglycan complex via β-Sarcoglycan as well as to Sarcospan. Sarcospan levels are markedly reduced in the absence of the Sarcoglycan complex (Araishi *et al.*, 1999; Duclos *et al.*, 1998), while the Sarcoglycan complex is stable in the absence of Sarcospan (Ozawa *et al.*, 2005), suggesting that the Sarcoglycan complex is required for correct Sarcospan localization.

Mutations in α-, β-, γ- or δ-Sarcoglycan result in limb girdle muscular dystrophy (LGMD2C, LGMD2D, LGMD2E or LGMD2F, respectively; reviewed in Laval & Bushby, 2004). These muscular dystrophies may be relatively severe, with a DMD-like onset in childhood, but show a wide variety of symptoms and severities. Cardiac and respiratory involvement is common and patients often develop cardiomyopathy (Politano *et al.*, 2001). Absence of ε-Sarcoglycan leads to myoclonus-dystonia syndrome, a movement disorder characterized by muscle jerks and often also psychological disorders as panic attacks and obsessive-compulsive behavior (Zimprich *et al.*, 2001; Muller *et al.*, 2002). The precise function of the Sarcoglycan complex is not known, but it appears to have mechanical and signaling roles (Barton, 2006). The extracellular part of the Sarcoglycans is homologous to a region in the extracellular domain of the epidermal growth factor (Rando, 2001), which transduces signals upon binding to its ligand.
Mice deficient for α-, β-, γ- or δ-Sarcoglycan develop severe muscular dystrophy. All Sarcoglycan deficiencies, except α-Sarcoglycan deficiency, also result in cardiomyopathy. This is caused by the absence of the Sarcoglycan complex in smooth muscle cells, where the complex consists of ε-, β-, γ-, and δ- Sarcoglycan. The loss of α-, β- or δ- Sarcoglycan leads to loss of the Sarcoglycan/Sarcospan complex as well as destabilization of the Dystroglycan complex (Durbeej & Campbell, 2002). Symptoms in mice with a γ- Sarcoglycan deficiency, where the Sarcoglycan/Sarcospan complex is destabilized, but the Dystroglycan complex is still present, also suggest a signaling function for the Sarcoglycan complex. Although the mechanical link between the ECM and the cytoskeleton is still intact and these mice show no evidence for contraction-induced injury after exercise, they have severe muscular dystrophy and cardiomyopathy.

Syntrophin
There are five genes encoding different Syntrophin family members, α1-, β1-, β2-, γ1-, and γ2-Syntrophin (Haenggi & Fritschy, 2006). In skeletal muscle, β2-Syntrophin is restricted to the NMJ, α1-, β1- and γ2- Syntrophin are also found along the sarcolemma (Haenggi & Fritschy, 2006). The γ-Syntrophins are predominantly expressed in the brain. Syntrophins are also expressed in other tissues, such as retina, kidney, and liver. Mediated by the Syntrophin unique C-terminus and the adjacent pleckstrin homology domain (Kachinski *et al.*, 1999), α1- and β1-Syntrophin bind to Dystrophin, β2-Syntrophin binds Utrophin, and Syntrophin binds Dystrobrevin (Ahn *et al.*, 1996; Butler *et al.*, 1992; Kramarcy *et al.*, 1994; Ahn & Kunkel, 1995; Dwyer & Froehner, 1995; Suzuki *et al.*, 1995; Yang *et al.*, 1995). The DGC has four Syntrophin binding sites in close proximity (Newey *et al.*, 2000), two on Dystrophin and two on Dystrobrevin (Figure 1), suggesting that Syntrophins may cluster signaling molecules for assembly of a larger signaling machine (Albrecht & Froehner, 2002). The Syntrophin PDZ domain interacts with ion channels, such as $Na_v1.4$, $Na_v1.5$, and other signaling proteins, such as nNOS, SAPK3 (also known as ERK6 or p38γ), syntrophin-associated serine/threonine kinase, its homologue, microtubule-associated serine/threonine kinase-205kD, and diacylglycerol kinase-ζ (Albrecht & Froehner, 2002).

No human disease has yet been associated with mutations in the *syntrophin* genes. Knockout mice for Syntrophin or nNOS, do not develop muscular dystrophy, but they do have abnormal NMJs with reduced clustering of AChRs (Adams *et al.*, 2000; Crosbie *et al.*, 1998). In Dystrophin-deficient muscle, α-Syntrophin is not located at the sarcolemma, but it does localize at the NMJ, where it possibly binds to Utrophin (Adams *et al.*, 2000).

Sarcospan
Sarcospan binds to the Sarcoglycan complex (Ervasti, 2007). Mutations in Sarcospan have not been associated with any human myopathy so far and Sarcospan null mice do not have a muscle phenotype (Lebakken *et al.*, 2000). However, Sarcospan overexpression has recently been shown to cause a severe muscular dystrophy similar to congenital muscular dystrophy (Peter *et al.*, 2007) probably due to destabilization of α-Dystroglycan.

### 1.3 The DGC in other animal models

Zebrafish
Two isoforms of *dystrophin*, *β-dystroglycan* and three *sarcoglycans* (*β-sarcoglycan*, *γ-sarcoglycan* and *δ-sarcoglycan*) have been identified in zebrafish (*Danio rerio*) (Guyon *et al.*, 2003; Parsons *et al.*, 2002; Chambers *et al.*, 2003). Dystrophin, Dystrobrevin and Sarcoglycan are expressed in zebrafish skeletal muscle and localized to the myosepta (Guyon *et al.*, 2003; Parsons *et al.*, 2002; Guyon *et al.*, 2005), which are homologous in function to myotendinous junctions in mice. Loss of Dystrophin (sapje mutant of *dmd* locus) results in muscle degeneration caused by the failure of embryonic muscle end attachments (Bassett *et al.*, 2003). Loss of β-Dystroglycan results in a "bent" embryonic phenotype and muscle degeneration. It is unclear what causes the "bent" phenotype, but a similar phenotype has been found in mutations affecting notochord development (Guyon *et al.*, 2003) and it is reminiscent of the kyphosis (a progressive curvature of the spine) found in *mdx/utr* double mutant mice (Guyon *et al.*, 2003). Reduction of δ-Sarcoglycan expression using morpholinos resulted in disorganized myofibers and uninflated swimbladders in zebrafish embryos (Guyon *et al.*, 2005). The δ-Sarcoglycan zebrafish morphant also portrayed a bending phenotype, which reflects a compensatory posture subsequent to muscle weakness, but might also be an effect of the failure of the swimbladder to inflate (Guyon *et al.*, 2005).

*Caenorhabditis elegans*
Of the DGC members, at least one *dystrophin* (*dys-1*), one *dystrobrevin* (*dyb-1*), one *dystroglycan* (*DGN-1*), three *sarcoglycans* (*α-sarcoglycan* (H22K11.4), *β-sarcoglycan* (K01A2.1) and *δ/γ-sarcoglycan* (F07H5.2)), and two *syntrophins* (*γ-syntrophin* (F27D9.8) and *α/β syntrophin* (*stn-1*)) have been identified in *C. elegans* (Bessou *et al.*, 1998; Grisoni *et al.*, 2002; Grisoni *et al.*, 2003; Cox & Hardin, 2004). All genes, except the *dystroglycans* and the neuron specific *γ-syntrophin* ortholog, are expressed in the muscle. A lack of Dystrophin, Dystrobrevin, or Syntrophin results in hyperactive worms, that have a tendency to hypercontract and are hypersensitive to acetylcholine. *Dys-1* and *dyb-1* mutant muscles are more excitable than normal (Bessou *et al.*, 1998; Gieseler *et al.*, 2001).
Screens for this *dystrophin* phenotype has led to the discovery of several other genes possibly involved in the same pathway, since they also cause hyperactivity when absent. These genes are *dyc1* (homologous to the mammalian nitric oxide synthetase binding protein CAPON), *SLO1* (a $Ca^{2+}$ activated $K^+$ channel), and the acetylcholine transporter *snf-6* (Gieseler *et al.*, 2000; Ségalat, 2002; Carre-Pierrat *et al.*, 2006; Kim *et al.*, 2004). In the *SLO-1* and *snf-6* mutants the hyperactivity can possibly be explained by the lack of $K^+$ channels and clearing of acetylcholine resulting in hyperexcitability of the muscle membrane. The role of *dyc-1* is unclear, since there is no known nNOS homologue in *C.elegans* (Gieseler *et al.*, 2000).

Muscle degeneration does not occur in *dys-1* mutants unless the background is sensitized by removing the *MyoD* homolog, *hlh-1* (Bessou *et al.*, 1998; Grisoni *et al.*, 2003). This indicates that, similar to MyoD function in mice, hlh-1 can somehow prevent or repair muscle degeneration in the absence of Dystrophin. Interestingly, *dyb-1/hlh-1* double mutants or *stn-1/hlh-1* double mutants do not show muscle degeneration even in this sensitized background (Grisoni *et al.*, 2003). This might mean that the hyperactivity, seen in *dys-1*, *dyb-1* and *stn-1*, and the muscle degeneration, seen only in *dys-1/hlh-1* double mutants, may represent two distinct mechanisms.
The muscle degeneration in *dys-1/hlh-1* double mutants has been shown to be a $Ca^{2+}$ dependent process. The *dys-1* hyperactivity phenotype suppressed in a mutant of the major voltage-gated calcium channel, *egl-19*, as shown in *dys-1/egl-19* double mutants, and RNAi against *egl-19* in *dys-1/hlh-1* mutants prevents the onset of muscle degeneration (Mariol & Ségalat, 2001). Conversely, a gain-of-function mutation in *egl-19* induces muscle degeneration in non-sensitized *dys-1* mutants, indicating a role for calcium in the onset of muscle degeneration.

The *C. elegans dystroglycan* gene is not apparently expressed in skeletal muscle, but is present in the epithelium and peripheral nervous system, where it is involved in the organization of the epithelium and in motoneuron axon guidance, respectively (Johnson *et al.*, 2006).

## 2. *Drosophila* as a model system

In recent years *Drosophila melanogaster* has often been used as an animal system for the study of numerous human diseases. This is mainly due to the fact that the fly has a short generation time and is easy to study due to its segmental body plan and simple morphology. Another advantage is that over 70% of the human disease genes can be identified in highly conserved form in the fruit fly. Fortunately, unlike as in mammals, fly genes generally lack, or have fewer, partially redundant homologues, which often complicate the dissection of the function of a gene. A large array of genetic tools, such as the UAS-GAL4 system or the use of transgenic RNAi also greatly facilitates genetic manipulation. All these advantages have led to the unraveling of many important signaling pathways, for example the role of segment-polarity genes, which could easily be translated to the vertebrate system. *Drosophila* is also widely used in the study of complex mechanisms as learning and memory, as well as of complex diseases, such as Alzheimer's, Parkinson, and heart diseases. Research on fruit flies has also shed light on developmental pathways and differentiation. Very detailed descriptions have been made on the development of the musculature and the nervous system in which many conserved counterparts have been found to be involved in similar processes in vertebrates.

### 2.1 The DGC in Drosophila melanogaster

Almost all DGC proteins have been identified in *Drosophila melanogaster* (Figure 2). This includes Dystrophin, Dystroglycan, Dystrobrevin, three Sarcoglycans and two Syntrophins (Greener & Roberts, 2000). A Sarcospan homologue is not present in the *Drosophila* genome.

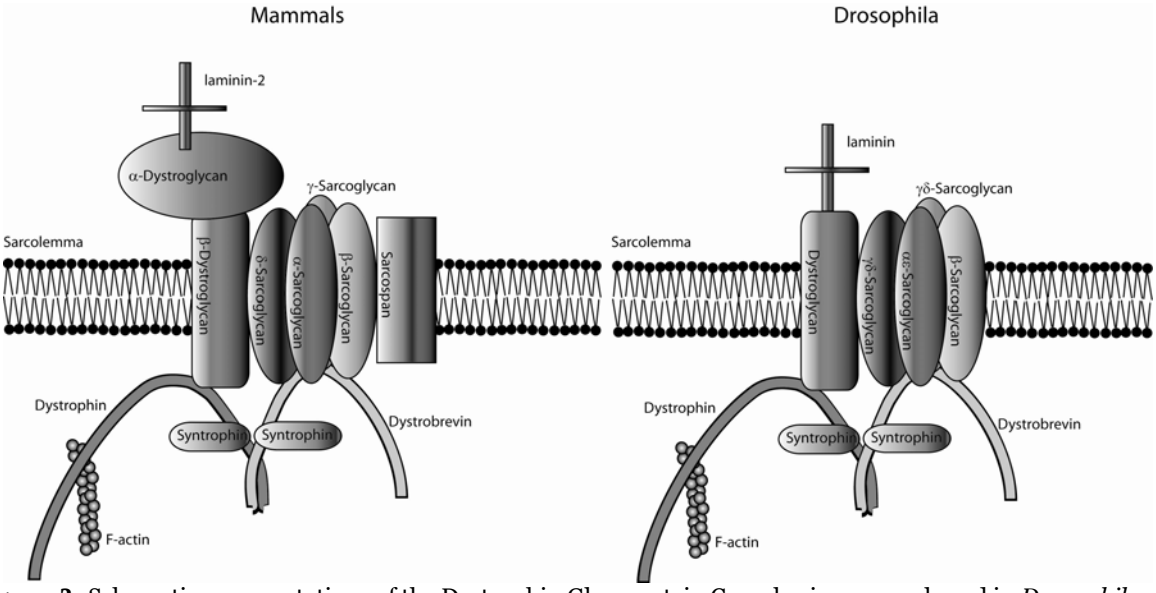


**Figure 2:** Schematic representations of the Dystrophin Glycoprotein Complex in mammals and in *Drosophila*.

Dystrophin

*dystrophin* is the second largest gene in the *Drosophila* genome, even though it has less than half the amount of exons as the vertebrate gene, spanning at least 130kb (Roberts, 2001). It encodes at least six different isoforms, three long isoforms DLP1, DLP2, and DLP3 of approximately 400kDa, and three shorter isoforms, Dp205, Dp186, and Dp117, transcribed

from internal promotors located in large introns (Neuman *et al.*, 2001; Neuman *et al.*, 2005). The Dystrophin protein contains the four main domains also present in vertebrate Dystrophin, namely the actin-binding domain at the N-terminus, the spectrin-repeat rod domain, the cysteine rich domain and the common carboxy terminal. The most highly conserved region is the cysteine-rich domain with a WW-domain, four EF-hands and a ZZ domain, and the C-terminal region (Greener & Roberts, 2000). There is no second actin-binding domain in the spectrin rod domain of *Drosophila* Dystrophin (Amann *et al.*, 1998).
Mutants for the DLP2 isoform of Dystrophin have been shown to develop age-dependent muscle degeneration and mobility defects (Shcherbata *et al.*, 2007). Reduction of Dp117 in the muscle has been shown to result in muscle degeneration in third instar larvae and adult flies (van der Plas *et al.*, 2007 and Chapter 5 of this thesis). Furthermore, Dystrophin is involved in proper axon pathfinding in both photoreceptor neurons and the targeting glial cells (Shcherbata *et al.*, 2007). DLP2 has also been shown to be involved in proper retrograde control of neurotransmitter release at the NMJ (van der Plas *et al.*, 2006 and Chapter 3 of this thesis). A similar role has been found for Dp186 at central neuron synapses (Chapter 4 of this thesis). Finally, a deficiency line, which among a couple of other genes, lacks the entire d*ystrophin* locus, has been shown to develop dilated cardiomyopathy in adult flies (Wolf *et al.*, 2006).

Dystrobrevin
The protein structure of the single fly Dystrobrevin gene, DmDYB, is similar to both its vertebrate counterparts, containing four EF-hands, a ZZ-domain, and a Syntrophin binding region (Greener & Roberts, 2000). The *dystrobrevin* gene is predicted to encode five isoforms (Dystrobrevin A-E). Dystrobrevin is expressed in muscle at muscle attachment sites, the NMJ and at the sarcomeric I-band (Chapter 6 of this thesis). Furthermore, Dystrobrevin is located in the neuropile, at the optic tectum and in both the eye- and wingdisc. Dystrobrevin localization at the muscle attachment sites, in the neuropile and in the eye-disc depends on the presence of Dystrophin (Chapter 6 of this thesis). Reduction of Dystrobrevin expression has shown that Dystrobrevin is involved in maintaining muscle integrity in third instar larvae, development in the pupal stage and proper wing formation (Chapter 6).

Dystroglycan
A single *dystroglycan* gene has been identified in *Drosophila*. The Dystroglycan protein contains all the functional domains of mammalian Dystroglycan; a mucin-like domain, a transmembrane domain and a C-terminal region with a WW-domain (the Dystrophin-binding site), SH2-domains and SH3-domains (Deng *et al.*, 2003). The C-terminal half is the best conserved domain. The *dystroglycan* gene expresses five different isoforms (Deng *et al.*, 2003). Analysis of *dystroglycan* mutants have shown it to be involved in apical-basal polarization of epithelial cells in oocytes, where it acts together with Perlecan (Pcan) and is regulated by EGF receptor signaling (Deng *et al.*, 2003; Schneider *et al.*, 2006; Poulton & Deng, 2006). Pcan and Dystroglycan have a role in maintaining epithelial organization at the basal side of the *Drosophila* follicular epithelium (Schneider *et al.*, 2006).
Dystroglycan and Dystrophin are interdependent for their localization in the basal membrane of the follicle-cell epithelium (FCE) (Schneider *et al.*, 2006) and in wing imaginal discs, suggesting that the interaction between both proteins, present in vertebrates, is conserved in *Drosophila*. Dystroglycan is required to exclude Neurexin IV from the basal membrane domain in the *Drosophila* FCE (Schneider *et al.*, 2006). Dystroglycan is required for stabilizing Discs-large (Dlg) at the lateral membrane, which in turn prevents apical markers and zonula adherens (ZA) components from invading the basolateral membrane domain (Schneider *et al.*, 2006).

Sarcoglycan
Three *sarcoglycans* genes have been found in *Drosophila*, *α/ε-sarcoglycan* (DmSCG-αε; orthologue for *α-* and *ε-sarcoglycan*), *β-sarcoglycan* (DmSCG-β), and *γ/δ-sarcoglycan*

(DmSCG-γδ; orthologue of both *γ-* and *δ-sarcoglycan*). DmSCG-αε is a type-I transmembrane protein and DmSCG-β and DmSCG-γδ are both type-II transmembrane proteins like their vertebrate counterparts (Greener & Roberts, 2000).
Absence of DmSCG-β in the fly eye causes a rough eye phenotype accompanied by defects of ommatidial rotation and production of ectopic cells in the retina (Hashimoto & Yamaguchi, 2006a). This phenotype could be suppressed by reduction of the Egfr signaling related gene rhomboid-1, leading to the hypothesis that DmSCG-β downregulates rhomboid and thereby ERK, and that in the absence of DmSCG-β, rhomboid is ectopically expressed. Furthermore, the localization of βPS integrin was disrupted in flies with reduced *dscgβ* expression (Hashimoto & Yamaguchi, 2006a). DmSCG-β has also been suggested to play a role in onset or progression of mitosis in association with tubulin, due to its changing localization during cell cycle progression (Hashimoto & Yamaguchi, 2006b).

Syntrophin
Two *syntrophin* genes have been identified in *Drosophila*, *Dmsyn-1* and *Dmsyn-2* (Greener & Roberts, 2000). Both proteins contain the characteristic PH domain, interrupted by a PDZ domain and followed by a second PH domain. DmSYN-1 is most homologous to human α1-, β1- and β2-Syntrophin, and DmSYN-2 is most similar to γ1- and γ2-Syntrophin.

## 3. Muscle formation in *Drosophila*

The most prominent functions of Dystrophin are in the muscle and most of the work presented in this thesis focuses on processes in the muscle. Therefore, this section reviews what is known about muscle formation and patterning in *Drosophila*.

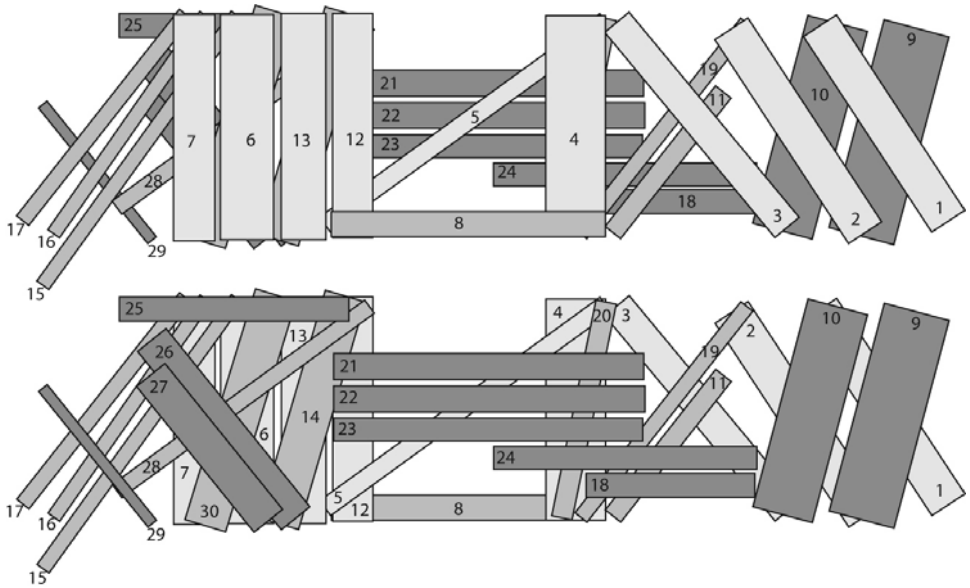


**Figure 3:** A schematic of the Drosophila embryonic muscle pattern viewed from both sides. The dark grey muscles are located closest to the epidermis. Ventral is to the left, dorsal is to the right.

### 3.1 Embryonic muscle formation

Muscle development is extensively studied in *Drosophila* due to its relatively simple architecture and segmentally reiterative muscle fiber pattern (Figure 3). The *Drosophila* embryo and larva contain 30 muscles in each hemisegment, each traceable back to one original founder cell (reviewed in Baylies *et al.*, 1998; Olsen & Klein, 1998). Although all cells in the layer of mesodermal cells initially express Twist (Thisse *et al.*, 1988) and its targets Tinman (Bodmer *et al.*, 1990; Yin *et al.*, 1997) and DMef2 (Lilly *et al.*, 1994; Nguyen *et al.*, 1994; Taylor *et al.*, 1995), they relatively quickly differentiate into units which give rise to different mesodermally derived tissues (Borkowski *et al.*, 1995; Azpiazu *et al.*, 1996;

Riechmann *et al.*, 1997). This division in cell fate is determined partially by secreted proteins, like Wingless and Dpp, emanating from different regions of the overlying ectoderm (Bate & Baylies, 1996; Azpiazu *et al.*, 1996; Tajbakhsh & Cossu, 1997). For instance, Dpp is expressed in a dorsal band of the ectoderm and maintains the expression of Tinman, while blocking expression of ventrally expressed proteins such as pox meso. At the same time, expression of segmentation genes *even-skipped* (*eve*) and *sloppy paired* (*slp*) divides the mesoderm along the anterior-posterior axis of the embryo, separating each segment into two domains that give rise to two different progenitor populations. The *eve* expressing domain gives rise to visceral mesoderm and fat body progenitors and the *slp* expressing domain determines the somatic muscles and the heart progenitors (Baylies *et al.*, 1998).

Furthermore, the level of *twist* expression is also a determining factor; high *twist* expression in mesodermal cells marks them as somatic muscle primordium, whereas low *twist* expression marks visceral and cardiac primordium (Baylies and Bates, 1996). In cells with high *twist* expression, clusters of *lethal of scute* (*l'sc*) expressing cells ensue with only one or two cells in this cluster expressing high levels of *l'sc*. These high expressing cells are the muscle progenitors, while the other cells are fusion competent myoblasts (FCMs) (Schnorrer and Dickson, 2004).
At late stage 11, the muscle progenitors divide once more, resulting in one muscle founder cell and one cell that can be either another muscle founder cell, a progenitor for adult muscle, or it can adopt another undefined fate (Carmena *et al.*, 1998; Ruiz-Gomez and Bate, 1997). These differences in cell fate are the result of asymmetric segregation of Numb between the sibling cells, which results in suppression of the Notch signaling pathway (Ruiz-Gomez & Bate, 1997).

The founder cell hypothesis was first put forward by Bate (1990). Details of subsequent events of myoblast fusion during development *in vivo* was described by Doberstein *et al.* (1997). According to the founder cell hypothesis, the muscle fate is determined by the fate of the muscle founder cells. The FCMs were thought to be naïve and only help with the growth of the muscle (Rushton *et al.*, 1995), but the discovery of the FCM specific genes, *myoblasts incompetent* (*minc*; Ruiz-Gomez *et al.*, 2002) and *lame duck* (*lmd*; Duan *et al.*, 2001) have shown that these cells also have their own pathways of differentiation.
The muscle progenitors are patterned by the influence of Wingless and Dpp from the ectoderm and subsequent RTK signaling (Baylies & Michelson, 2001; Frasch, 1999; Halfon *et al.*, 2000). Important for fusion is the expression of Dumbfounded (Duf also called Kirre) by the founder cells, which acts both as an attractant as well as an adhesion molecule (Ruiz-Gomez *et al.*, 2000; Strünkelnberg *et al.*, 2001), and the corresponding expression of Sticks and stones (Sns) by the FCMs (Bour *et al.*, 2000). Similar results are obtained with Irregular-chiasm-C/Roughest (IrreC/rst) and Hibris (Hbs), both paralogues of Duf and Sns, respectively (Ramos *et al.*, 1993; Strünkelnberg *et al.*, 2001; Artero *et al.*, 2001; Dworak *et al.*, 2001; Dworak & Sink, 2002). IrreC/rst is expressed in both founder cells and FCMs, while Hibris is only expressed in FCMs.
Initial fusion mediated by these proteins is followed by further signaling inside both the founder cells and FCMs to mediate complete fusion. Some of these signaling pathways are founder specific. The discovery of these founder specific cytoplasmic proteins indicates that the asymmetry of the fusion process does not simply reside with surface molecules, but extends deeper in the molecular machinery of the cell (Taylor, 2002). It is thought that similar specific cytoplasmic proteins likely regulate fusion in the FCMs, although they have not yet been identified.

After the fusion process is completed, the body wall muscles attach to epidermal tendon cells, which are analogous to the tendon cells that attach vertebrate skeletal muscles to bone. Tendon cells are located at the segment borders, but also within segments, called segmental and intrasegmental attachment sites, respectively. The first identified tendon cell marker was

the zinc-finger transcription factor Stripe (Piepenburg *et al.*, 2000; Volk and VijayRaghavan, 1994). Tendon cell development is biphasic: first, Stripe expression is induced solely by epidermal signals (Becker *et al.*, 1997), while maturation cannot take place without a signal in the form of the secreted protein Vein, from an approaching myotube (Yarnitzky *et al.*, 1997). Reception of Vein in a tendon cell results in expression of β1-Tubulin or Delilah, markers for terminally differentiated tendon cells. Tendon cells that do not attach to a muscle dedifferentiate and disappear (Becker *et al.*, 1997; Buttgereit, 1996; Yarnitzki *et al.*, 1997). Stripe expression in tendon cells has both an attractive role for myotubes as well as a role in stopping the migration of myotubes at the appropriate place, since in *stripe* mutants, migration to a tendon cell becomes erratic after stage 14 (Becker *et al.*, 1997; Vorbruggen and Jackle, 1997) and some muscles overshoot their target tendon cell (Frommer *et al.*, 1996). In order for each muscle to find the right tendon cell, it appears that each tendon cell expresses specific guidance cues, which attract specific muscles to their correct targets, probably homologous to how specific FCMs find their correct founder cell (Beckett & Baylies, 2006).

### 3.2 Imaginal muscle formation

As described above, not all Twist expressing cells during embryogenesis develop into embryonic muscle. Some cells, born as sister cells to embryonic muscle founder cells, are set aside to become adult muscle precursors (Ruiz-Gomez & Bate, 1997). These cells delay differentiation, maintain Twist expression and proliferate during larval stages. During pupal morphogenesis, these cells contribute to the development of adult muscles. Adult precursors can be found in segment-specific arrangements, eg. in the abdomen, six Twist expressing mesodermal cells are found in each hemisegment (1 ventral, 2 lateral, and 3 dorsal) associated with the peripheral nervous system (reviewed in Roy and VijayRaghavan, 1999).

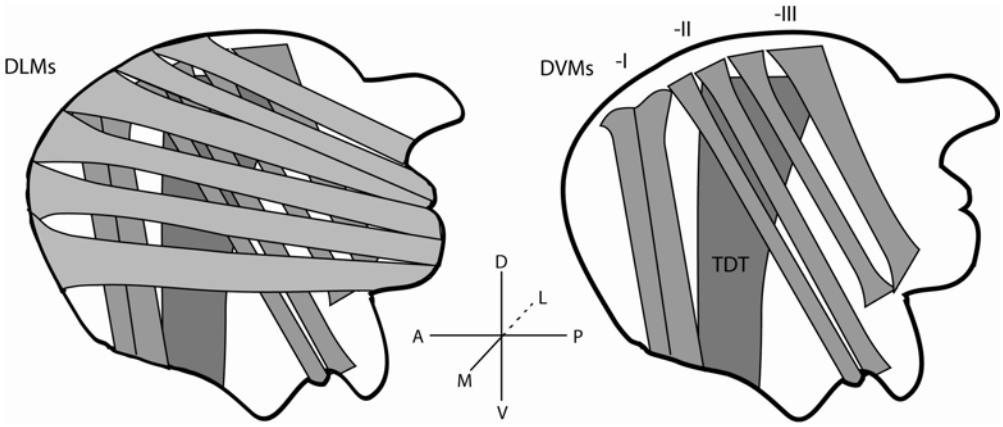


**Figure 4:** A schematic of the thoracic muscle pattern in adult flies viewed once with and once without the overlying DLMs (Dorsal longitudinal muscles). DVMs: Dorso-ventral muscles. TDT: tergal depressor of trochanter. Anterior is to the left, dorsal is up.

During larval stages, the adult precursors divide actively, forming pools of cells in interstices of adult muscles associated to peripheral nerves, and in thoracic segments also associated with imaginal discs at precise locations (Bate *et al.*, 1991; Fernandes *et al.*, 1996). The epidermal cells that will act as attachment sites for the muscles developing from these, imaginal discs associated, myoblasts are characterized by the expression of Stripe in third instar larvae and overly the myoblasts.

Adult myogenesis is best described for the mesothoracic and abdominal segments. Most muscles in the mesothorax belong to the indirect flight muscles (IFMs), which can be divided into two groups: 1) the dorsal longitudinal muscles (DLMs) and 2) the dorsoventral muscles (DVMs) (Figure 4; Roy and VijayRaghavan, 1999). The six DLMs develop from wing disc associated myoblasts using three persistent larval muscles as scaffolds (LOM 1-3) (Fernandes *et al.*, 1991). The DVMs are also derived from myoblasts associated with imaginal discs, but

are formed de novo, without larval muscles as a scaffold (Fernandes *et al.*, 1991). Most of the indirect and direct flight muscles are derived from wing imaginal disc associated myoblasts, whereas myoblasts associated with the mesothoracic leg discs contribute to leg muscles and the tergal depressor of trochanter (TDT) or jump muscle (Lawrence, 1982).
Although this organization implies that each myoblast is predetermined to become a certain muscle early on, transplanted disc associated myoblasts can fuse with abdominal myoblasts to contribute to abdominal muscles, showing that myoblasts are not necessarily dedicated to become part of a specific fiber (Lawrence & Brower, 1982; Roy & VijayRaghavan, 1997). Environmental signals, therefore, apparently help determine a myoblasts' fate, so that when myoblasts are taken out of context and the external signals change, they can contribute to other muscles. It is unclear if the myoblasts that are associated with the peripheral nervous system (Bate *et al.*, 1991; Fernandes & VijayRaghavan, 1993) contribute to particular muscles during myogenesis.

Early in development, IFMs form filopodia with which they search for defined positions on the pupal epidermis to make contact with (Fernandes *et al.*, 1996). The specialized junctions between muscle and epidermis that arise from this contact are called the myotendon junctions (Reedy & Beall, 1993).

## 4. Synapse formation of *Drosophila* motoneurons

The approximately 30 *Drosophila* muscles are innervated by approximately 30 (Ruiz-Canada & Budnik, 2006) to 40 (Koh *et al.*, 2000) motoneurons, whose cell bodies are located in the CNS cortex in the ventral ganglion (Ruiz-Canada & Budnik, 2006). *Drosophila* motoneurons are unipolar in that 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 target. Motoneuronal axons exit the CNS in one of seven different nerve roots that are formed in each hemisegment (Figure 5; the ISN, SN, and TN nerve roots).

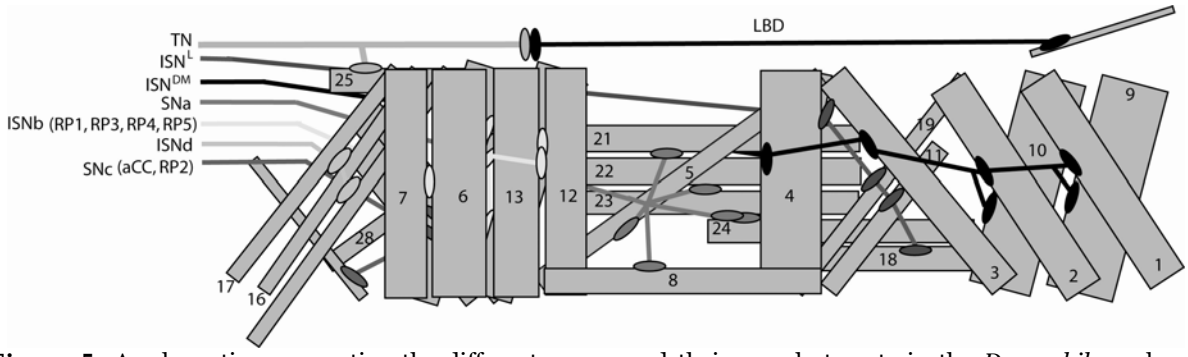


**Figure 5:** A schematic representing the different nerves and their muscle targets in the *Drosophila* embryo. Ventral is to the left, dorsal is to the right.

### 4.1 Axon pathfinding and target recognition

Nerve roots contain axons of motoneurons that innervate certain regions of the body wall muscles (reviewed in Ruiz-Canada & Budnik, 2006). These axons fasciculate together (regulated by cell adhesion molecules such as FasII, FasIII, connectin, and DN-Cadherin (Chiba *et al.*, 1995; Iwai *et al.*, 1997; Iwai *et al.*, 2002; Lin & Goodman, 1994; Lin *et al.*, 1994; Nose *et al.*, 1992; Nose *et al.*, 1997) up to certain critical "decision" points where they defasciculate and bifurcate, using mechanisms mediated by eg. FasII (Lin & Goodman, 1994; Lin *et al.*, 1994), Beat Ia (Fambrough & Goodman, 1996; Pipes *et al.*, 2001), and Sema 1a (Yu *et al.*, 1998). These decisions are likely coordinated by certain cues from the musculature, but these have yet to be defined.

When axons reach the area of their potential targets, target recognition can take place likely mediated by cell surface proteins. For instance, motoneurons expressing a certain cell adhesion molecule, like FasIII or Connectin, will eventually find and contact muscles that also express the same cell adhesion molecule (Ruiz-Canada & Budnik, 2006). However, this does not mean that target recognition is dependent on only one cell adhesion molecule. Instead it is mediated by multiple positive and negative homophilic molecules that together ensure target recognition (Jin, 2002). Synapse formation takes place between axonal filopodia and myopodia from the muscle. If they have found the correct partner, precise synapses form between the cells (Ruiz-Canada & Budnik, 2006).
Usually, at least two motoneurons innervate each muscle, one for innervation by the primary excitatory neurotransmitter glutamate (type Ib and type Is boutons; Jan and Jan, 1976a; Johansen *et al.*, 1989b), and one which has a more neuromodulatory function by releasing molecules such as octopamine or a variety of neuropeptides as well as glutamate (type II and type III boutons; Monastirioti *et al.*, 1995; Cantera & Nassel, 1992; Choi *et al.*, 2004; Gorczyca *et al.*, 1993; Hoang & Chiba, 2001; Landgraf *et al.*, 2003).

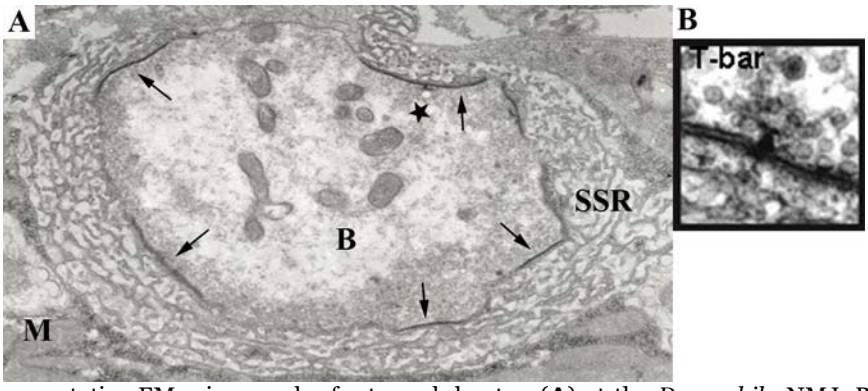


**Figure 6:** A representative EM micrograph of a type 1b bouton (**A**) at the *Drosophila* NMJ. B: bouton (nerve end), M: muscle, SSR: Subsynaptic reticulum, arrows indicate the darker membrane areas representing active zones, asterisk: T-bar structure on an active zone. (**B**) enlargement of a T-bar.

The type I terminals are the most extensively studied termini at the *Drosophila* NMJ (Figure 6). Type-I boutons are surrounded by the subsynaptic reticulum (SSR), which is homologous to a postsynaptic fold in that it is a structure of highly complex invaginations of the muscle plasma membrane around the bouton (reviewed in Ataman *et al.*, 2006). The surface area of the SSR is correlated to muscle size. Glutamate receptors (GluR) cluster specifically at the SSR membrane opposite active zones in the presynaptic membrane (Marrus & DiAntonio, 2004), but other synaptic proteins, such as Shaker-type $K^+$ channels, FasII, Spectrin, DLG, Scribble, Bazooka, and Lin-7 are localized throughout most of the SSR (Ataman *et al.*, 2006). The functions of the SSR are not known, but it is thought to be involved in glutamate uptake (Faeder & Salpeter, 1970), in concentrating large amounts of important molecules for synapse development and function, in rapid transport of molecules required during growth, in local translation of GluRs (Sigrist *et al.*, 2000), and in dividing the $Ca^{2+}$ waves needed for muscle contraction and the $Ca^{2+}$ transients involved in maintaining homeostasis (Frank *et al.*, 2006).
The presynaptic side of a synapse contains a number of synaptic proteins also found on the postsynaptic side, like FasII, Dlg, Shaker $K^+$ channels and another type of GluR receptor, metabolic GluR (mGluR). It also contains proteins required for vesicle loading, release and recycling, such as synaptotagmin, syntaxin, and synaptobrevin (for review see Ataman *et al.*, 2006; DiAntonio, 2006; Schwarz, 2006).

Active zones are the release sites for neurotransmitter containing vesicles. In *Drosophila*, active zones are electron dense areas on the bouton membrane, mostly lined with a specialized electron dense T-like shape, called a T-bar (Figure 6), which is surrounded by vesicles. The composition of T-bars is unknown, but the recent discovery of the Bruchpilot

protein, recognized by the nc82 monoclonal antibody, has identified one crucial component (Wagh *et al.*, 2006; Kittel *et al.*, 2006). *Bruchpilot* mutants lack T-bars and have reduced neurotransmitter release, indicating that although not absolutely required for vesicle release, T-bars are important for normal neurotransmitter release at the *Drosophila* NMJ. Furthermore, *bruchpilot* mutants show a reduction in $Ca^{2+}$ channel density and short-term plasticity is altered (Kittel *et al.*, 2006). Bruchpilot protein seems to cluster $Ca^{2+}$ channels and vesicles to allow efficient transmitter release, supporting the theory that T-bar structures are involved in elevating the probability of local neurotransmitter vesicle release.

### 4.2 Dendrite pathfinding and target recognition

While the axon almost reaches its peripheral target, the dendritic arbor starts to form in the neuropile from the primary neurite (Kim & Chiba, 2004; Sanchez-Soriano *et al.*, 2005; reviewed in Landgraf & Thor, 2006). This starts as the emergence of fine neurites that gradually becomes more elaborate and adopts a treelike pattern. The dendritic arbor of motoneurons is located in the dorsal part of the neuropile, in contrast to the sensory input region, which is located ventrally (Sanchez-Soriano *et al.*, 2005). The location of the dendrites can be described as a neural map, which represents the distribution of the target muscles in the periphery. This conceptual map consists of two dendritic domains at different anteroposterior positions in the neuropile representing the motoneurons innervating the internal muscles, which axons project through the ISN, and the motoneurons extending to the external muscles, which axons project through the SN and TN nerves (Landgraf & Thor, 2006). It is thought (but not demonstrated) that the distribution of the motoneuron dendrites reflects the difference in presynaptic input. Most likely, target recognition and pathfinding in the CNS is mediated by similar groups of proteins as is the case for axons. However, few dendritic guidance cues have been identified to date (Landgraf & Thor, 2006).

### 4.3 Synaptic plasticity during synapse development

The synapse is a dynamic structure which integrates many signals from the neuron and the muscle to determine size and activity throughout development, but also during adult life. The process of constant adaptation of synaptic activity to internal and external signals is called synaptic plasticity and is a vital characteristic for synapse maturation and growth. During development from embryo to larva, the muscle increases dramatically in size. In order to maintain proper innervation of the muscle for contraction during growth, the motoneuron must extend its synaptic contacts to compensate for this increase in muscle volume. The muscle in turn will have to respond to the motoneuron endings being formed by making its own new postsynaptic specialized compartments opposite the new nerve endings. Furthermore, it has been shown in third instar larvae that increasing or decreasing muscle activity also has a profound influence on size and activity of the NMJ (Sigrist *et al.*, 2003). This process of adjusting NMJ size and activity has proven to be dependent on different types of signals from both the nerve and the muscle. For instance, cell adhesion molecules such as FasII, ion channels such as Shaker-type $K^+$ channels, signaling molecules such as members of the BMP pathway, scaffolding proteins such as Dlg and molecules involved in local translation have all been implicated in regulating synaptic plasticity.
Plasticity can be divided into two different types: 1) structural plasticity, where the change in the magnitude of the synaptic transmission is caused by gross structural changes requiring new protein synthesis, and 2) functional plasticity, where the change in synaptic strength is caused without gross structural change, through, for instance, creation of larger neurotransmitter vesicles, an increased probability of release of neurotransmitter vesicles, or a change in distribution of postsynaptic glutamate receptors. Under these conditions, the homeostatic response can be very fast, within minutes (Frank *et al.*, 2006).

Plasticity may be mediated by several different mechanisms, such as synaptic activity and a number of proteins and peptides, for example glutamate, octopamine, amnesiac, insulin, FMRF-amide, BMPs, Wingless, FasII, integrins, and proteoglycans (reviewed in Griffith &

Budnik. 2006). The role of synaptic activity was shown by mutants affecting channel proteins. Hyperexcitable mutants with mutations in potassium channels or their regulatory subunits (Hyperkinetic (Hk), Shaker (Sh), and ether-a-go-go (eag)) or by duplication of the $Na^+$ channel gene *paralytic* (*para*) have increased branching and an increase in bouton number (Budnik *et al.*, 1990). Furthermore, these mutants show vesicle depletion and changes in the number of synaptic densities (Jia *et al.*, 1993). Hypoexcitability can be induced by disrupting *para* splicing with *no action potential* ($nap^{ts}$) mutants. These mutants have a slight reduction in number and complexity of branches and are capable of suppressing the hyperexcitability phenotypes mentioned above. Because $N^+$ channels are only present at the presynaptic side in *Drosophila* NMJs, the above mentioned observations suggest that presynaptic activity signals the changes in NMJ arborization (Griffith & Budnik. 2006).
Perturbations of NMJ function inducing changes in plasticity can result in the activation of several intracellular effectors, such as cAMP, cGMP, PKC, CaMKII, and the RAS/MAPK pathway. However, the exact mechanisms by which plasticity is induced and mediates the changes mentioned above are yet to be unraveled.

### 4.4 Synaptic homeostasis and retrograde signaling

Synaptic homeostasis ensures that synaptic excitation can achieve the physiologically appropriate level. In the case of the NMJ, the appropriate level is enough to make the muscle contract when an action potential reaches the motoneuron.
Synaptic homeostasis was first described in studies of myasthenia gravis (Hughes *et al.*, 2004; Plomp *et al.*, 1992; Xu *et al.*, 1998) where the dysfunction of the acetylcholine receptors was compensated by increasing neurotransmitter release to achieve appropriate levels of excitation. Decreased innervation of the muscle by the motoneuron is somehow sensed in the muscle and this information is transmitted back to the motoneuron causing a compensatory increase in neurotransmitter release. This transmission of information about the level of excitation of the muscle from the muscle to the motoneuron is known as retrograde signaling, the counterpart of anterograde signaling (from nerve to muscle), which is achieved by for instance neurotransmitter or other molecules, such as adhesion molecules or trophic factors, released from the presynaptic side. A retrograde signal is inferred when a postsynaptic defect is compensated by a presynaptic change. It is unclear, whether the retrograde signal is only turned on when homeostasis is disrupted or if the signal is constitutively active and turned off when homeostasis is disrupted. The presynaptic change can be a visible morphological change like synapse growth or increase in active zone size or number, but can also be a functional change, for instance increased probability of release of neurotransmitter containing vesicles.

In *Drosophila*, a retrograde signal for homeostasis has been shown to be active in mutants for the DGluRIIA glutamate receptor subtype. Absence or reduction of DGluRIIA causes a decrease in quantal size. To compensate for this reduction, the quantal content is increased, so that the EJPs, required for proper innervation of the muscle, remain at normal levels (Petersen *et al.*, 1997). However, this retrograde signal seems to respond only to a decrease in excitation, since overexpression of DGluRIIA results in an increase in quantal size, an increase in quantal content and larger than normal EJPs (DiAntonio *et al.*, 1999).

What is the sensor mechanism for homeostasis/retrograde signaling (reviewed in Marques & Zhang, 2006)? One suggestion is that membrane depolarization is sensed. The study of Paradis *et al.*, (2001) shows that membrane depolarization or changes in membrane resting potentials may be sensors for retrograde signaling. This study shows that hyperpolarizing the muscle membrane by overexpressing a 'leaky' potassium channel kir 2.1 leads to a decrease in quantal size, which is compensated by increasing transmitter release to achieve normal depolarization (EJP). However, another study focusing on a modified Shaker $K^+$ channel (EKO) also caused hyperpolarization and decreased quantal size, but failed to show a compensatory response in quantal content (White *et al.*, 2001). This could be due to the less

severe hyperpolarization in EKO overexpressors or possibly signaling molecules involved in retrograde signaling are associated with Kir 2.1, but not with EKO. This second option would suggest that the sensor for retrograde signaling does not sense the depolarization or membrane potential (Marques & Zhang, 2006). Furthermore, Frank *et al.* (2006) have stated that the fast synaptic homeostasis they describe, is not triggered by evoked depolarization or motoneuron activity.

Another possibility is that the molecular composition of glutamate receptors or glutamate receptor density triggers the retrograde signal. Glutamate receptors in *Drosophila* muscle can be one of two types: glutamate receptor A or B. The difference in the two lies in only one of the four different subunits that make up a receptor: DGluRIII (also called DGluRIIC), DGluRIID, DGluRIIE and either DGluRIIA or DGluRIIB (DiAntonio *et al.*, 1999; Featherstone *et al.*, 2005; Marrus *et al.*, 2004; Petersen *et al.*, 1997; Qin *et al.*, 2005; Schuster *et al.*, 1991; reviewed in DiAntonio, 2006). Functionally, this makes a difference in the kinetics of the channels. DGluRIIB quickly desensitizes and is therefore open for a very short time, letting in only small amounts of $Ca^{2+}$. DGluRIIB channels therefore have a marginal effect on muscle depolarization. Depolarization is largely the result of opening of DGluRIIA type channels. Thus, a change in the amount of DGluRIIA versus DGluRIIB channels has an effect on the amount of depolarization (DiAntonio *et al.*, 1999; Petersen *et al.*, 1997).
A low increase in DGluRIIA versus DGluRIIB results in an overcompensated increase of quantal content and larger than normal EJPs (DiAntonio *et al.*, 1999). Likewise, postsynaptic modification of PKA activity levels influences the amount of DGluRIIA versus DGluRIIB. When PKA is deactivated, quantal size increases, but this is not compensated by a decrease in synaptic release, resulting in larger than normal EJPs (Davis *et al.*, 1998). This could indicate that the composition of glutamate receptors is somehow involved in the sensing mechanism. However, a decrease in quantal size through a reduction of DGluRIIA receptors or enhanced activity of PKA is readily compensated for by an increase in transmitter release, indicating that the change in glutamate receptor composition does not hinder the sensing mechanism of the retrograde signal. It is possible that compensation for too little activation of the muscle is much more important (since it is also a result of muscle growth), than correcting overstimulation of the muscle. Perhaps, the retrograde signal or homeostasis in general is not equipped to sense overstimulation, but only registers when stimulation is insufficient for correct muscle function.

A third, and at this point most favoured, possibility is that the retrograde signal is triggered by changes in $Ca^{2+}$ levels. $Ca^{2+}$ enters through the glutamate receptors upon binding of glutamate, causing the depolarization of the muscle membrane. It is possible that this $Ca^{2+}$ entry activates $Ca^{2+}$ sensitive molecules such as Calmodulin or $Ca^{2+}$/Calmodulin-dependent kinase II (CaMKII) and through these molecules activates a signaling pathway that either transduces the retrograde signal or suppresses it. However, when the muscle is depolarized, the depolarization spreads along the membrane and opens other depolarization sensitive $Ca^{2+}$ channels, like the L-type $Ca^{2+}$ channel Ca-α1D, which is located in the T-tubules opposite Ryanodine receptors in the sarcoplasmic reticulum (SR). Opening of these L-type $Ca^{2+}$ channels triggers $Ca^{2+}$ release from the internal stores in the SR, which causes the muscle to contract. It is possible that the amount of $Ca^{2+}$ entry through these L-type $Ca^{2+}$ channels is monitored for homeostasis. This has also been suggested for vertebrate L-type $Ca^{2+}$ channels (Thiagarajan *et al.*, 2005).

A number of molecules involved in retrograde signaling have been identified in the last few years, including Gbb (Aberle *et al.*, 2002; Marques *et al.*, 2002; McCabe *et al.*, 2004; Rawson *et al.*, 2003), CaMKII (Haghighi *et al.*, 2003; Kazama *et al.*, 2003; Morimoto-Tanifuji *et al.*, 2004), Syt4 (Littleton *et al.*, 1999; Adolfsen *et al.*; Yoshihara *et al.*, 2005), PKA (cAMP dependent protein kinase (reviewed by Davis, 1995; Davis *et al.*, 1998)), Amphiphysin

(Razzaq *et al.*, 2001; Zelhof *et al.*, 2001; Mathew *et al.*, 2003), eIF-4E and PABP (Sigrist *et al.*, 2000), and Dystrophin (van der Plas *et al.*, 2006). However, apart from their ability to somehow regulate the retrograde signal, their exact roles remain mysterious.
An alternative candidate for the NMJ retrograde signal is nitric oxide (NO). NO has been shown in *Drosophila* to induce cGMP at the presynaptic side of the NMJ, leading to $Ca^{2+}$-independent vesicle release (Wildemann & Bicker, 1999a; Wildemann & Bicker, 1999b). A caveat to these findings is that it is unclear where NO is formed to induce this pathway. The postsynaptic muscles surrounding the NMJ were observed to lack NADPH staining used to detect NO production.

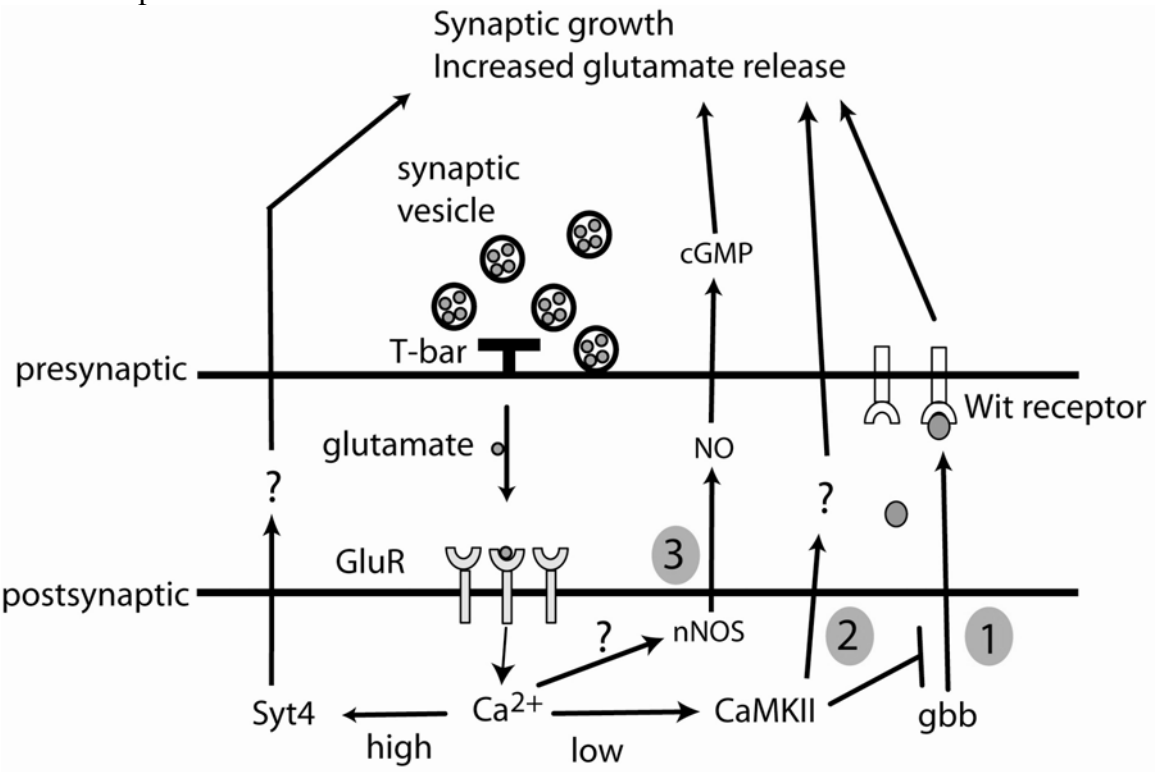


**Figure 7:** A schematic representation of the current model for retrograde signaling.
Pathway 1: Gbb is an inductive signal for retrograde signaling, inhibited by CaMKII.
Pathway 2: Gbb is a permissive signal, CaMKII regulates the unknown retrograde messenger.
Pathway 3: NO is the retrograde messenger.

The current working model for retrograde signaling (Figure 7) involves $Ca^{2+}$ influx through glutamate receptors (Chang *et al.*, 1994), followed by activation of CaMKII (Haghighi *et al.*, 2003; Kazama *et al.*, 2003; Morimoto-Tanifuji *et al.*, 2004) or Syt4 (Littleton *et al.*, 1999; Adolfsen *et al.*, 2004; Yoshihara *et al.*, 2005). However, whereas low activity of CaMKII caused by low activity of glutamate receptors triggers the retrograde signal, Syt4 activates the signal as a response to high $Ca^{2+}$ influx from glutamate receptors (Marques & Zhang, 2006). The activity of Syt4 could be required not for maintaining homeostasis, but for strengthening or weakening the synapse in response to environmental demands, since elevated postsynaptic $Ca^{2+}$ and Syt4 mediate a fast increase in neurotransmitter release during continuous muscle activity (Yoshihara *et al.*, 2005). CaMKII could mediate the response to low activity of glutamate receptors and the resulting low $Ca^{2+}$ influx (Haghighi *et al.*, 2003). Sigrist *et al.* (2003) have shown that increased muscle activity leads to increased glutamatergic neurotransmission at the NMJ and that this is dependent on DGluRIIA and perhaps also on CaMKII and Gbb.

A likely candidate pathway for transmitting the signal that indicates insufficient levels of muscle excitation is the BMP (bone morphogenetic proteins) pathway (Aberle *et al.*, 2002; Marques *et al.*, 2002; McCabe *et al.*, 2004; Rawson *et al.*, 2003). The current working model

for retrograde signaling is that Glass bottom boat (Gbb) is secreted by the muscle either constitutively or as a response to reduced muscle contraction/depolarization. Binding of Gbb to the BMP receptor (a dimer of one type-I receptor, Saxophone or Thick veins, and one type-II receptor Wishful thinking (Wit)) on the presynaptic membrane leads to activation of the BMP pathway, meaning the phosphorylation of Mothers against Dpp (MAD) and activation of Medea (a co-SMAD), which signal the motoneuron to increase synaptic size and the amount of neurotransmitter released (McCabe *et al.*, 2004; Rawson *et al.*, 2003).

Re-expression of Gbb in muscle in *gbb* mutants, reestablishes the nuclear accumulation of MAD in the motoneuron and the NMJ size, which neuronally expressed Gbb cannot. However, it does not rescue the reduced EJP amplitude. Re-expression of Gbb in the motoneuron however, does return the EJP amplitude to normal, despite the small NMJ size. This could mean that Gbb is required in the motoneuron for the retrograde signal to work and that this is independent of NMJ size regulation. However, another possibility is that effectivity of the retrograde signal is dependent on synaptic activity. Motoneuronal Gbb has been shown to be required for signaling retrogradely to its innervating interneurons (Baines, 2004). Reduced transmission from the interneuron to the motoneuron in *gbb* mutants may lead to reduced motoneuron output, regardless of the retrograde signal from the muscle (Marques & Zhang, 2006).

The significant reduction in NMJ size in *gbb* and *wit* mutants have led to discussion on whether the BMP pathway is part of the retrograde signal, or if perhaps it is required for synapse maturation and growth and is thus a permissive signal (Sanyal *et al.*, 2004; van der Plas *et al.*, 2006). Even though it has been shown for DGluRIIA mutants, inhibition of CaMKII and reduction of Dystrophin that the Wit receptor is required for the increase of quantal content seen, activation of the BMP pathway as evidenced by increased P-MAD staining in motoneuron nuclei was not observed (van der Plas *et al.*, 2006). However, there is evidence that the Wit receptor activates a P-Mad-independent pathway, in parallel to the canonical pathway, by way of dLIMK1 (Derynck & Zhang, 2003; Eaton & Davis, 2005). Another possibility is that the BMP pathway is required for the retrograde signal to be possible via another route, or possibly, synapses may become unresponsive to retrograde signals when they are inappropriately formed as in *wit* mutants (Marques & Zhang, 2006).

The presynaptic pathway for the increase in neurotransmitter release as a result of retrograde signaling is still unclear. However, a recent study on a fast type of homeostatic regulation has revealed the requirement for a presynaptic $Ca^{2+}$ channel, Cacophony (Frank *et al.*, 2006). It is unclear, whether fast synaptic homeostasis operates through the same pathway as slow synaptic homeostasis.

## 5. Electrophysiology in *Drosophila*

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. First of all, since the fruit fly nerves do not have to extend so far in order to reach the muscle, *Drosophila* neurons do not need the characteristic myelin sheath.
Secondly, active zones in *Drosophila* have a unique T-shaped electron dense structure called a T-bar (Figure 6), not present in mammals, which is hypothesized to be involved in vesicle release due to the fact that these structures are surrounded by vesicles.
Thirdly, the principal neurotransmitter at the NMJ in *Drosophila* is glutamate. Acetylcholine is used as neurotransmitter in synapses in the CNS (as well as glutamate). This is in contrast to the mammalian system, where acetylcholine is the principal neurotransmitter at the NMJ, while glutamate is used at certain synapses in the CNS, for example in Hippocampal neurons and Purkinje cells.

Fourthly, in mammals the postsynaptic fold is a series of invaginations of the muscle membrane which has acetylcholine receptors at the crests opposite the active zones and $Na^+$ channels at the troughs of these pits. Binding of acetylcholine to its receptor, initiates a depolarization, which in turn opens the $Na^+$ channels in the troughs. The result is an action potential which is propagated over the muscle membrane. In *Drosophila*, the postsynaptic membrane is called the subsynaptic reticulum (SSR) and is a vast maze of curvatures of the muscle membrane, hypothesized to help in distinguishing between the $Ca^{2+}$ waves needed for muscle contraction and the $Ca^{2+}$ transients that might be involved in maintaining homeostasis (Frank *et al.*, 2006). The glutamate receptor fields are located opposite the active zones. When glutamate binds its receptor, the receptor allows $Ca^{2+}$ to enter the muscle, which causes the muscle membrane to depolarize. There is no evidence for $Na^+$ channels in *Drosophila* muscles (Pichon & Ashcroft, 1985; Hille, 1992; Kraliz & Singh, 1996) and there is no action potential generated upon depolarization of the membrane. The absence of an action potential in the *Drosophila* muscle is one of the reasons why it is a desired 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. Furthermore, *Drosophila* muscles and NMJs are much easier to access and dissection is relatively simple. *Drosophila* third instar larval NMJs can be measured at room temperature, maintaining their electrical properties for many hours.

Electrophysiology was performed to study the function of the *Drosophila* NMJ. In order to record from the muscle, a glass electrode filled with KCl is inserted in the muscle cell. To stimulate the motoneuron innervating the muscle, the nerve is cut at the neuropile and taken up by a suction electrode connected to a pulse generator (see Figure 8 for setup). Two types of signals can be recorded from a muscle in this way: 1) spontaneous small depolarizations of the muscle membrane, called miniature excitatory junction potentials or mEJPs. These are hypothesized to represent the activation of glutamate receptors as a result of the spontaneous release of a single neurotransmitter vesicle or quanta from the motoneuron, therefore also called the quantal size. The amplitude of the mEJPs gives information about the amount of neurotransmitter in a vesicle and the amount of glutamate receptors able to respond. 2) large depolarizations of the muscle membrane evoked by stimulation of the motoneuron, called (evoked) excitatory junction potentials or (e)EJPs. These are fired when an action potential in the motoneuron triggers the release of many neurotransmitter containing vesicles. These two measurements are often combined to calculate the quantal content, which is the amount of vesicles released upon stimulation of the motoneuron. The quantal content can be calculated by dividing the EJPs by the mEJPs (amount of vesicles released = amount of depolarization by multiple vesicles/amount of depolarization by one vesicle). However, since the measurements are done in current clamp, meaning that the amount of current flowing through the recording electrode is constant, and we therefore measure membrane depolarization directly in voltage, we have to add a correction to the calculation to compensate for nonlinear summation. This is based on the observation that the amount of neurotransmitter needed to achieve a certain membrane potential change is dependent on the membrane potential. When the membrane potential (normally in *Drosophila* muscle in HL3 buffer at -60mV) rises toward 0, the amount of neurotransmitter in a vesicle will cause a smaller and smaller depolarization. In order to correct for this underestimation of the total amount of vesicle released, a formula has been created by Martin (1955) for mouse NMJs and adapted by B.A. Stewart (personal communication) for the *Drosophila* NMJ:

$$EJP'' = EJP'/(1-f*(EJP'/Vr-Vm)) = EJP'/(1-0.4*(EJP'/50))$$

Where: EJP'' represents the average EJP amplitude corrected for nonlinear summation
EJP' represents the average EJP amplitude normalized to a Vm of -60mV
F = 0.4
Vr represents the reversal potential = -10mV
Vm represents the membrane potential = -60mV

This correction is only necessary in current clamp mode. It is also possible to measure in voltage clamp. In this method, a second electrode is inserted into the muscle, which injects current into the muscle when it depolarizes to keep the membrane potential constant. In this case, not the change in potential, but the amount of current needed to keep the membrane potential constant is measured.

Similar electrophysiological techniques can be used at CNS interneuronal synapses as well (Chapter 4 of this thesis; Baines & Bate, 1998; Baines, 2003). In that case, a patch electrode is inserted in aCC or RP2 motoneuronal cell bodies located in the neuropile of first instar larvae. Whole cell voltage clamp is used to measure the depolarization caused by spontaneous signals from the innervating interneuron, EJCs, which are comparable to the EJPs recorded at the NMJ. Tetrodotoxin (TTX) is added to the medium to reduce actionpotential formation in order to measure spontaneous miniature currents, mEJCs, comparable to mEJPs at the NMJ.

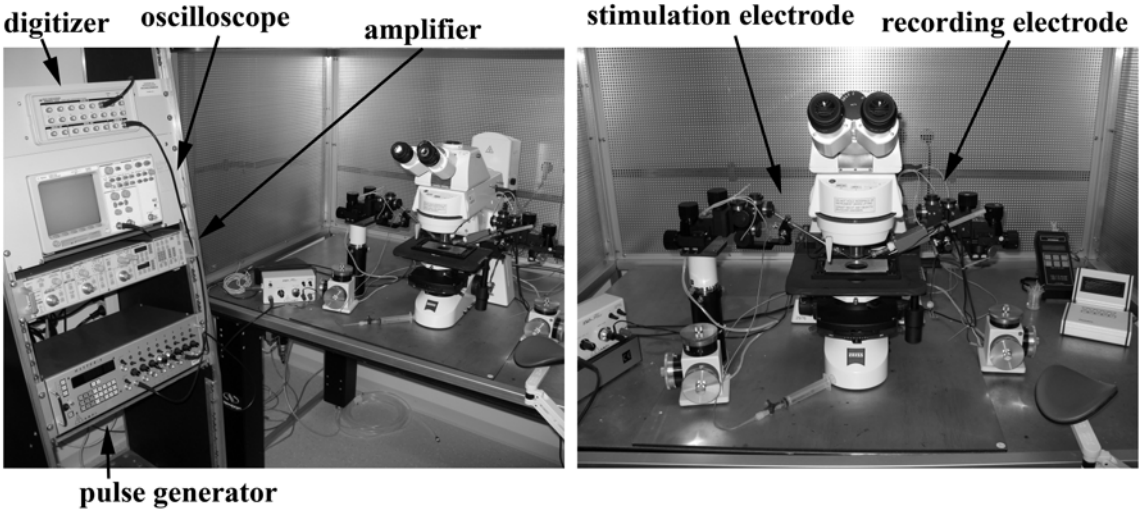


**Figure 8:** The set up for the electrophysiology measurements. A stimulus is sent from the pulse generator via the stimulation electrode to the NMJ of a third instar larva. The muscle depolarization is measured with the recording electrode, which sends a signal to the amplifier to make the depolarization visible on the oscilloscope. Meanwhile the signal is also sent to the digitizer to convert the analog signal to a digital signal so it can be recorded on a computer for analysis.

## 6. Outline of this thesis

In this thesis, our findings on the function of *dystrophin* in the musculature and nervous system of *Drosophila* are described. In the second Chapter, the expression of known DGC member homologues in *Drosophila* is examined using RNA in situ hybridization. It is shown that most of the DGC members in *Drosophila* are expressed in similar tissues as in the mammalian system and that many are coexpressed in these tissues. In the third Chapter, the role of the long Dystrophin isoform, DLP2 at the NMJ is studied. In the absence of DLP2 in the muscle, the quantal content increases, indicating a novel function of Dystrophin in retrograde signaling. It is shown that the BMP receptor Wit is required for the increase in quantal content in the absence of DLP2, although activation of the downstream effector Mad could not be detected in DLP2 mutants. The fourth Chapter describes the finding that Dp186 performs a similar function at CNS synapses as DLP2 does at the NMJ. The fifth Chapter describes the onset of activity dependent muscle degeneration in *Drosophila* body wall muscles in the larval and adult stage when Dp117 expression is reduced. Furthermore, it describes a requirement for Dp117 in survival. The sixth Chapter summarizes our findings on the function of Dystrobrevin in *Drosophila*.

In summary, this thesis describes the function of Dystrophin in *Drosophila* and shows that the large amount of *dystrophin* isoforms present in *Drosophila* can have both a similar function in different tissues, as well as different functions in the same tissues. This can give us important information about crucial protein domains for the exertion of different functions as well as clues as to the roles of Dystrophin in mammalian muscle where also many different isoforms are present as well as some other homologous genes. For instance, the different roles uncovered in this thesis for DLP2 and Dp117 in the muscle could be indicative of the different roles of Dystrophin and Utrophin in mammalian muscle. Furthermore, the role of Dp186 in CNS synapses can be reminiscent of the role of Dp71 in the occurrence of mental retardation in a portion of DMD patients. Further studies of these roles in *Drosophila* could therefore give new insight into the onset and progression of DMD and also help to uncover new therapies.

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