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

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CHAPTER 6

Discussion

Does a Dystrophin Glycoprotein Complex (DGC) exist at the *Drosophila* NMJ?

A number of the conserved orthologs of the mammalian DGC are present in the central nervous system and the musculature of *Drosophila* (this study; [1–3]). In mammals, the main components of the DGC, Dystrobrevin (DYB), Dystrophin (DYS) and Dystroglycan (DG), are in a complex in most tissues (reviewed in the Introduction of this thesis). Until the studies presented in this thesis, it was not clear whether a DGC exist in *Drosophila*, however, all the members of the mammalian DGC where known to be present in highly conserved form in the *Drosophila* genome. In contrast to the mammalian DGC members, in *Drosophila* there is usually only one gene for each DGC member, however, many of the fly's DGC components have, similarly to their mammalian counterparts, multiple distinct protein isoforms. For example, *Drosophila Dyb* encodes 7 isoforms; DYB-PA, DYB-PC, DYB-PD, DYB-PF, DYB-PG, DYB-PH and DYB-PI. Nothing is known about the expression pattern of the individual DYB isoforms, since the anti-DYBCO_2H that we generated, binds to the carboxyterminal part of the protein that is common to all isoforms. The fly's *Dys* gene encodes at least six different protein isoforms, three long isoforms, DLP1, DLP2, and DLP3 and three shorter isoforms, Dp205, Dp186, and Dp117 [2]. Some DYS isoforms, such as DLP2, are present at the postsynaptic side of the NMJ, while Dp186 is present in central neurons in the brain. *Dg* encodes three isoforms, DG-RA, DG-RB and DG-RC [4]. In the embryo, DG-RC is expressed in the central and peripheral nervous system whereas DG-RB is predominantly present at the dorsal median cells, gut and peripheral glia and at muscle attachment sites [5].

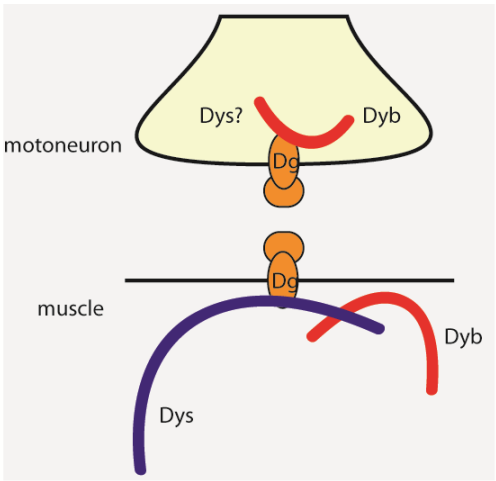

Figure 1. A Model for a DGC complex at the *Drosophila* NMJ.

Do the *Drosophila* DGC members form a protein complex? In **Chapter 3.1 and 4**, I show that DYB localization is dependent on the presence of DYS and that DYS and DYB localization are both are dependent on DG. From these results, I conclude that there is a complex present at the *Drosophila* NMJ that contains, at least, DG, DYS and DYB. Furthermore, I conclude that DG is the main component that anchors DYS and DYB at the synapse. I have also shown that DYB and DG are present at both pre- and postsynaptic compartments, while the DYS long isoform DLP2 previously has been shown to be present at the postsynaptic site [2]. DLP1 is unlikely to be the DYS isoform responsible for localizing DYB at the presynaptic site of NMJ, since it cannot be detected at this site with the available antibodies. Other DYS isoforms may be present there and have roles at the motoneuron terminal such as Dp186 or Dp117. Van der Plas et al. [6] showed that muscle integrity was affected only when Dp117 was reduced by RNA interference at the postsynaptic site, however, it is conceivable that Dp117 localized at the presynaptic membrane acts as a docking molecule for DYB. Since we do not have Dp117-specific antisera, we cannot determine its exact location at this moment.

Electrophysiological studies show that DG and DYB function both pre- and postsynaptically. Previously, Bogdanik and colleagues [7] argued that DG is required mainly postsynaptically at the NMJ. Our results, however, also indicate a presynaptic role for this protein. Our, in part, conflicting data is possibly caused by the fact that we used different ectopic expression tools for the rescue and RNA interference experiments. Bogdanik and colleagues reduced DG levels in

the presynaptic compartment by using a pan-neuronal driver (elav-GAL4) which also drives expression in interneurons. We previously showed that the lack of Dp186 at the postsynaptic motoneuron, led to increased neurotransmitter release from the interneurons [3]. It is likely that reducing DG levels using the elav-GAL4 driver interferes with the recordings of muscle depolarization at the NMJ. In our study, we used the OK6-GAL4 driver which only drives transgenic expression at the motoneuron site of the NMJ and we found that decreased DG expression (by RNA interference) either in the pre- or postsynaptic compartments leads to a decrease in the neurotransmitter release. In addition, we performed rescue experiments using the *Dg* mutant and found that overexpression of the DG-RB spliced isoform postsynaptically, restores the neurotransmitter release to the wild type level. Overexpression of the DG-RC spliced isoform presynaptically also rescued the *Dg* mutant. These results suggest that the pre- and postsynaptic roles of DG might be performed by distinct isoforms.

In conclusion, we would like to propose that at least some of the conserved members of the mammalian DGC co-localize at the *Drosophila* pre- and postsynaptic side of the NMJ to from a DGC-like complex. DG and DYB have roles both at the pre- and postsynaptic sites (**Fig. 1**), while the DYS DLP2 isoform functions at the postsynaptic side to control neurotransmitter release. The DYS isoform located and playing a potential role at the presynaptic site might be Dp117 or Dp186. Besides the main three DGC components described and studied in my PhD project, there are other potential DGC members in *Drosophila*, such as the Sarcoglycans and the Syntrophins, whose location and roles at the NMJ are at present largely unknown.

DYB acts through the Rho GTPase and CaMKII signaling pathways to modulate transmitter release at the NMJ

The increase in neurotransmitter release observed in the *Dys* mutant is likely caused by the absence of another DGC member, DYB. The work presented in this thesis provides 4 pieces of evidence why this might be the case. Firstly, in the *Dys* deficiency (in which all DYS isoforms are absent) DYB protein is not localized at the NMJ, while at the *Dyb* mutant NMJ, DYS is still localized. Secondly and importantly, the *Dyb* mutant exhibits increased neurotransmitter release despite the presence of DYS at the NMJ, suggesting that *Dyb* is downstream of *Dys*. Thirdly, expression of a DYB fusion protein that is targeted to bind to the Disc-large protein in the subsynaptic reticulum, rescues the DYS-deficient NMJ back to wild type levels. Finally, we show that both *Dyb* and *Dys* genetically interact with members of a Rho-GTPase signaling pathway, indicating that DYB and DYS likely signal through the same pathway (**Chapter 3.1**).

Rho-GTPase signaling pathways have been implicated in actin cytoskeleton remodeling, vesicle trafficking and many other biological processes. In **Chapters 2 and 3.1**, we demonstrate that *Dys* and *Dyb* interact with Crossveinless-C (*cv-c*). CV-C is a member of the Rho-GAP family, which, together with the Rho-GEFs and Rho-GDIs, controls the nucleotide state of the Rho GTPases that cycle between an inactive GDP-bound and an active GTP-bound state. CV-C regulates the Rho pathway by converting the active form of CDC42 (CDC42-GTP) into the inactive form (CDC42-GDP) (**Fig. 2A**). Both the $Dys^{DLP2\ E6}$ and Dyb^{11} mutants display genetic interactions with the $cv\text{-}c^{1}$ mutant. How a lack of DYS affects the level or activity of CV-C is not yet clear. It is possible that DYS or DYB is required for CV-C localization at the NMJ. However, we cannot examine this possibility by immunohistochemistry at present since no anti-CV-C antibodies are available. From genetic interaction studies using a defined *Dys* mutant wing phenotype and from electrophysiology analyses, we conclude that *Dys* and *cv-c* likely work in the same or parallel pathways. The working model we propose is that the lack of DYS or DYB leads to the accumulation of active CDC42, which either directly or indirectly activates retrograde signaling resulting in increased neurotransmitter release (**Fig. 2B**). This model is

supported by our studies that show that constitutively-active Rho GTPase CDC42 expressed at the postsynaptic site in an otherwise wild type background results in increased neurotransmitter release. The increase in the neurotransmitter release when activated CDC42 is expressed can be counteracted by expression of constitutively-active CaMKII, a protein which has been shown by others previously to be involved in retrograde signaling [8].

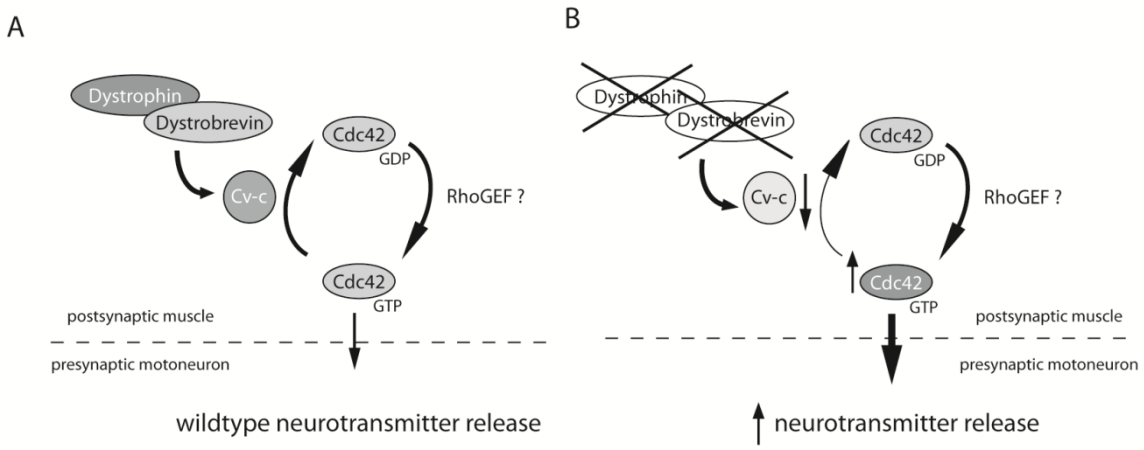

Figure 2. Rho GTPase signaling is downstream of *Dys* and *Dyb*. (**A)**. In the wild type animal, CV-C (a Rho-GTPase activating protein), increases the catalytic rate of the CDC42 protein resulting in increased conversion of the active CDC42-GTP protein into the inactive CDC42-GDP form, leading to normal neurotransmitter release. **(B)** In the absence of DYS or DYB Cv-c cannot reduce the activity of CDC42, resulting in an abnormal accumulation of active GTP-bound CDC42, which leads to increased presynaptic neurotransmitter release.

Another study that likely supports the role of Rho-signaling at the NMJ is that describing the function of dPix [9]. dPix, a *Drosophila* Rho-type guanine nucleotide exchange factor (Rt-GEF), is localized to the PSD and coverts the inactive-Rho-GDP to the GTP-bound active form. The *dpix* mutants exhibit a decrease in the level of neurotransmitter release and therefore show the opposite phenotype to that of the *cv-c* mutant, emphasizing the importance of Rho signaling at the NMJ.

Other potential substrates of CV-C, i.e., Rho and Rac, did not show any genetic interactions, such as that exhibited by CDC42, with *cv-c* indicating substrate specificity of this Rho-GTPase in this pathway. Rho-GTPase signaling does not only play roles in the postsynaptic compartment, since there is evidence that it is also required at the presynaptic side. It has been reported that *cdc42* interacts genetically with the presynaptic Rho-GEF gene, *ephexin* (*exn*), whose protein product functions downstream of the Ephrin receptor to regulate the Cacophony (Cac) calcium channel which is required for the compensatory increases in neurotransmitter release observed in the *GluRIIA* glutamate receptor mutant [10]. The potential roles of DYB in Rho-GTPase signaling in the presynaptic compartment remain to be elucidated.

Both CDC42 and CaMKII are important for the role of DYB in presynaptic neurotransmitter release (**Chapter 3.1**). Decreased levels of CDC42 or expression of constitutively-active CaMKII suppress the increase neurotransmitter release observed in the Dyb^{11} mutant. The increase in the neurotransmitter release when activated CDC42 is expressed is blocked by the expression of constitutively-active CaMKII. However, the lack of CDC42 does not suppress the increased neurotransmitter release observed when CaMKII function is inhibited. We conclude, therefore, that CaMKII is acting downstream of CDC42 to control transmitter release. Since there is no biochemical data to support a direct interaction between CaMKII and CDC42, it is not possible at this point to conclude that CaMKII is a substrate of CDC42. Many other questions remain, such as, when and at what side of the synapse is the activation of CDC42

required for appropriate synaptic homeostatic endpoints, how does *CaMKII* inhibit retrograde signaling, what are CDC42's postsynaptic substrates, etc.?

Other signaling molecules associated with DYB at the *Drosophila* NMJ

We generated a *Dyb* mutant by homologous recombination that lacks DYB protein (**Chapter 3.1**). Analysis of this mutant reveals interesting similarities in the postsynaptic roles of DYS and DYB in the regulation of presynaptic neurotransmitter release. However, the mechanism for controlling synaptic homeostasis is likely to be quite different. Synaptic homeostasis at the *Drosophila* NMJ in response to muscle growth is maintained by at least two known mechanisms which are a) modulation of the number of synaptic boutons and b) regulation of the number of active zones that release neurotransmitter. The increased neurotransmitter release in the Dys^{DLP2E6} mutant is likely caused by an increase in the number of active zone with T-bars, since no change in the bouton numbers is observed in these mutants. Conversely, the number of active zones with T-bars is not changed in the *Dyb* mutant, but the number of bouton is increased. This increased number of boutons results into an enlargement of the NMJ synaptic terminal. The mechanisms that underlie the enlargement of the NMJ have been studied in detail in *Drosophila* and some of the molecules involved in growth and homeostasis of the synaptic terminal have been identified.

One of these signals is cyclic adenosine monophosphate (cAMP). NMJ growth is enhanced when cAMP levels are increased. The cAMP specific phosphodiesterase mutant *dunce* ([11], [12]) shows a very similar synaptic overgrowth phenotype [13]. This enzyme is important for cAMP degradation; the lack of function of this enzyme leads to elevated cAMP levels. Another member of the cAMP pathway, *rut* encodes a Calcium/calmodulin-responsive adenylyl cyclase which is important for cAMP synthesis. Therefore, *rut* mutants have a decreased cAMP production which leads in turn to the presence of fewer boutons ([12], [14], [15]). Since, cAMP is implicated in the activation of protein kinases such as Protein kinase A (PKA) and, since I have shown that DYB interacts with the regulatory subunit of PKA (PKA-R) (**Chapter 3.2**), it is conceivable that the absence of DYB affects cAMP dependent signaling at the NMJ. When cAMP binds to specific locations on the PKA-R subunit, it causes a dissociation between the regulatory and catalytic (C) subunits, thus activating the catalytic subunits and enabling them to phosphorylate a series of substrate proteins. The absence of DYB may lead to decreased levels of PKA-R, resulting in an increase of cAMP levels. At present, we have not yet examined the levels of cAMP or PKA-R in the *Dyb* mutant.

Binding of the PKA catalytic subunit to the PKA regulatory subunit not only inactivates its activity but the R subunit also protects the C subunit against degradation. In the $pka\text{-}RII^{EP(2)2162}$ mutant, RII levels are reduced by 95% and the cAMP-induced PKA catalytic activity is reduced by 60%, suggesting that the C subunit is degraded in this mutant [16]. A result that supports the existence of a possible low level of PKA activity in the *Dyb* mutant is the observation that the phenotype of the heterozygous *Dyb* allele was enhanced by low activity of PKA (**Chapter 3.2**). Electrophysiological recordings also indicate that the *Dyb* mutant may have decreased PKA activity since increasing PKA activity in the *Dyb* mutant background restores the high QC back to the wild type level. In conclusion, the roles of DYB in synaptic plasticity may prove to be mediated by cAMP-PKA signaling.

Another molecule implicated in modulation of NMJ growth and size is Fasciclin II (FasII). FasII is a cell adhesion molecule located at both the pre- and postsynaptic membrane. FasII controls presynaptic sprouting by functioning downstream of cAMP [17], [18] . However, since FasII

staining was not affected in the *Dyb* mutant there are, at this point, no indications for an interaction between these two genes.

Ultrastructural examination of larval NMJs that lack DYB reveals an abnormal accumulation of polysomes, clusters of ribosomes, within the subsynaptic reticulum (SSR) (**Chapter 3.1**). Polysomes are part of the translational machinery and are possibly involved in local translation of mRNAs at the synapse. Increased accumulation of polysomes was also found when the translation initiation factors eIF4E and poly(A)-binding protein (PABP) were overexpressed at the larval NMJ [19]. The eIF4E and PABP proteins were found to aggregate throughout the SSR while the presynaptic compartment was free of polysomes. Larvae also exhibited increases in NMJ size and of the amount of neurotransmitter released as was observed in the *Dyb* mutant. Therefore, increased accumulation of the components of the translation machinery at the SSR seems to correlate with an increased number of synaptic boutons.

Another mutant in which enlarged synaptic terminals at the larval NMJ are observed is the *PAR-1* mutant [20]. PAR-1 kinase encodes a conserved Ser/Thr kinase that can phosphorylate the Discs-large protein (DLG). DLG is a member of the membrane-associated guanylate kinase (MAGUK) family and serves, together with PSD95, as a scaffold for many synaptic proteins [21–24]. Loss of *PAR-1* leads to increased synapse size (more boutons) and elevated synaptic transmission. PAR-1 phosphorylation of DLG negatively regulates its mobility and targeting to the postsynaptic side of the NMJ. DLG is less likely to be involved in the NMJ enlargement in *Dyb* mutants since we did not observe a change in the DLG staining pattern in the *Dyb* mutant.

Model for Signaling via a Putative Dystrophin Glycoprotein Complex at the *Drosophila* NMJ

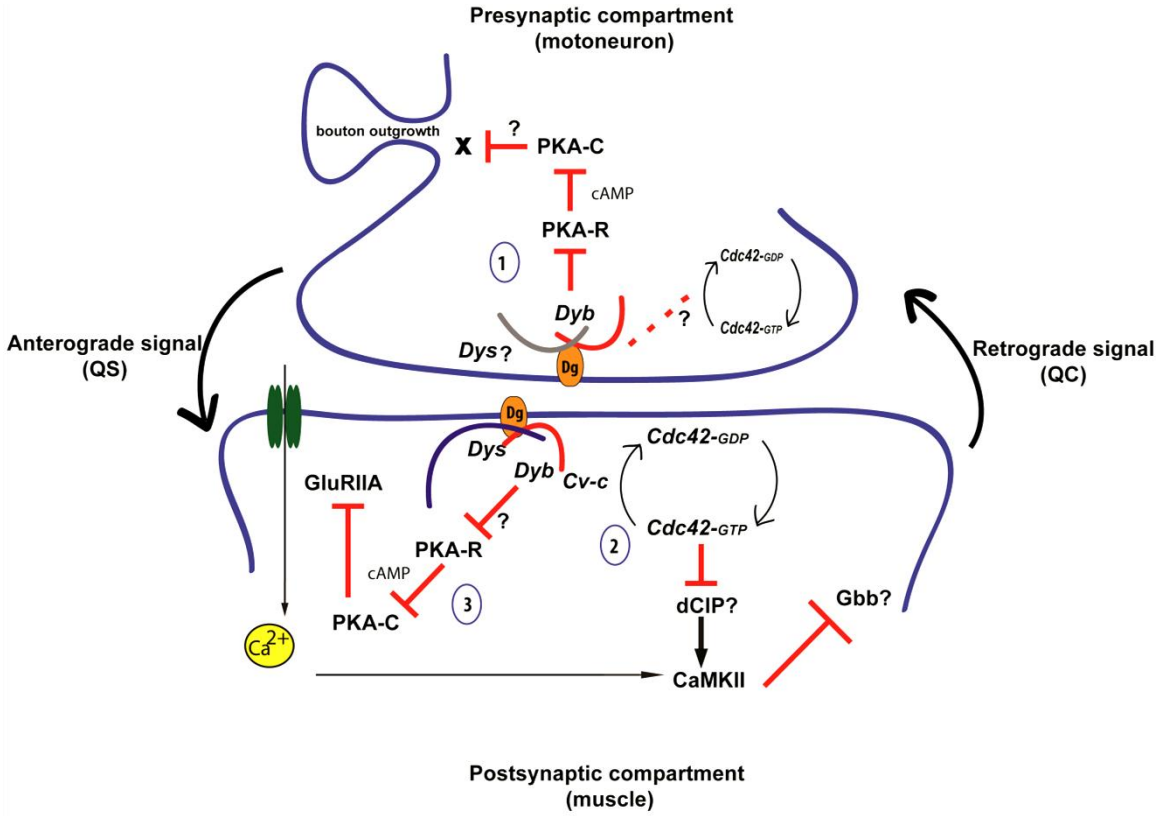

Figure 3. A **schematic representation of the possible roles of several DGC members at the *Drosophila* NMJ. Pathway1**: DYB at the presynaptic side interacts with the cAMP and the PKA cascade to regulate synaptic

strength. **Pathway 2 and 3**: DYB at the postsynaptic side mediates retrograde control of transmitter release via the PKA, Rho-GTPase and CaMKII signaling pathways.

During larval development, the muscle increases in size approximately 100 fold. This growth is depending on bi-directional communication between the motoneuron and the muscle mediated by anterograde and retrograde signaling pathways. Wingless, a ligand of the Wnt family, acts as one of the developmental regulators for anterograde signaling required for synaptic growth, while the TGF-β members Glass bottom boat (Gbb) and Wit are the primary developmental signals for the retrograde pathway controlling synapse size (reviewed in [25]).

Retrograde signaling emanating from the postsynaptic side to regulate the amount of transmitter release is believed to play an important part in regulating synaptic homeostasis. However, the identity of the diffusible molecule transported from the post to the presynaptic side (or potential trans-synaptic protein) is still unclear. A protein implicated in retrograde signaling is Gbb, known as the bone morphogenetic protein (BMP) homolog Glass bottom boat. Gbb is thought to act as a muscle-derived signal acting through a presynaptic receptor complex consisting of Wishful thinking (wit), Thickveins (Tkv) and Saxophone (Sax) [26–28]. Receptor activation leads to phosphorylation of mothers against decapentaplegic (Mad) which in turn regulates the transcription of a battery of target genes.

In this thesis, we reported that a pathway acting downstream of the DGC in the postsynaptic compartment is the Rho-GTPase signaling pathway (**Fig. 3, pathway 2**). The lack of either DYB or DYS apparently leads to an increase of the active form of CDC42 resulting in increased neurotransmitter release. In **Chapter 3.1**, we showed that the increase in neurotransmitter release by overexpression of CDC42 is blocked by CaMKII overexpression. This could be interpreted to mean that CDC42 negatively regulates CaMKII activity. Consistent with these findings are reports by others showing that reduced levels of CaMKII activity stimulate retrograde signaling [8].

Even in the absence of direct evidence showing that CaMKII inhibits Gbb, a model in which a Cdc42-dCIP4-Wsp mediated pathway that attenuates retrograde signaling has been proposed ([29], [30]). Nahm et al. showed that the *Drosophila* CDC42-interacting protein 4 (dCIP4) functions postsynaptically downstream of CDC42 to activate Wasp, a protein which has been implicated in the regulation of endocytosis. A decrease in the dCIP4 and Wsp activities result in increased levels of extracellular Gbb. Thus, the CDC42-dCIP4-Wsp-Arp2/3 pathway may play an inhibitory role in Gbb secretion and CaMKII may regulate this pathway. One study of single dendritic spine plasticity showed that CDC42 activation depends on CaMKII activity [31]. Taking the results of these studies together, a possible pathway controlling retrograde signaling emerges including CDC42, dCIP, CaMKII and GBB with DYS and DYB acting as essential scaffolds to localize some members of this pathway.

PKA

A postsynaptic role for PKA at the *Drosophila* NMJ has been previously uncovered [32]. Since we demonstrated an interaction of PKA-R and *Dyb*, this pathway may also be involved in altering the synaptic homeostatic endpoint in the *Dyb* mutant (**Fig. 3; pathway 3**). The authors of the previous study reported that low PKA activity in the postsynaptic compartment resulted in increased quantal size (mEJP amplitudes). This increased quantal size was diminished in the *DGluRIIA* mutant background and it was proposed that phosphorylation of GluRIIA by PKA modulates quantal size by reducing the receptor's activity. Alterations in presynaptic PKA levels did not suppress the increase in quantal size. Interestingly, decreased presynaptic activity of PKA resulted in increased EJPs and an increase in quantal content.

The presynaptic role of PKA is clearly different from its role at the postsynaptic side. The question remains as to whether changes in PKA activity at the presynaptic side regulate the amount of vesicle release.

The role of the nervous system specific dystrophin isoform, Dp186, in the olfactory system

One in three DMD patients present with mental retardation, an aspect of the disease that seems to be unrelated to the severity of muscle degeneration but does correlate with poor prognoses for the disease progression. The absence of the short Dystrophin isoform, Dp71 is commonly believed to be linked to these cognitive impairments in DMD patients [33], [34]. Little is still known, however, about the molecular mechanisms that underlie the cognitive symptoms of DMD. We have started to study the roles of the DYS Dp186 CNS specific isoform in the *Drosophila* olfactory system. The *Drosophila* olfactory system is an excellent system to study the establishment, maintenance and plasticity of neural circuits. In **Chapter 5**, we describe a role for Dp186 in synaptic transmission of primary olfactory neurons and in olfactory avoidance behavior. We show that Dp186 is localized at primary olfactory synapses and that $Dys^{Dp186\ 166.3}$ mutants exhibit a decreased output from these neurons in electroantennograms. This defect in transmission is accompanied by a reduced response to adverse odors in an odor avoidance behavioral assay. Interestingly, we do not find any clear morphological abnormalities in the patterns of the olfactory sensillae in the antennae, nor in the axon guidance patterns of the olfactory neurons and their synaptic terminals. These results further indicate important roles of the DGC in synaptic efficacy and might shed further light on the mental deficits present in DMD patients.

Future Perspectives

Future experiments elucidating the DGC's role at the synapse will include the identification of the CDC42 postsynaptic targets whose deregulation affects transmission at the *dystrophin* and *cv-c* mutant NMJs. Rho-GTPases do not covalently modify their targets but act, at least in part, by relieving auto-inhibitory protein interactions. Thus, conventional proteomic approaches cannot be employed to identify the postsynaptic targets of CDC42. Therefore, we can start by performing a candidate-based screen for potential CDC42 targets by testing the interactions of genes, previously shown to interact with *cdc42* in other *Drosophila* tissues, with *cdc42* at the NMJ.

Furthermore, the putative Rho-GEF(s) that counteracts the Rho-GAP CV-C in the regulation of CDC42 at the NMJ can possibly be identified using a similar genetic approach. We can, for example, express RNA interference transgenes targeting individual known Rho-GEFs in the *cv-c*, *dystrophin* or *dyb* mutant backgrounds. Upon reduction of the relevant Rho-GEF, we expect to observe suppression of the increased quantal content seen in the mutant background, since there should be a significant reduction in the conversion of the inactive GDP-bound CDC42 to the active GTP bound state. One promising candidate for a postsynaptic CDC42-specific Rho-GEF is Ephexin which has been shown to interact with CDC42 and the Ephrin receptor in a *Drosophila* presynaptic homeostatic pathway regulating Ca^{2+} influx [9]. There is the possibility, however, that multiple Rho-GEFs may target CDC42 postsynaptically and they might therefore not be identified using such a single gene-directed approach.

DLP2 and Dp186 mutants display similar phenotypes at the NMJ and the brain interneuronal synapse, respectively, increased evoked presynaptic neurotransmitter release when the

postsynaptic Dystrophin isoform expression levels are reduced. We have not yet observed any visible adult phenotypes in the Dp186 mutants, thus genetic screens for Dp186-interacting genes in other tissues like the wing or the eye are not feasible at this moment. However, as discussed above, DLP2 and Dp186 may likely interact with the same or similar proteins at both synapses. Thus, to further elucidate the roles of Dp186 at brain interneuronal synapses, we will ascertain the possible roles of newly uncovered DLP2-interacting genes in maintaining wild type interneuronal synaptic physiology in a candidate-based screen, similar to that described above for the NMJ.

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