Muscle Dystroglycan Organizes the Postsynapse and Regulates Presynaptic Neurotransmitter Release at the Drosophila Neuromuscular Junction

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Abstract

Background: The Dystrophin-glycoprotein complex (DGC) comprises dystrophin, dystroglycan, sarcoglycan, dystrobrevin and syntrophin subunits. In muscle fibers, it is thought to provide an essential mechanical link between the intracellular cytoskeleton and the extracellular matrix and to protect the sarcolemma during muscle contraction. Mutations affecting the DGC cause muscular dystrophies. Most members of the DGC are also concentrated at the neuromuscular junction (NMJ), where their deficiency is often associated with NMJ structural defects. Hence, synaptic dysfunction may also intervene in the pathology of dystrophic muscles. Dystroglycan is a central component of the DGC because it establishes a link between the extracellular matrix and Dystrophin. In this study, we focused on the synaptic role of Dystroglycan (Dg) in Drosophila.

Methodology/Principal Findings: We show that Dg was concentrated postsynaptically at the glutamatergic NMJ, where, unlike in vertebrates, it controls the concentration of synaptic Laminin and Dystrophin homologues. We also found that synaptic Dg controlled the amount of postsynaptic 4.1 protein Coracle and alpha-Spectrin, as well as the relative subunit composition of glutamate receptors. In addition, both Dystrophin and Coracle were required for normal Dg concentration at the synapse. In electrophysiological recordings, loss of postsynaptic Dg did not affect postsynaptic response, but, surprisingly, led to a decrease in glutamate release from the presynaptic site.

Conclusion/Significance: Altogether, our study illustrates a conservation of DGC composition and interactions between Drosophila and vertebrates at the synapse, highlights new proteins associated with this complex and suggests an unsuspected trans-synaptic function of Dg.

Introduction

In muscle fibers, the Dystrophin-glycoprotein complex (DGC) is thought to provide an essential mechanical link between the intracellular cytoskeleton and the extracellular matrix. This complex comprises Dystroglycan subunits (α and β), Dystrophin or Utrophin, sarcoglycans subunits, syntrophin subunits, and dystrobrevin subunits. Dystroglycan (Dg) is a central component of the DGC because it establishes the transmembrane link between Laminins and Dystrophin [1,2]. Dg is encoded by a single gene (dag1) [3], and is expressed as a propeptide that gets cleaved in two fragments: α and β-Dg, which associate non-covalently in mature skeletal muscle. The extracellular Dg α-subunit is heavily glycosylated, and interacts with extracellular Laminin, whereas the transmembrane β-subunit interacts with subsarcolemmal Dystrophin, which itself links the actin network. The maintenance of this structural link provides stability of the sarcolemma, especially upon muscle contraction. In Duchenne muscular dystrophy, mutation and subsequent loss of Dystrophin destabilizes the other DGC members [4], which further leads to mechanical damage of the muscle cell membrane [5,6]. There are no known dystrophies associated with a mutation in the dystroglycan gene, probably because of the importance of the encoded protein in other cellular types. Indeed, loss of Dg in mice leads to early embryonic death [7]. However, mutations in enzymes responsible for the glycosylation of α-Dg are the cause of congenital myopathies such as the Walker-Warburg syndrome, the muscle-eye-brain disease and Fukuyama-type congenital muscular dystrophy, for review [8,9].

Most members of the DGC are found to be concentrated at the cholinergic neuromuscular junction. Moreover, mouse studies revealed abnormalities of NMJs lacking DGC components [10-15].
suggesting a role for a defective neurotransmission in some dystrophies [16,17]. For example, NMJs without muscle Dg show decreased levels of synaptic Laminin and Urothrin, as well as smaller acetylcholine receptor clusters [17–20]. A more general function of the DGC in synaptic transmission is further supported by the localization of DGC components in central brain synapses [21–23] and the occurrence of mental retardation in myopathic patients that do not show any major brain structural defect [24]. In this study we focused on the synaptic role of Dg, using the Drosophila glutamatergic larval NMJ. This synapse is not only a neuromuscular junction, but also a well-established model to study the development, the structure and the function of a glutamatergic synapse [25]. We showed that Dg is concentrated postsynaptically at this NMJ. We analyzed the status of Dg partners at this synapse and found that 1) like in vertebrates, Dg controls synaptic Laminin and Dystrophin concentration, 2) Dg also controls the amount of postsynaptic 4.1 protein Coracle, the postsynaptic spectrin cytoskeleton, and the relative subunit composition of glutamate receptors, and 3) reciprocally, both Dystrophin and Coracle are required for Dg concentration at the synapse. Finally, electrophysiological analysis shows that loss of muscle Dg leads to a functional defect, i.e. a decrease in presynaptic glutamate release.

Materials and Methods

Fly stocks

yw CS flies were used as a control in all experiments with dg or cora mutants. dg-RNAi/+ or UAS-Dg-C/+ flies were used as controls when studying crosses with these transgenes. The 24B-Gal4 line was used for all muscle expression experiments [26]. dg 2K3 and dg 2K2 alleles as well as UAS-Dg-C transgenic flies have been previously described [27]. The genotype of larvae overexpressing Dg-C in our experiments is 24BGal4/UAS-Dg-C. We also used the piggybac insertion 2K3 [28]. The 2K directed RNAi construct [27] originally on chromosome 3 was remobilized on chromosome 2 (line n12), and flies containing the two RNAi transgenes over wild-type chromosomes were used for all immunocytochemistry experiments. We created a UAS-Dg-C-GFP construct using the Gateway system and by inserting the PCR amplified sequence of Dg (LD11619) in pWG. The Dys-COOH RNAi transgenic flies were described in [29]. The hypomorph cora 2 allele was described in [30]. This mutation leads to the replacement of R1607 by a stop codon. The null cora [k08713] allele was analyzed in [31]. This mutation leads to the replacement of R1607 by a stop codon [30]. This mutation leads to the replacement of R1607 by a stop codon.

Generation of polyclonal antibody

The Dystroglycan C-terminal polyclonal antibody (LG5) was raised in New Zealand rabbits by repeated intra dermal injections. Synthetic peptide of the last 7 C-terminal amino acids of Human Dystroglycan (PPPVYPP) was conjugated via a cysteine residue to hexanoic acid to the keyhole limpet hemocyanin and then purified. The purified peptide was used as antigen according to a previously described protocol. The resulting polyclonal antibody was purified and characterized as previously described [32]. Note the Drosophila 7 C-term sequence is PPPVYSP.

Immunocytochemistry

Larvae were dissected in PBS 1x, EDTA 1mM, and then fixed in fresh 4% paraformaldehyde (Sigma, L’Isle d’Abeau, France) for 20 min. for all stainings, apart from GluRIIA and GluRIIC ones. For these stainings, preparations were fixed for 15 min. in Bouin’s fixative (Sigma, L’Isle d’Abeau, France). Antibody incubations were performed in PBS 1x buffer with 0.3% Triton X100 and 0.2 to 1% BSA. The following antibodies were used: Goat anti-HRP (Sigma, L’Isle d’Abeau, France, 1:1000), mouse monoclonal anti-alpha-Spectrin 3A9 (1:25), mouse monoclonal anti-Dg 4F3 (1:100), mouse monoclonal anti-DGlurRIIA 8B0D2 (1:50), mouse monoclonal anti-Fas2 1D4 (1:20), all four were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); rabbit anti-GluRIIC (gift from A. DiAntonio, 1:3000) [33], rabbit anti-Dgex3 (gift from M. Schneider, 1:1000) [29], rabbit anti-Dys (gift from A. Wodarz, 1:1000) [29], rabbit anti-Laminin (gift from L. Fessler, 1:1000) [34] and guinea-pig anti-Cora raised against a 2–2KB fragment from the 3` half of the coding sequence (bp 2193–2425 of cDNA1, see [35]), (gift from R. Fehon, 1:2500). Alexa 488, Cy3 or Cy5-conjugated anti-rabbit, anti-guinea pig, anti-goat or anti-mouse were obtained from Molecular Probes and Jackson ImmunoResearch, (WestGrove, PA) and used at dilutions ranging from 1:1000 to 1:250. Images were acquired with a Biorad 1024 or a Zeiss LSM 510 Meta confocal microscope. Quantification of DGlurRIIA and DGlurRIIC staining intensities was performed with imageJ. For each channel, a threshold was set and used for all images. The sum of pixel intensities (S) above threshold was calculated for each channel and the ratio of S (DGluRIIA)/S(DGluRIIC) calculated for each image.

Co-immunoprecipitation and immunoblot analysis

Drosophila heads from 24B Gal4+; UAS-Dg-C-GFP/+ flies where homogenized in lysis buffer (50mM Tris pH8, 100mM NaCl, 1% NP40, 1mM EDTA, protease inhibitor cocktail) during 45’ at 4°C. The lysate was centrifuged at 16000 g at 4°C for 20 min. The supernatant was incubated with anti-GFP antibody (A6455, Molecular Probes) O/N at 4°C. Next, we added protein-A conjugated sepharose beads (ProteinA Sepharose CL-4B, GE Healthcare) at 4°C for 3 hours. After incubation, the complex was precipitated at 4000g for 2 min., washed 4 times in lysis buffer, eluted in Laemmli buffer and separated on a 4–12% SDS-PAGE gel (4–12% GelBage, Interchim) followed by transfer onto nitrocellulose membrane (Hybond-C, Amersham Biosciences). Membranes were incubated overnight at 4°C with primary antibody diluted in TBS-T (50mM Tris pH 7.4; 150 mM NaCl and 0.2% Tween 20) containing 5% nonfat dried milk. Dilutions were performed in PBS 1x buffer with 0.3% Triton X100 and 0.2 for 1% BSA. The following antibodies were used: Goat anti-HRP (Sigma, L’Isle d’Abeau, France, 1:1000), mouse monoclonal anti-shaggy (4G-1E, Upstate). Membranes were washed three times for 10 min. with TBS-T and incubated with secondary antibodies for one hour at room temperature. Membranes were finally washed three times in TBS-T and signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

For analysis of the specificity of the Cora antibody, Drosophila larvae from the following genotypes: Canton S, P[E(yg2) cora (YW7390);+/4; 24B Gal4/+] and P[lacW]cora 2K3 (YW7390;+/4) were dissected in PBS 1x, EDTA 1mM and then homogenized in lysis buffer (50mM Tris pH8, 100mM NaCl, 1% NP40, 1mM EDTA, protease inhibitor cocktail). Proteins were separated by SDS-PAGE (8%) and transferred electrophoretically onto nitrocellulose membranes (Hybond-C, Amersham Biosciences). Membranes were incubated overnight at 4°C with primary antibodies: anti-Cora, Guinea-pig, 1:10000, then anti-Tubulin DM1A, Mouse, 1:1000, and the procedure continued as described above.
Quantitative PCR analysis

Dg (CG18250) transcript levels were measured to compare the strength of different dg mutant conditions. The following genotypes were tested Canton S, dg^{PBac{RB}}/dg^{PBac{RB}e01554}, and dg^{PBac{RB}e01554}/dg^{PBac{RB}e01554} transheterozygotes. cDNAs were generated from 1 μg total RNAs treated with DNase I by using random hexamers and Moloney murine leukemia virus reverse transcriptase (LTI). Real-time PCR was done using Applied Biosystems (Courtaboeuf, France) SYBR Green PCR mix according to the manufacturer’s instructions. PCR was done as follows: 10 minutes at 95°C followed by 40 cycles: 15 seconds at 95°C, 60 seconds at 60°C. Housekeeping genes used to normalize dg expression levels were RPL13, TBP and PGK. Sequences of the primers are: RPL13

Electrophysiology

Two-electrode voltage clamp recordings were obtained at 22°C from VLM 6 in segments A2 and A3, of late third instar larvae, as previously described in [36]. All cells selected for analysis had resting potentials between ~60 and ~70 mV and the input resistance was ≥4 MΩ. Student’s tests were performed for statistical analysis.

Results

Dystroglycan is a postsynaptic component of the Drosophila NMJ

In order to identify proteins of the Dystrophin-glycoprotein complex conserved at the Drosophila NMJ, and to study these molecules in a model organism, we undertook a screen of many molecules in a model organism, we undertook a screen of many mutants. Since known null mutants of dg are lethal at the late embryonic, first instar larval stages, we used a housekeeping genes used to normalize dg. The Piggybac element insertion PBac{RB}e01554 in dg led to a 90% decrease in all dg transcripts (Fig. S1). In third instar larvae transheterozygous for dg^{PBac{RB}e01554} and the dg null allele dg^{PBac{RB}e01554}, the Dg synaptic staining was strongly reduced with the LG5 antibody (Fig. 1C) and with the Dgex8 antibody as well (Fig. S1). Finally, when we overexpressed the Dg-C isoform in muscle cells, we observed a large increase in NMJ Dg staining (Fig. 1D), as was already previously reported [39], confirming the synaptic localization of this protein. Overexpressed Dg also aggregated in discrete patches (arrows in Fig. 1D). Altogether, these data show that Dystroglycan, notably the Dg-C isoform containing the mucin-like domain, is endogenously concentrated at the larval NMJ, mainly on the postsynaptic side.

To look in more detail at the fine synaptic localization of Dystroglycan, we expressed a Dg-C-GFP tagged isoform in the muscle. Like the PSD-95 homologue Discs-Large (Dlg) [40], Dg-C-GFP is partly excluded from the postsynaptic densities facing the active zones (Fig. 1E). These latter were labelled with an antibody directed against Bruchpilot (BRP) [36,41] (Fig. 1). This suggests that Dg-C localization is mainly perisynaptic.

Dystroglycan controls varicosities Laminin concentration

In vertebrates, loss of Dg in muscle cells was reported to suppress Laminin concentration at the cholinergic NMJ [19]. We tested if this was also the case at the Drosophila glutamatergic NMJ. First, we investigated whether Laminin was indeed present at the Drosophila NMJ. Laminin is a heterotrimer consisting of three chains, A, B1 and B2. We used a polyclonal antibody against Laminin heterotrimer [34]. We could detect some Laminin (Lam) staining at the muscle surface, like in mammalian muscle cells, and could detect Lam immunoreactivity at the NMJ (Fig. 2A). This could detect Lam immunoreactivity at the NMJ (Fig. 2A). At this level of optical resolution, the NMJ localization of Lam fits with a trans-synaptic localization of this component of the extracellular matrix. Containing with the active zone marker Bruchpilot revealed that, like Dg, Lam was excluded from synapses (Fig. 2A) and restricted to the perisynaptic zones. Lam were present around varicosities as well as around the inter-varicosities connectives (Fig. 2B). In larvae expressing dg-RNAi in muscles, the Lam content around the varicosities was decreased so that the varicosities were less distinguishable compared to the connectives (Fig. 2C). This situation was similar and even more pronounced in dg mutants (Fig. 2D). We also analyzed the consequences of Dg overexpression in the muscle cell on the amount and localization of Lam. We used the Dg-C isoform, containing the mucin domain, which is supposed to play a critical role in Lam binding of Dg [29,42]. Lam patches, never observed in control conditions, appeared extrasynaptically and colocalized with Dg-C-GFP (Fig. S2). This indicated that the Dg-C isoform could indeed recruit Lam in the muscle cell. At the NMJ, the pattern of Lam localization was more irregular, with some perisynaptic areas larger, and more intense in Lam immunoreactivity, compared to a more homogenous situation in controls (Fig. 2E). Altogether, these data show that Laminin localization around varicosities is partly dependent on Dg. We then tested the interaction between Dg and its second well-known partner, Dystrophin, at the synapse.

Dystroglycan controls postsynaptic Dystrophin concentration

Dg was shown to be partly responsible for the synaptic concentration of Utrophin at the vertebrate cholinergic NMJ [18]. In Drosophila, there is only one gene homologous to utrophin and dystrophin genes, called dystrophin [43]. This gene encodes for different protein isoforms. Large isoforms are concentrated postsynaptically at the NMJ [44]. To test whether synaptic
Dystrophin (Dys) localization was dependent on Dg, we used an antibody directed against a C-terminal sequence of Dys, which detects all known Dys isoforms [29]. As previously described with an antibody directed against large isoforms of Dys, we observed a postsynaptic localization of Dys at the NMJ (Fig. 3A). Dg mutants displayed a strongly reduced Dys staining (Fig. 3B1-3). This reduction was not due to a general absence of the postsynaptic apparatus since postsynaptic marker Discs-Large (Dlg) staining was present in these mutants (Fig. 3B4). This indicates that Dg is required for the normal postsynaptic Dys localization. To test whether Dg was also sufficient for this Dys localization, we overexpressed postsynaptically Dg-C. In these NMJs, Dystrophin

**Figure 1. Localization of Dg at the NMJ.** A-D) Dg (A2, B2, C2, D2) and HRP (A3, B3, C3, D3) immunoreactivity at the NMJ of muscle 4. Merge of both images with Dg in magenta and HRP in green (A1, B1, C1, D1). The LGS antibody was used to analyze Dg localization. (A) In wild-type (ywCS) flies, Dg concentration is clearly visible at type Ib boutons. Dg immunoreactivity is much larger than motoneuron-specific HRP immunoreactivity, indicating that Dg is present in the muscle cell, i.e. postsynaptically. (B) In larvae expressing dg-directed RNAi in the muscle using the 24B Gal4 driver, the NMJ LGS staining was decreased in intensity. (C) In larvae mutant for dg (dg0135/dg323), the NMJ LGS staining was more strongly affected. (D) In larvae overexpressing Dg-C isoform in the muscles using the 24B Gal4 driver, a clear increase in LGS immunoreactivity was observed (laser intensity was decreased to avoid too much signal saturation in this genotype) both at the NMJ, and also in distant patches (arrows). (E) Two serial sections (every 0.7 μm) of a synaptic varicosity of a larva expressing a Dg-C-GFP construct in the muscle cells (with the 24B Gal4 driver). GFP fluorescence (E2) is present in the subsynaptic reticulum (SSR) (arrow) and is partially excluded from the sensus-stricto synapses (arrowhead). Synapses are labelled with the active zone marker Bruchpilot (BRP)(E3). Merge of both images with Dg-GFP in green and BRP in magenta is shown in E1. (F) Dg staining with anti-Dgex8 antibody, specific of the Dg-C isoform (F2) and HRP (F3). The Dgex8 immunoreactivity is similar to the LGS immunoreactivity (A2). Again, Dgex8 immunoreactivity is much larger than motoneuron-specific HRP immunoreactivity (F1), indicating that Dg-C protein is present at the postsynapse. In all panels, a muscle 4 NMJ is shown. Scale bar is 10 μm in A–D, 5 μm in E and 10 μm in F.
immunoreactivity was strongly enhanced around synaptic boutons (Fig. 3C), indicating that postsynaptic Dg can indeed recruit or stabilize Dys. Altogether, these data show that Dg controls Dys concentration at the glutamatergic NMJ. Dystrophin is known to be required for Dg sarcolemmal localization in vertebrate muscles [4]. We wondered whether Dys was also required for normal Dg localization at the Drosophila NMJ.

To test this hypothesis, we used RNAi interference to target all

Figure 2. Laminin localization at the drosophila NMJ varicosities is influenced by dg. A) Serial sections of two adjacent varicosities (every 0.7 μm) labelled for BRP (green) and Lam (red) to analyze Lam localization. There is no colocalization between the active zone marker BRP and the Lam staining, indicating that Lam is mainly perisynaptically localized. B–D) Lower magnification of NMJ stained for Lam (B2,C2,D2) and HRP (B3,C3,D3). The merge image is shown in B1,C1,D1 (Lam in red and HRP in blue). (B) In wild-type (ywCS) larvae, Lam is present both in varicosities and inter-varicosities connectives. (C) In larvae expressing dg-directed RNAi in the muscle using the 24B Gal4 driver (dg-RNAi/+; 24B Gal4/dg-RNAi), the varicosity Lam staining was decreased in intensity, whereas the connective staining remained unchanged. (D) In larvae mutant for dg (dge01554/dg323), the Lam varicosity staining was strongly affected, but not the connective staining. (E) In larvae overexpressing Dg-C isoform with the 24B Gal4 driver, no clear increase in Lam immunoreactivity was observed at the NMJ. However, larger Lam stretches were observed (see arrows in E1, E2 and in the insert showing a double BRP (green), Lam (red) staining of a synaptic bouton in this genotype). In addition Lam patches (arrowhead) appeared in this genotype. N.B. an immunoreactive trachea is visible in E1 and E2 (asterisk). In all panels, a muscle 4 NMJ is shown. Scale bar is 10 μm.

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Figure 3. Dg controls synaptic Dys localization. Triple staining for HRP (blue), Dys (red) and Dlg (green) in control (ywCS) (A), dg mutant (B) and Dg-C overexpressing (C) larvae. Merge images are shown in A1, B1, C1. Single channel stainings are shown in A2, B2, C2 for HRP, A3, B3, C3 for Dys and A4, B4, C4 for Dlg. In dg00154/dg223 mutant larvae, the Dys staining (B3) is almost absent compared to wild-type larvae (A3), although the Dlg staining is still present (compare A4 and B4). When Dg-C is overexpressed with the 24B Gal4 driver, Dys staining is strongly enhanced around HRP positive boutons, and also in the entire muscle cell (compare C3 and A3). In all panels, a muscle 4 NMJ is shown. Scale bar is 10 μm.

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Expression of the RNAi transgene led to a very strong decrease in postsynaptic Dys immunoreactivity (Fig. 4A–C). This lack of postsynaptic Dystrophin was accompanied by a decrease in postsynaptic Dg (Fig. 4D, F), although not a complete loss of Dg. Hence, at the *Drosophila* NMJ, Dys partially controls Dg synaptic concentration. Altogether, our data illustrate the presence of a synaptic Lam/Dg/Dys complex, like in vertebrate neuromuscular junctions. What can be the functional consequences of the loss of this complex in absence of Dg? At the cholinergic NMJ in mammals, Dg is involved in synapse function through rapsyn-mediated anchoring of the nicotinic acetylcholine receptors [19,45,46]. We wondered whether Dg is also required for the clustering of glutamate receptors and if so, through which cytoplasmic scaffold. We first looked at the synaptic protein Coracle, reported to control some glutamate receptor subunits localization at the *Drosophila* NMJ [31].

Dystroglycan also controls intracellular Coracle NMJ concentration

Coracle (Cora) is the *Drosophila* 4.1 protein homologue. It has been shown to be concentrated at the NMJ and to control the amount of GluRIIA subunit in postsynaptic glutamate receptors [31] in late embryos and first instar larvae. The *cora* gene encodes four protein isoforms (ranging from 699 to 1698 amino acids) [35]. In Chen et al. [31], the 9C monoclonal antibody directed against the FERM domain of all Cora isoforms [35] was used, and no clear Cora immunoreactivity could be detected at the NMJ of late larval developmental stages. Here, we demonstrated that in third instar larvae, Cora was concentrated around the NMJ (Fig. 5A), using a polyclonal guinea-pig antibody directed against a sequence present only in large Cora isoforms [35]. We tested the specificity of the stainings we obtained in flies with genetically altered levels of Cora protein, where the intensity of the staining should parallel the amount of protein. We used the *cora*14 hypomorph mutant [30] and the null mutation *cora* k08713 [31] as well as flies overexpressing *cora*14. In *cora*14/*cora* k08713 third instar larvae, the NMJ Cora staining was strongly reduced, indicating that this staining was specific and that Cora was actually present postsynaptically in the domain rich in subsynaptic reticulum (SSR) (Fig. S3). We also overexpressed Cora in muscles, using the 24B Gal4 driver and the P element insertion P{EPgy2}cora[EY07598] upstream of the translation start of all Cora isoforms. This produced an increased staining in the SSR as well as in the whole sarcolemma (Fig. S3). These data further confirmed the ability of this antibody to recognize Cora. We observed that in larvae expressing a *dg* directed RNAi and in *dg* mutants, the synaptic concentration of Cora was markedly affected (Fig. 5 B,C,E,F). No clear change in intensity was observed for the sarcolemmal immunoreactivity. These data showed that Dg was required for the NMJ localization of Cora. We further tested whether Dg was also sufficient to concentrate Cora at the synapse, by overexpressing Dg-C in the muscle. This led to a very large increase in the amount of postsynaptic Cora (Fig. 5D), but not to an overall homogenous increase of Cora immunoreactivity at the muscle surface. However, many cytoplasmic Cora clusters could be found, in a way similar to Laminin clusters when Dg-C was overexpressed (arrows in Fig. 5D). In Dg-C-GFP overexpressing larvae, the Cora

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**Figure 4. Dys controls Dystroglycan postsynaptic concentration.** (A–C) Double staining for Fas2 (A green, B), Dys (A magenta, C) on control (1) and UAS-dys-RNAi flies crossed with 24B Gal4 (2). In larvae expressing the dys-RNAi (dys-RNAi/+; 24B Gal4/+), there is almost no Dys immunoreactivity detectable at the NMJ. (D–G) Triple staining for Fas2 (D blue, E), Dystroglycan (D green, F) and Coracle (D red, G) in the same genotypes. Dg postsynaptic labelling is reduced in absence of postsynaptic Dystrophin. Coracle immunoreactivity is also reduced, but to a much lower extent compared to the loss of postsynaptic Dg. (H) alpha-Spectrin immunostaining on the same genotypes. Scale bar is 10 µm. In all panels, a muscle 4 NMJ is shown. doi:10.1371/journal.pone.0002084.g004

Dystrophin isoforms in the muscle [29]. Expression of the RNAi transgene led to a very strong decrease in postsynaptic Dys immunoreactivity (Fig. 4A–C). This lack of postsynaptic Dystrophin was accompanied by a decrease in postsynaptic Dg (Fig. 4D, F), although not a complete loss of Dg. Hence, at the *Drosophila* NMJ, Dys partially controls Dg synaptic concentration. Altogether, our data illustrate the presence of a synaptic Lam/Dg/Dys complex, like in vertebrate neuromuscular junctions. What can be the functional consequences of the loss of this complex in absence of Dg? At the cholinergic NMJ in mammals, Dg is involved in synapse function through rapsyn-mediated anchoring of the nicotinic acetylcholine receptors [19,45,46]. We wondered whether Dg is also required for the clustering of glutamate receptors and if so, through which cytoplasmic scaffold. We first looked at the synaptic protein Coracle, reported to control some glutamate receptor subunits localization at the *Drosophila* NMJ [31].

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patches were localized at positions where Dg-C-GFP could be found, exactly like the Lam patches (Fig. S2). This further confirms the ability of Dg-C to recruit the Cora protein. Altogether, these data show that Dg controls synaptic Cora localization.

Dystroglycan controls the abundance of Spectrin cytoskeleton at the NMJ

The 4.1 proteins are described as spectrin-actin binding proteins [47]. The prototypical 4.1 protein is the erythrocytal 4.1 protein (4.1R), which is essential for the submembranous actin-spectrin cytoskeleton. The protein domain of 4.1R protein responsible for this interaction has been delineated and is called the SAB domain. This domain is not conserved in the Drosophila homologue Cora [35], which is probably not able to bind actin and spectrin. The spectrin cytoskeleton is composed of heteromers of alpha and beta spectrin subunits. Previous studies have shown that this cytoskeleton is also present at the NMJ [48]. Since we observed a clear colocalization of Cora and Spectrin at the NMJ postsynaptic side (Fig. S3), we looked whether loss of Cora in the dg mutant conditions influenced the synaptic spectrin cytoskeleton. In dg loss of function conditions, there was a decrease in the postsynaptic staining intensity of the spectrin cytoskeleton, as assessed with an anti-alpha Spectrin antibody (Fig. 6B, C) compared to the wild-type situation (Fig. 6A). However, this cytoskeleton was still clearly visible. In addition, Dg postsynaptic overexpression led to an increase in postsynaptic alpha-Spectrin immunoreactivity, which was not completely penetrant (Fig. 6D). Hence, our data show that Dg controls postsynaptic Cora concentration, and, to a lesser extent, the postsynaptic spectrin cytoskeleton.

To address whether Cora and Spectrin proteins belonged to the protein complexes interacting with Dg, we performed immunoprecipitation of a Dg-GFP protein expressed in muscles. Both Cora and alpha-Spectrin proteins co-immunoprecipitated with Dg-GFP, whereas a control protein like the GSK-3 homologue Shaggy (Sgg) did not co-immunoprecipitate (Fig. 6E). In conclusion, Cora and alpha-Spectrin are part of a protein complex including Dg.

Dg effect on Cora and alpha-Spectrin is not merely a consequence of the loss of Dystrophin

The most parsimonious way to explain how Dg can affect Cora and alpha-Spectrin NMJ concentration is: Dg controls the F-actin cytoskeleton via Dystrophin; Coracle as well as alpha-Spectrin, known to be actin binding protein, are delocalized when the postsynaptic F-actin cytoskeleton is disrupted in absence of Dystrophin in the dg loss of function condition. To test this hypothesis, we tried to look at the postsynaptic F-actin cytoskeleton with rhodamine-phalloidin staining, but could not reliably see its size due to the underlying actin-myosin contractile apparatus. Still, some postsynaptic F-actin cytoskeleton could be

Figure 5. Dg controls postsynaptic Cora concentration. Double staining for Cora (Magenta) and HRP (green)(1) in (A) control (yw-)/ larvae, (B) larvae expressing muscle dg-RNAi (dg-RNAi/+ ; 24B Gal4/dg-RNAi), (C) dg01554/dg323 mutant larvae and (D) larvae overexpressing Dg-C in muscles (24B-Gal4/UAS-DgC). Arrows indicate patches of Cora protein. Single stainings for Cora and HRP are shown respectively in (2) and (3). In all panels, a muscle 4 NMJ is shown. Scale bar is 10 μm. 3D views of the preparations shown in A1 and C1 are shown in E and F: we look at the NMJ from the inside of the muscle cell. Cora is in magenta and HRP in green.

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observed in dg mutants (data not shown). We thus tested whether the loss of Dystrophin also led to a decrease in Cora and alpha-Spectrin postsynaptic immunoreactivity. We performed this experiment, keeping in mind that the loss of Dystrophin also affected Dg immunoreactivity (Fig. 4). We could see that an almost complete loss of NMJ Dystrophin obtained when expressing a Dys RNAi construct always had smaller effects on Cora and \( \alpha \)-Spectrin immunoreactivity compared to a loss of Dg function, which affected only partially Dys staining (compare Fig. 4 and Fig. 5). These results suggest that Dg controls postsynaptic Cora and \( \alpha \)-Spectrin concentration independently of Dystrophin (Fig. S4).

Coracle reciprocally controls Dg localization

If Dg controls in parallel Dys and Cora synaptic concentration, and Dys controls reciprocally Dg, we wondered whether Cora also played such a reciprocal function on Dg. We looked at the Dg NMJ staining in the cora\(^{14/}\)cora\(^{468713}\) mutant. This mutant reproduced the dg phenotype at the NMJ, in that postsynaptic Cora concentration was markedly affected (Fig. 7B), although presynaptic HRP staining (Fig. 7A) and postsynaptic Dlg staining (Fig. 7C) did not change intensity. In this cora hypomorph mutant, postsynaptic Dg staining was reduced (Fig. 7E). This indicated that Cora controlled Dg postsynaptic localization and that the functional interactions between Dg and Cora were reciprocal. Associated with the postsynaptic Dg loss, we could observe, as expected, a decrease in alpha-Spectrin immunoreactivity (Fig. 7D) and in Dystrophin staining (Fig. 7E).

Dg influences the amount of GluRIIA subunits in postsynaptic glutamate receptor clusters

The Drosophila NMJ is a glutamatergic synapse. The postsynaptic glutamate channel receptors are thought to be composed of four subunits: GluRIIC, IID, IIE and either the GluRIIA or GluRIIB subunit [33,49–52]. Since Cora was shown to control the amount of GluRIIA, and since the amount of postsynaptic Cora was largely reduced in dg mutants, we expected to see a decrease in the amount of GluRIIA subunit present in the postsynaptic glutamate receptors. We quantified the immunoreactivity for GluRIIA compared to the immunoreactivity for GluRIIC and could indeed see a significant decrease in the relative amount of GluRIIA subunit (Fig. 8), although we could never see a complete loss of this subunit. These data show that Dg influences the relative subunit composition of glutamate receptors at the

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**Figure 6.** Dg controls postsynaptic Spectrin concentration and both Cora and Spectrin co-immunoprecipitate with Dg. Double staining for alpha-Spectrin (Magenta) and HRP (green) in (A) control (ywCS) larvae, (B) larvae expressing muscle dg-RNAi (dg-RNAi/+; 24B Gal4/dg-RNAi), (C) dg\(^{07554/dg^{272}}\) mutant larvae and (D) larvae overexpressing Dg-C isoform in the muscles (24B-Gal4/UAS-DgC). Single stainings for alpha-Spectrin are shown in (2). Scale bar is 10 µm. (E) Co-immunoprecipitation was performed with a polyclonal anti-GFP antibody on protein extracts from flies expressing Dg-C-GFP. S corresponds to the supernatant and P to the pellet. Cora and alpha-Spectrin co-immunoprecipitate with Dg-C-GFP, but not Shaggy (Sgg), a cytoplasmic protein kinase.

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NMJ. We then analyzed electrophysiologically the consequences of the loss of postsynaptic Dg.

Loss of postsynaptic Dystroglycan leads to a decrease in synaptic quantal content

We performed intracellular, two-electrode voltage clamp recordings from muscle 6 of segments A3 at the larval NMJs of WT and dge01554 homozygous mutants, or of 24B/+ and dge-RNAi/+; 24B/+ larvae (Fig. 9). In dge mutants, or in flies expressing dge-RNAi in muscles, the evoked junctional currents (EJCs) were decreased by approximately 40% (control: 73.95±3.47 nA, n = 11; dge mutant: 53.86±3.87 nA, n = 10; 24B/+ control: 72.80±4.45 nA, n = 10; dge-RNAi/+; 24B/+: 36.02±2.75 nA, n = 11) (Fig. 9A and 9B). This effect could be due either to a decrease in the sensitivity of the postsynaptic glutamate receptor field, which should be reflected in a decrease of miniature junctional currents amplitudes (mEJCs). On the other hand, the number of vesicles released per action potential (“quantal content”) could be reduced. mEJC amplitude was not diminished when Dg was decreased (control: 0.92±0.034 nA, n = 11; dge mutant: 1.11±0.064 nA, n = 10; 24B/+ control: 0.80±0.024 nA, n = 9; dge-RNAi/+; 24B/+: 0.76±0.036 nA, n = 10) (Fig. 9C). On the contrary, there was a slight enhancement in the dge mutant, but not reproduced in dge-RNAi larvae. However, estimation of quantal content (EJC/mEJC) revealed a reduction of about 40% in dge mutant and dge-RNAi larvae (control: 81.1±4.1, n = 11; dge mutant: 49.2±3.8, n = 10; 24B/+ control: 89.8±6.4, n = 9; dge-RNAi/+; 24B/+: 50.6±5.2, n = 10) (Fig. 9D). Hence, postsynaptic Dg seems to be able to positively control the amount of presynaptic vesicles released upon stimulation. We further verified that the effect observed in dge-RNAi/+; 24B/+ flies was not due to a non specific presynaptic expression of the RNAi, by comparing this result with flies expressing the RNAi in neurons with the elav-Gal4 driver. In elav/+; dge-RNAi/+ larvae, the evoked junctional currents (EJCs) were only slightly decreased compared to control genotypes (elav/+; dge-RNAi /+: 63.65±5.41 nA, n = 9) (Fig. 9B). In these larvae, the mEJCs amplitude was not affected (0.86±0.025 nA, n = 5) (Fig. 9C) and the quantal content was not significantly diminished compared to both the w and the 24B/+ control genotypes (71.86±11.40 nA, n = 5) (Fig. 9D). Since larvae expressing dge-RNAi in motoneurones do not show as strong a phenotype as larvae expressing dge-RNAi in muscles, this indicates that postsynaptic Dystroglycan controls presynaptic vesicle release.

Discussion

Synaptic localization of Dg

The widely accepted hypothesis about the function of the DGC complex is its protective role in the sarcolemma against muscle contraction induced size changes. Here we analyzed the synaptic function of a core member of the DGC, Dystroglycan. We showed that Drosophila Dg was concentrated at the NMJ, and that most Dg immunoreactivity at the NMJ was postsynaptic. We also showed that a proportion of synaptic Dg contained the munc-18-like domain (MLD), which is the most heavily glycosylated domain in vertebrate Dg. Haines et al reported recently that the MLD containing Drosophila Dg isoform was indeed glycosylated [39]. Thus, like the vertebrate cholinergic NMJ, the Drosophila NMJ is enriched in Dg, and notably in glycosylated forms of this protein. These data are in accordance with the previously described

Figure 7. Cora controls Spectrin, Dystroglycan, Dystrophin but not Dlg postsynaptic concentration. (A–C) Triple staining for HRP (A), Cora (B) and Dlg (C) in control (ywCS) (1) and cora14/coraACTM4 larvae (2). Cora concentration at the NMJ is largely decreased in the cora mutant, whereas synaptic HRP and Dlg stainings show the same signal intensity. This indicates that Cora synaptic loss in the cora mutant is not the direct consequence of a total disruption of NMJ structure. (D–F) Stainings for alpha-Spectrin (D), Dgex8 (E) and Dystrophin (F) in control (1) and cora14/cora14 larvae (2). In cora mutants, alpha-Spectrin postsynaptic concentration decreases, but does not completely disappear, like in dge mutants. (E) Dystroglycan staining appears thinner in cora mutants. Inserts show higher magnification of a synaptic bouton with presynaptic HRP in magenta and Dg staining in green. The thinner appearance of the NMJ in cora mutants is due to a decrease in postsynaptic Dg staining compared to WT larvae. (F) In cora mutants, Dys labelling is reduced, but is still visible, contrarily to cora mutants, or in flies expressing coraRNAi flies expressing the RNAi in neurones with the elav-Gal4 driver. In elav/+; coraRNAi/+ larvae, the evoked junctional currents (EJCs) were only slightly decreased compared to control genotypes (elav/+; coraRNAi /+: 63.65±5.41 nA, n = 9) (Fig. 9B). These data are in accordance with the previously described
concentration of Dystrophin at the Drosophila NMJ [44], suggesting the presence of all DGC members at the postsynapse.

It is possible that the NMJ defects observed in the dg mutants used in this study are a consequence of a general muscle dysfunction, due to the loss of Dg at extrasynaptic sites. Indeed, muscle dysfunction has been observed in dg null mutants that are lethal at the embryonic and first instar larval stage [39]. However, the mutants analyzed here are hypomorphs and the allelic combination used, 

\[ \text{dge01554/dg323} \]

is viable. The larvae crawl, pupate and give rise to fertile adults, which do not show any wing position phenotype corresponding to flight muscle degeneration. Although we cannot rule out that there are some subtle muscle defects at extrasynaptic sites, our data illustrate that synaptic electrophysiological and morphological defects are already present in these mild loss of function conditions.

Laminin-Dg-Dys complex is conserved at the Drosophila glutamatergic NMJ

A function of the \textit{lanA} gene, encoding a Laminin A subunit, in stabilizing the initial motoneuron/muscle contact during synaptogenesis was published earlier [53]. Here, we show that Laminin is still present during late larval stages, and that it is concentrated around synapses in varicosities. Our data indicate that, like in mice where Dg is required for synaptic Utophin, Laminin γ5 and Laminin γ1 concentration [13], \textit{Drosophila} Dg controls synaptic Laminin and Dystrophin concentration. In addition, Dystrophin was known to be required for Dg sarcomemal localization in vertebrate muscles [4], and both Dystrophin and Utophin account for part of the clustering of Dg at the NMJ [54,55]. Here, we show that Dystrophin also controls synaptic Dg concentration. Thus the interdependence between Laminin, Dg and Dystrophin at the NMJ seems to be conserved phylogenetically. Importantly, in dystrophin/utrophin double mutants, a significant amount of Dg remains at the synapse, indicating that other proteins control, in parallel, its synaptic localization [54,55].

Our observations indicate that, similarly, the Utophin-Dystrophin homologue in flies does not account for the whole synaptic localization of DG, and we identify Coracle as a new, additional synaptic anchor for Dg.

\section*{The 4.1 protein Cora at the NMJ}

Looking for any new potential partners of Dg, we studied Cora localization in late larval stages at the NMJ. A previous report described a function for Cora in early larval stages [31], but did not show any clear synaptic localization of Cora in late larval stages, using a monoclonal antibody [35] recognizing all Cora isoforms. Instead, a strong immunoreactivity in NMJ associated glial cells was reported. We used a polyclonal antibody recognizing only the large Cora isoform [35]. With this antibody, we had no immunoreactivity in any NMJ associated glial cell, but we could easily detect a postsynaptic concentration of Cora, which partially disappeared in a \textit{cora} hypomorph mutant, and was increased when Cora was overexpressed in the muscle. These data indicated that the observed staining was indeed Cora. The Localization of protein 4.1 members in vertebrate muscle fibers is not well documented. It has been shown that protein 4.1R isoforms were indeed present in the muscle cells, notably at the cell periphery (probably the sarcolemma) [56]. Interestingly, in DMD patients, the peripheral localization of protein 4.1R isoforms was lost, although the sub-sarcomemal spectrin cytoskeleton was still

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Dg influences glutamate receptor subunit composition. Triple staining for HRP (1), DGIUIIC (2) and DGluRIIA (3) in control (yw\textsuperscript{CS}) (A) and \textit{dg}\textsuperscript{01354/dg}\textsuperscript{225} mutant larvae (B). DGluRIIA immunoreactivity is reduced in the \textit{dg} mutant whereas DGIUIIC and HRP immunoreactivities are unchanged. (C) Quantification of the ratio of DGIUIIC versus DGluRIIC staining intensities (\(n = 6\) for yw\textsuperscript{CS} and \textit{dg} mutant, and \(n = 8\) for dg-RNAi/+ and dg-RNAi/+: 24B Gal4/+). \(\ast \ p < 0.05; \ \ast\ast \ p < 0.01\). Error bars represent SEM. doi:10.1371/journal.pone.0002084.g008}
\end{figure}
present [56]. This set of data already indicated that protein 4.1 sarcolemmal localization was dependent on the DGC complex. Here, we show that this is the case at the NMJ, and that Dg is the principal component involved in Cora localization, since loss of postsynaptic Dys gave much weaker phenotypes compared to loss of postsynaptic Dg. In addition, we show that Cora co-immunoprecipitates with Dg, indicating the presence of the two proteins in the same complex, although further biochemical analysis will be required to assess whether they interact directly or indirectly.

Unexpectedly, we observed that Cora was required for the normal postsynaptic localization of Dg and, to a lesser extent, of Dys. This result was observed using a hypomorph cora mutant in which the C-terminal domain is partially deleted. In this mutant, synaptic amount of Cora was strongly reduced (Fig. 7 and Fig. S3). Further structure-function studies will be required to understand 1) which domain of Cora is required for its synaptic localization and for its interaction with Dg, 2) which part of Dg C-terminal tail is involved in Cora interaction. Previous studies have shown that the juxtamembrane region of the C-terminal Dg tail was interacting with Ezrin, a protein containing a FERM domain, like Cora [57,58]. It is possible that the same Dg domain interacts with Cora.

Since Cora was known to control synaptic GluRIIA abundance [31], an expected consequence of the loss of synaptic Cora in dg mutant NMJ was a reduction in the amount of GluRIIA subunit at the NMJ. We found such a reduction, but to a mild degree (relative quantification to the amount of GluRIIC subunit had to be done to decrease the effect of preparation variance). This small effect may be due to the fact that dg-induced reduction of synaptic Cora is not as strong as a complete cora loss of function, which was the situation analyzed originally. The small effect observed on DGluRIIA probably explains why there was no change in the amplitude of mEJCs in dg loss of functions. Indeed, DGluRIIA is the dominant subunit compared to DGluRIIB and a significant loss of DGluRIIA should lead to a decrease in mini amplitude [51].

**Dystroglycan and the spectrin cytoskeleton**

Loss of synaptic Dystroglycan resulted in a clear decrease in postsynaptic spectrin cytoskeleton, as assessed with alpha-Spectrin immunoreactivity. Although the spectrin defect may be a consequence of the loss of synaptic Cora, a more direct interaction between Dg and the spectrin cytoskeleton remains a possibility. Hence, the link between Dg, Cora and spectrin cytoskeleton remains to be further defined. The postsynaptic spectrin cytoskeleton was shown to play a role in the repartition of postsynaptic receptor fields [48]. Indeed, loss of postsynaptic immunoreactivity for both alpha and beta-Spectrin led to a disorganization of postsynaptic receptor fields. We looked for such a defect in the dystroglycan loss of function conditions, but could not find any. This is probably due to the fact that the loss of spectrin immunoreactivity in these mutants was not complete.

**Presynaptic glutamate release defect in postsynaptic dg deficient larvae**

We demonstrated that Dg played a functional role in neuromuscular synaptic transmission. Indeed, the glutamate release was decreased by approximately 40% in absence of muscle Dg. The main specificity of the insect NMJ, compared to the vertebrate NMJ is the presence of glutamate as a neurotransmitter instead of acetylcholine. Hence, these synapses are not only NMJ models, but also models of glutamatergic synapses, which are by far the most frequent synapses found in the vertebrate brain. A previous study of Dg function in brain synapses illustrated an

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**Figure 9. NMJ electrophysiological defects due to the loss of dg function.** A) Example traces of evoked postsynaptic currents (EJCs) from NMJs of wild type (wt) and dg mutant dg01554 larvae. B) Mean EJC amplitude for wild type (wt larvae), dg mutant dg01554, Canton S crossed with 248 Gal4 (248 Gal4/+), dg-RNAi crossed with 248 Gal4 (dg-RNAi/+; 248 Gal4/+) and dg-RNAi crossed with elavC155 Gal4 (elav Gal4/++; dg-RNAi/+). B) mini EJC amplitudes and C) quantal content for the same genotypes. The number of measured larvae is indicated within each histogram bar. (* p<0.05; *** p<0.001). Error bars represent SEM. doi:10.1371/journal.pone.0002084.g009
alteration of LTP in DG−/− CNS mice, but no modification of the amplitude of synaptic responses evoked by low frequency stimulation of Schaeffer collaterals, and no changes in paired-pulse facilitation [59]. Here, we could detect a reduced synaptic response at low frequency, indicating a function of Dg in basal glutamatergic synaptic transmission.

One surprising result in our electrophysiology experiments was the fact that defects in quantal content of the dg mutant were also present, with the same intensity in flies expressing a 24B Gal4 driven dg-RNAi. This indicated that loss of postsynaptic Dg led to a functional change in the other synaptic compartment, the presynapse. Such a presynaptic effect associated with postsynaptic modifications is not new, since the NMJ function displays homeostasis, and that decrease in postsynaptic responsiveness is often associated to increase in neurotransmitter release and vice versa in order to maintain constant EJCs [60]. The molecular mechanisms involved in this homeostatic control are largely unknown. Here, in dg mutants, homeostatic control is likely absent since mini amplitude (receptor field) is not altered in absence of postsynaptic Dg, but glutamate release is modified. This suggests that Dg-deficient muscles inappropriate signals to the presynaptic release machinery. A similar trans-synaptic effect of loss of muscle Dystrophin onto presynaptic quantal content was observed [44]. What can be the mechanisms involved? One possibility is that postsynaptic Dg directly controls the levels of synaptic ECM molecules such as Laminin. These proteins, by interacting with presynaptic receptors, would affect the structure of the presynapse, e.g. the amount, size or molecular composition of active or periactives zones. This hypothesis is strongly supported by the finding in mouse, that a synaptic Laminin-calcium channel interaction organizes active zones in motor nerve terminals [61]. Another postsynaptic Laminin receptor could be the synaptic vesicle protein SV2 [62]. Looking for modifications at the presynapse, we did not detect any obvious change in the number and size of active zones, using Bruchpilot immunoreactivity as a marker (unpublished observations) and did not detect any modification in the immunoreactivity of the periactive zone marker Fas2 (unpublished observations). Still, the regulation of synaptic Laminin by Dg that we demonstrate here, together with the postsynaptic electrophysiological phenotype we observed, make the hypothesis of Laminin bridging postsynaptic Dg and the presynapse, at least in periactive zones, very likely.

These findings, i.e. the new components of a Dystroglycan complex, as well as the unexpected trans-synaptic role of Dg pave the way for understanding the role of the DGC in the formation, maintenance and plasticity of glutamatergic synapses.

### Supporting Information

**Figure S1**  
DG gene structure, DG mutants and qRT-PCR analysis  
A) Gene structure of DG gene compiled from flybase data and [27]. The two null alleles dg323 and dg62 are deletions comprising the first exon. The Piggybac element Pbac[RB]e01554 is localized within the ninth exon, subjected to alternative splicing. B) To analyze how the Piggybac element altered DG transcription, we performed qRT-PCR experiments with couples of oligonucleotides designed against sequences common to all transcripts, either in the extracellular domain (couple 1), or in the intracellular tail (couple 2). In both the homozygote condition, or in transheterozygote with the null alleles, the Piggybac element led to a loss of more than 90% of transcripts. C) and D) Specificity of NMJ DG staining observed with the anti-Dgex8 antibody on muscle 4 NMJs. Dgex8 (panels 1 and 2) or Discs-Large (panels 1 and 3) immunoreactivity in ywCS control (C) and dg1554/dg323 mutant larvae (D). The Dgex8 NMJ immunoreactivity almost completely disappears in the mutant condition whereas Discs-Large immunoreactivity is still present. Scale bar is 10 µm.

**Figure S2**  
DG-GFP can recruit Lam and Cora at extrasynaptic patches (A-C) Double staining against GFP (A, C) and Lam (B, A, B) in larvae overexpressing DG-GFP in the muscles with the 24B Gal4 driver. In all panels of this figure, a single confocal optical section, which crosses the sarcolemma, is taken (D). The bottom right part of each panel corresponds the extracellular space and the up left part to the sarcolemma. Lam colocalizes with DG-GFP patches at the sarcolemma (see arrows in A). (E-G) Double staining against GFP (E, G) and Cora (E, F) in larvae overexpressing DG-GFP in the muscles with the 24B Gal4 driver. Cora colocalizes with DG-GFP patches at the sarcolemma (see arrows in E). (H-J) Double staining against Cora (H, I) and Lam (H, J) in wild-type larvae, without any DG overexpression. There are no extrasynaptic patches of Cora and Lam in these larvae. Scale bar is 10 µm.

**Figure S3**  
CorA is concentrated at the third instar larval NMJ and colocalizes with Spectrin. Double staining for Cora (red) and HRP (blue)/1 on (A) WT larvae, (B) corak14/corak08713 mutant larvae and (C) P[EPgy2]cora[EF07598]/++; 24B Gal4/+ larvae, which overexpress all Cora isoforms in muscles. Muscle 4 NMJs are shown. Single stainings for Cora and HRP are shown respectively in (2) and (3). Scale bar is 10 µm. (D) Immunoblot of proteins isolated from ywCS, cora14/corak08713 and P[EPgy2]cora[EF07598]/++; 24B Gal4/+ larvae. This blot was stained with the polyclonal Guinea-pig anti-Cora antibody. The wild-type larva display 2 bands of about 210 and 240 kDa. The signal intensity is clearly reduced in cora hypomorph mutants, whereas it is enhanced when Cora is overexpressed with the 24B-Gal4 driver. A second staining of the same blot with an anti-Tubulin antibody indicated that the protein loading was similar in all lanes. Relative molecular mass size markers (kDa) are indicated at right. (E) Triple staining for HRP (blue), Cora (red) and alpha-Spectrin (green) on WT third instar larvae (E1). A muscle 4 NMJ is shown. Single stainings for HRP, Cora and alpha-Spectrin are displayed respectively in E2, E3 and E4. A plot of the intensity of each labelling along a line crossing a terminal bouton (see in E1) is represented in E5. Note that the extent of alpha-Spectrin and Cora stainings around the synaptic bouton -labelled with HRP- is similar. Scale bar is 10 µm.

**Figure S4**  
Model of protein interactions with DG at the postsynaptic side of the Drosophila NMJ.

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**Author Contributions**

Conceived and designed the experiments: JB SS MP LB YG. Performed the experiments: MP LB BF BF AF. Analyzed the data: SS MP LB BF BF AF. Contributed reagents/materials/analysis tools: DM. Wrote the paper: MP.
Dystroglycan at Drosophila NMJ


