# A Drosophila model for Duchenne muscular dystrophy

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# CHAPTER 4

# The Dp186 Dystrophin Isoform Mediates Regulation of neurotransmitter Release at Central Synapses in *Drosophila*

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# The Dp186 Dystrophin Isoform Mediates Regulation of Neurotransmitter Release at Central Synapses in *Drosophila*.

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## Abstract

The establishment of synaptic connections during the development of the nervous system is dependent on bi-directional communication between pre- and postsynaptic cells. Such antero- and retrograde signals are also essential for the further maturation and plasticity of these connections. Here, we show that a CNS-specific Dystrophin isoform, Dp186, is required for appropriate retrograde regulation of neurotransmitter release at central synapses in *Drosophila*. Dystrophin is encoded by the gene whose mutation in humans underlies Duchenne muscular dystrophy, a disease characterized by progressive muscle wasting. A number of Duchenne patients also exhibit poorly understood mental retardation, likely associated with loss of a brain-specific isoform. Furthermore, Dystrophin and the related Utrophin proteins have long been known to localize at synapses, however, their functions there are largely unknown. In *Drosophila*, we find that the CNS-specific Dp186 isoform localizes to the postsynaptic dendrites of larval motoneurons which receive input from upstream presynaptic cholinergic interneurons. In the absence of Dp186, evoked, but not spontaneous, presynaptic release is significantly enhanced. This increase in presynaptic release can be rescued to wild type levels by expression of a Dp186 transgene in the postsynaptic motoneuron, but not in presynaptic interneurons. The increase in neurotransmitter release observed in the Dp186 mutants requires the presence of the TGF-β ortholog, Gbb, a protein previously implicated in retrograde signaling at these synapses. Dp186 is, therefore, required to maintain appropriate retrograde control of synaptic function at interneuron-motoneuron synapses of the central motor circuitry.

## 1. Introduction

The precise connectivity of neural networks underlies complex behaviors, such as learning and memory acquisition. Whereas the initial stages of axon outgrowth and path finding are largely independent of neuronal activity, stabilization and refinement of synaptic connections relies on activity-dependent mechanisms (reviewed in Katz and Shatz, 1996; Tessier-Lavigne and Goodman, 1996). It has become clear recently that not only anterograde, but also

retrograde, signaling at the synapse is essential for the maturation and modification of electrical contact (Turrigiano, 1999; Davis and Bezprozvanny, 2001).

Here, we examine the role of the Dystrophin Dp186 isoform in synaptic transmission in the *Drosophila* central nervous system (CNS). Mutations in the human dystrophin gene cause Duchenne muscular dystrophy (DMD), a common human genetic disease, characterized by progressive muscle wasting (Hoffman *et al.*, 1987). A third of DMD patients also present cognitive defects that have been correlated with a specific set of mutations in the *dystrophin* gene (reviewed in Anderson *et al.*, 2002). This finding has raised the possibility that, in addition to their roles in the musculature, *dystrophin* and its partially functionally redundant ortholog, *utrophin*, also have critical functions in the nervous system. Consistent with this, several Dystrophin isoforms and Utrophin are localized to synaptic regions in the mammalian brain, retina and neuromuscular junction (NMJ) (reviewed in Blake *et al.*, 2002). Further indications suggesting that *dystrophin*-like proteins play roles at a variety of synapses include the observations that a) Utrophin-deficient mice display NMJ structural abnormalities (Deconinck *et al.*, 1997a; Grady *et al.*, 1997a), b) Utrophin/Dystrophin-deficient NMJs show a disruption of AChR clustering (Deconinck *et al.*, 1997b; Grady *et al.*, 1997b), c) absence of the postsynaptically localized *Drosophila* DLP2 Dystrophin isoform results in increased presynaptic neurotransmitter release at the larval NMJ (van der Plas *et al.*, 2006) and d) mice lacking brain-specific *dystrophin* isoforms exhibit deficits in both long-term spatial and recognition memory, reflecting increased long-term potentiation in the hippocampus and decreased long-term depression in the cerebellum (Vaillend *et al.*, 2004). These results suggest that alterations in synaptic plasticity, resulting from *dystrophin* deficiency, may underlie DMD-associated cognitive defects. The precise roles of Dystrophin at the synapse, however, remain to be elucidated.

We have employed *Drosophila melanogaster*, with its amenability to genetic analysis and highly orthologous single *dystrophin* gene, to examine the roles of Dystrophin at central synapses. We show that the CNS-specific isoform Dp186 is expressed in the embryonic ventral nerve cord and the larval CNS and that it accumulates in postsynaptic motoneuron dendrites. Electrophysiological studies of Dp186 mutants reveal that Dp186 is required for normal synaptic function at an identified interneuron-motoneuron synapse in the larval CNS. In the absence of Dp186, evoked release from presynaptic interneurons is significantly increased, while spontaneous release is unaffected. Post-, but not presynaptic expression of Dp186 in the mutants, restores synaptic currents back to wild type levels. The TGF-β ortholog, Gbb, a protein implicated previously in central synaptic retrograde signaling (Baines, 2004), is required for the increased evoked transmitter release in the Dp186 mutant, supporting a role for Dp186 in the retrograde control of presynaptic function.

## 2. Materials and Methods

### 2.1 Fly stocks

Flies were fed on apple juice agar supplemented with yeast. Wild type was Canton-S. RN2-Gal4 was used to selectively express UAS driven transgenes in aCC and RP2 (Baines, 2003; Fujioka *et al.*, 2003; Baines, 2004). Expression of RN2-Gal4 begins in early stage 16 embryos, preceding the onset of synaptogenesis (Baines and Bate, 1998). B19-Gal4 bears the Gal4 open reading frame downstream of the choline acetyltransferase promoter sequence and is selectively expressed in cholinergic neurons (Salvaterra and Kitamoto, 2001). The $gbb^{1}$ allele, described previously (Wharton *et al.*, 1999), was rebalanced over Cyo::GFP to allow unequivocal identification of mutant larvae. Ok6-Gal4 (Aberle *et al.*, 2002) drives expression in most, if not all, larval motoneurons and UAS-mCD8-GFP (Lee and Luo, 1999), when driven by Ok6-Gal4, localizes to the membranes of cell bodies, axons and dendrites of the

motoneurons (Sanchez-Soriano *et al.*, 2005). Elav-GAl4 drives expression throughout the embryonic and larval CNS (Luo *et al.*, 1994).

### 2.2 Generation of Dp186 mutants, UAS-RNAi-Dp186 and UAS-Dp186 transgenic fly lines

A P-element excision screen (Tower *et al.*, 1993) was used to generate Dp186 mutant fly lines. The GE20705 P-element line (obtained from GenExel, Daejon, South Korea) was used as a starting point for the excision screen; the transposon is inserted 250 bps 5' of the Dp186 initiator ATG (Figure 1A). Precise and imprecise excisions were generated, to remove genomic sequences flanking the original P-element, by crossing in a source of transposase. Two lines were used for further analyses, $dys^{Dp186\ 166.3}$, and $dys^{Dp186\ 30.3}$ (Figure 1A). Sequence analyses indicated that $dys^{Dp186\ 166.3}$ bears a 1.2 kb deletion removing the Dp186 ATG codon and most of the unique first exon of Dp186 and $dys^{Dp186\ 30.3}$ bears a 0.9 kb deletion. Both lines lack Dp186 expression (Figure 2E and data not shown).
To reduce Dp186 expression levels in a tissue specific manner, we generated transgenic fly lines that express double stranded (ds)-RNA targeting Dp186 unique sequences under Gal4 control. The UAS-RNAi-Dp186 construct contains the unique Dp186 sequences from basepairs 74 to 714 (Genbank Accession Number NM_169863) cloned into a pUAST (Brand and Perrimon, 1993) derivative bearing the mub intron (van der Plas *et al.*, 2006) between the Dp186 specific sequences. Multiple independent transgenic lines were generated using standard P-element transformation techniques and two lines were used in these studies.
To restore Dp186 expression in a tissue specific manner in the Dp186 mutant background, we generated fly lines expressing the full length Dp186 cDNA sequence under Gal4 control in the pUAST P-element vector. Multiple transgenic lines were obtained and two lines were used in these studies.

### 2.3 Immunohistochemistry

Anti-Dp186 rabbit antisera were raised against a histidine-tagged fusion protein containing unique Dp186 sequences (amino acids 15-170 of Genbank accession number AAK15257; (van der Plas *et al.*, 2006). Immunohistochemistry was performed as described previously (Dekkers *et al.*, 2004). Anti-Dp186 (1: 2500), anti-Synapsin (1:1000; Klagges *et al.*, 1996), anti-Bruchpilot Nc82 antibody (1:50; Hofbauer, 1991; Developmental Studies Hybridoma Bank), anti-GFP (1:1000; Roche Diagnostics, Almere, The Netherlands), Alexa Fluor 488 Phalloidin (1:200; Invitrogen, Breda, The Netherlands) and Alexa Fluor-conjugated secondary antibodies (1: 300; Invitrogen, Breda, The Netherlands) were used as indicated. The samples were visualized and photographed using confocal and/or standard epi-fluorescence microscopy.

### 2.4 Dissection of Larvae

First instar larvae (within 4 hrs of hatching) were dissected and central neurons accessed as described (Baines and Bate, 1998). Larvae were viewed using a water immersion lens (total magnification 800X) combined with Nomarski optics (BX51 WI microscope, Olympus Optical, Tokyo, Japan).

### 2.5 Electrophysiology

Whole cell voltage clamp recordings were performed using thick-walled borosilicate glass electrodes (GC100TF-10, Harvard, Edenbridge, UK), fire-polished to resistances of between 15 and 20mΩ. Recordings were made using an Axopatch-1D amplifier controlled by pClamp 8.1 via a Digidata 1322A A/D converter (Molecular Devices, Sunnyvale, CA). Cells were identified based on their invariant size and dorsal position in the ventral nerve cord. After breakthrough, currents were measured for a period of time ranging from between at least 2 and for no longer than 4 minutes from a minimum of 8 cells for each genotype tested. Although currents can be recorded for longer than 5 minutes, cells often begin to show increased leak currents after this duration of whole cell recording. Traces were filtered at 2

KHz and sampled at 20 KHz. Spontaneous miniature currents were recorded in the presence of TTX (0.1 μM, Alomone labs, Jerusalem, Israel). Miniature currents were identified based on a fast rise and slower decay and on having an amplitude of at least twice background (2-3 pA). Amplitudes of both evoked and miniature currents were measured using Minianalysis 5.2.8 (Synaptosoft, Decatur, GA). Composite averaged current amplitude was calculated by grouping all individual currents recorded in each genotype tested. All recordings were performed at room temperature (22- 24°C). Data were compared using a non-paired t-test. Results were deemed significant at $P \leq 0.05$ (*) or $P \leq 0.01$ (**). All values shown are mean ± SEM. External saline consisted of: (in mM) NaCl (135), KCl (5), $MgCl_2.6H_2O$ (4), $CaCl_2.2H_2O$ (2), N-Tris[hydroxymethyl]methyl-2-amonoethanesulfonic acid (TES, 5), sucrose (36), pH 7.15. Internal patch solution consisted of (in mM): $K^+$ methylsulfonate ($KCH_3SO_3$, 140), $MgCl_2.6H_2O$ (2), EGTA (2), KCl (5), HEPES (20), pH 7.4.

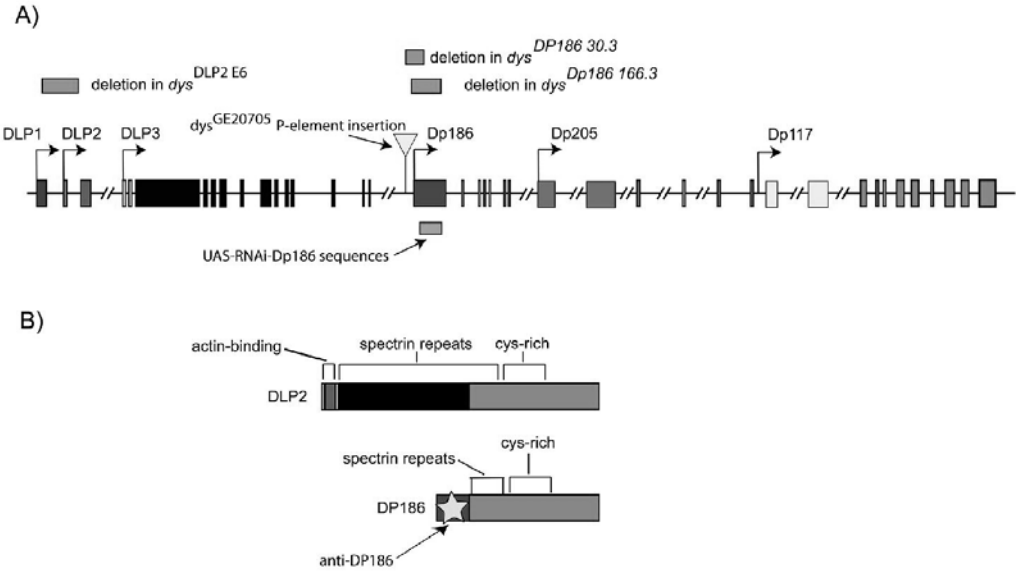


**Figure 1. The structure of the *Drosophila dystrophin* gene and the location of the deletions in the $dys^{DLP2\ E6}$, $dys^{Dp186\ 166.3}$ and $dys^{Dp186\ 30.3}$ mutants.**
(**A**) There are 6 known isoforms of *dystrophin* in *Drosophila*, DLP1, DLP2, DLP3, Dp186, Dp205 and Dp117, each likely expressed from their own promotors. The location of the isoform-specific deletions in the $dys^{DLP2\ E6}$, $dys^{Dp186\ 166.3}$ and $dys^{Dp186\ 30.3}$ mutants and the sequences used for the RNAi-Dp186 construct and the P-element used to generate the $dys^{Dp186\ 166.3}$ are shown. Exons are indicated as bars and introns as horizontal lines. (**B**) The unique and conserved protein domains of the DLP2 and Dp186 isoforms and the region used for the generation of the Dp186-specific rabbit polyclonal antibody (yellow star) are indicated.

# 3. Results

### 3.1 The Dystrophin Isoform Dp186 is expressed in the embryonic and larval neuropile and localizes to motoneuron dendrites

The *dystrophin* gene is a large highly conserved gene that encodes a number of isoforms expressed from distinct promotors (reviewed in Blake *et al.*, 2002). There are six known isoforms in *Drosophila* that are expressed predominantly in the musculature and nervous system (Greener and Roberts, 2000; Neuman *et al.*, 2001; Dekkers *et al.*, 2004; Neuman *et al.*, 2005; Figure 1A). The best-characterized *Drosophila* isoform, DLP2, is expressed at the postsynaptic side of the neuromuscular junction (van der Plas *et al.*, 2006). The large Dystrophin isoforms DLP1, DLP2 and DLP3, have an actin binding domain, 11 spectrin repeats and a highly conserved cysteine rich carboxyterminal domain (Figure 1). A shorter isoform, Dp186, lacks the actin binding domain, has a unique aminoterminal domain, only 4 spectrin repeats, but contains the conserved cysteine-rich domain present in all known isoforms. Previous RNA in situ analyses revealed that Dp186 is highly expressed in the embryonic CNS (Neuman *et al.*, 2001; Dekkers *et al.*, 2004). We raised rabbit polyclonal

antisera against sequences unique to Dp186 (Materials and Methods) and examined the localization of the Dp186 protein during the embryonic and larval stages. Dp186 protein was first detected in the embryonic CNS at stage 13 at which time it localizes to regions near the longitudinal connectives (Figure 2B). These regions likely represent the synapse-rich dorsal neuropile, since the presynaptic Bruchpilot protein, recognized by mAb Nc82 (Kittel *et al.*, 2006; Wagh *et al.*, 2006), is also present there (Figure 2A and C).

In 3rd instar larval CNS, Dp186 protein is found in the neuropile and accumulates in distinct areas in the two brain lobes (Figure 2D). In addition, the protein can be observed in three bilaterally symmetrical clusters, likely the thoracic neuromeres, which are located at the lateral sides of the neuropile. Staining is not evident in the $dys^{Dp186\ 166.3}$ mutant (Figure 2E). We performed double labeling of anti-Dp186 with an antibody recognizing the presynaptically-localized Synapsin protein (Klagges *et al.*, 1996). We found that Dp186 and Synapsin are present in close proximity in the synapse-rich neuropile (Figure 2F-H), but not at the presumptive thoracic neuromeres. We also examined a postsynaptic marker localized to motoneuron dendrites in double labelings with anti-Dp186. UAS-mCD8-GFP driven by a motoneuron-specific Gal4 driver (OK6-Gal4) results in the localization of GFP to the membranes of motoneuron cell bodies, dendrites and axons (Landgraf *et al.*, 2003; Sanchez-Soriano *et al.*, 2005). Double labeling with anti-Dp186 shows co-localization of GFP and Dp186 proteins in motoneuron dendrites (Figure 2IK), indicating that Dp186 is likely present at interneuron/motoneuron synapses. To further characterize Dp186 localization in the thoracic neuromeres, we used fluorescently-tagged phalloidin to stain F-actin in the larval neuropile, and found that Dp186 co-localizes with F-actin most strongly in the lateral cluster extremities (Figure 2L-N).

### 3.2 Lack of Dp186 results in an increase in synaptic currents in motoneurons

To study the role of the Dp186 Dystrophin isoform during CNS development, we generated mutant fly lines that lack Dp186 protein. We used a P-element mobilization strategy starting with a P-element, GE20705, inserted 250 bps upstream of the Dp186 ATG initiator codon to generate Dp186-specific deletions (Figure 1). Two lines were generated, $dys^{Dp186\ 166.3}$ and $dys^{Dp186\ 30.3}$, which both lack detectable levels of Dp186 protein (Figure 2E and data not shown).

Lack of the large, muscle-specific, DLP2 isoform of Dystrophin results in a significant increase in evoked release of neurotransmitter at the *Drosophila* NMJ (van der Plas *et al.*, 2006). To determine whether synaptic transmission in the CNS is similarly regulated by the Dp186 CNS-specific Dystrophin isoform, voltage clamp recordings (Vh –60mV) were made from either the aCC or RP2 motoneurons (no differences were observed between these neurons). In wild type backgrounds, such recordings show inward synaptic currents that are relatively long-lived (500-1000 msec) and have an average amplitude of 76 ± 3.3 pA (Figure 3C). These excitatory synaptic currents, which are cholinergic in nature and action potential-dependent, result from the synchronous activity of interneurons that are presynaptic to the motoneurons (Baines and Bate, 1998; Baines, 2003). Identical recordings in $dys^{Dp186\ 166.3}$, an allele which bears a deletion encompassing most of the unique first Dp186 exon, revealed that synaptic currents were significantly increased in amplitude (127 ± 4.4 pA, $P \leq 0.01$, Figures 3B, C). Cumulative probability plots of individual synaptic currents, that better show the range of current amplitudes recorded, revealed that there is a significant increase in amplitudes of the majority of the individual currents measured, compared to the wild type control (Figure 3D). Synaptic current amplitude was also significantly increased in $dys^{Dp186\ 30.3}$, a second independent imprecise excision allele lacking Dp186 (117 ± 3.5 pA, $P \leq 0.01$, Figure 3C). In contrast, synaptic currents in $dys^{DLP2\ E6}$, which lacks the DLP2 muscle-specific isoform, but expresses Dp186 at wild type levels (van der Plas *et al.*, 2006), were no different in amplitude compared to wild type (75 ± 2.5 pA, $P > 0.05$, Figures 3A and 3C). Evoked synaptic currents at the NMJ were, however, significantly elevated in $dys^{DLP2\ E6}$ (van der Plas

*et al.*, 2006). The frequency of action potential-dependent synaptic currents recorded in aCC/RP2 was not significantly altered in either $dys^{Dp186\ 166.3}$, $dys^{Dp186\ 30.3}$, or $dys^{DLP2\ E6}$ compared to wild type (data not shown), suggesting that the connectivity of the presynaptic interneurons with these two motoneurons remains unaltered.

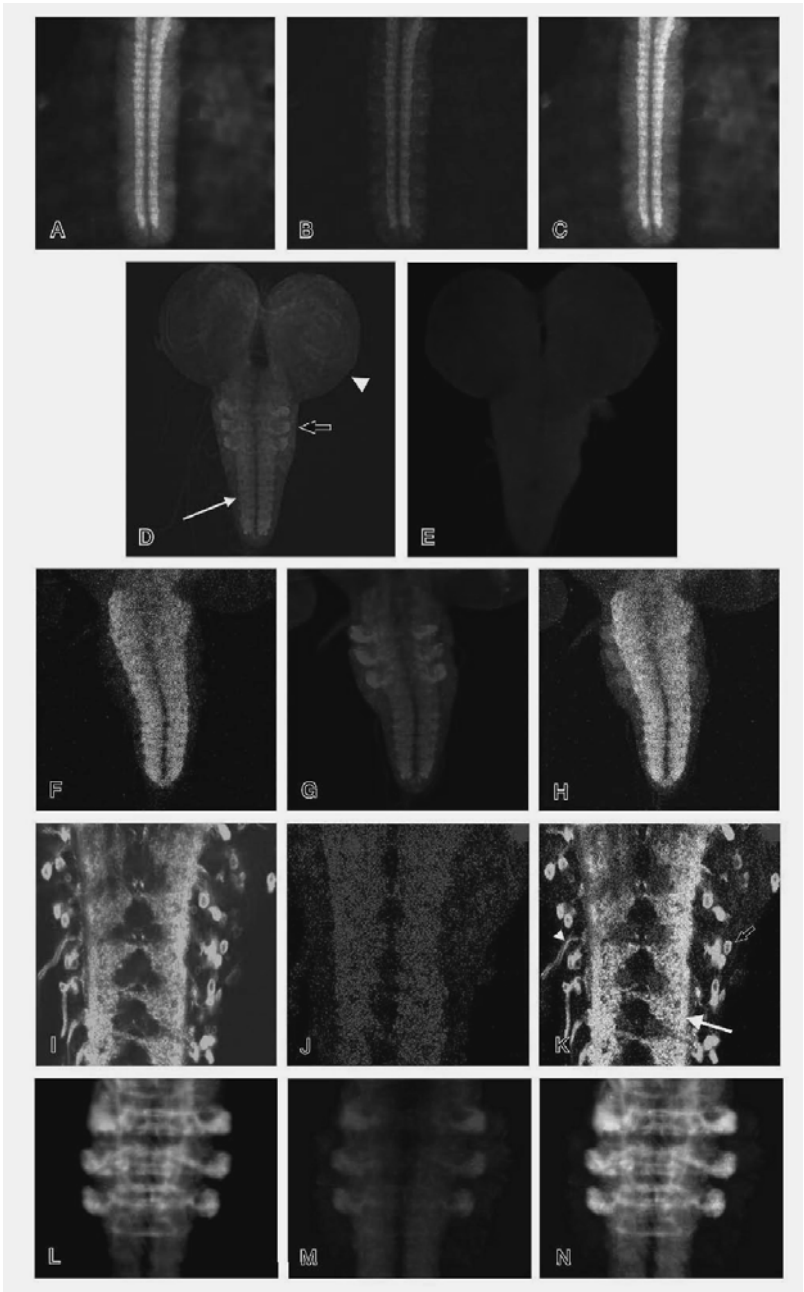


**Figure 2. Dp186 protein is expressed in synapse-rich regions of the embryonic and larval neuropiles, likely in motoneuronal dendrites.** A dissected stage 16 embryo ventral nerve cord (**A-C**), entire third instar larval central nervous systems (**D, E**) or only the neuropile (**F-H**) or a portion thereof (**I-N**) are shown. (**A**) The synapse-rich embryonic neuropile as detected by mAb nc82 which recognizes the presynaptic Bruchpilot protein. (**B**) antiDp186 staining of the ventral nerve cord. (**C**) The merge of the channels displayed in A and B. (**D**) Dp186 is expressed in the neuropile (arrow), the brain lobes (arrowhead) and in three lateral clusters, likely the thoracic neuromeres (open arrow, see also L-N) in third instar larvae. (**E**) Dp186 expression cannot be detected in the mutant $dys^{Dp186\ 166.3}$.(**F-H**) The presynaptic Synapsin protein (**F**) and Dp186 (**G**) are expressed throughout the larval neuropile. The merge of the channels shown in F and G is shown (**H**). (**I-K**) mCD8-GFP protein driven by OK6-Gal4 (**I**) colocalizes with Dp186 protein (**J**) in motoneuron dendrites (arrow), but not in cell bodies (open arrow), or axons (arrowhead)(**K**). (**L-N**) Alexa Fluor 488-conjugated phalloidin (**L**), which binds to F-actin, overlaps with Dp186 (**M**) at three lateral clusters, likely the thoracic neuromeres (**N**). Anterior is up in all preparations. See Appendix for color figure.

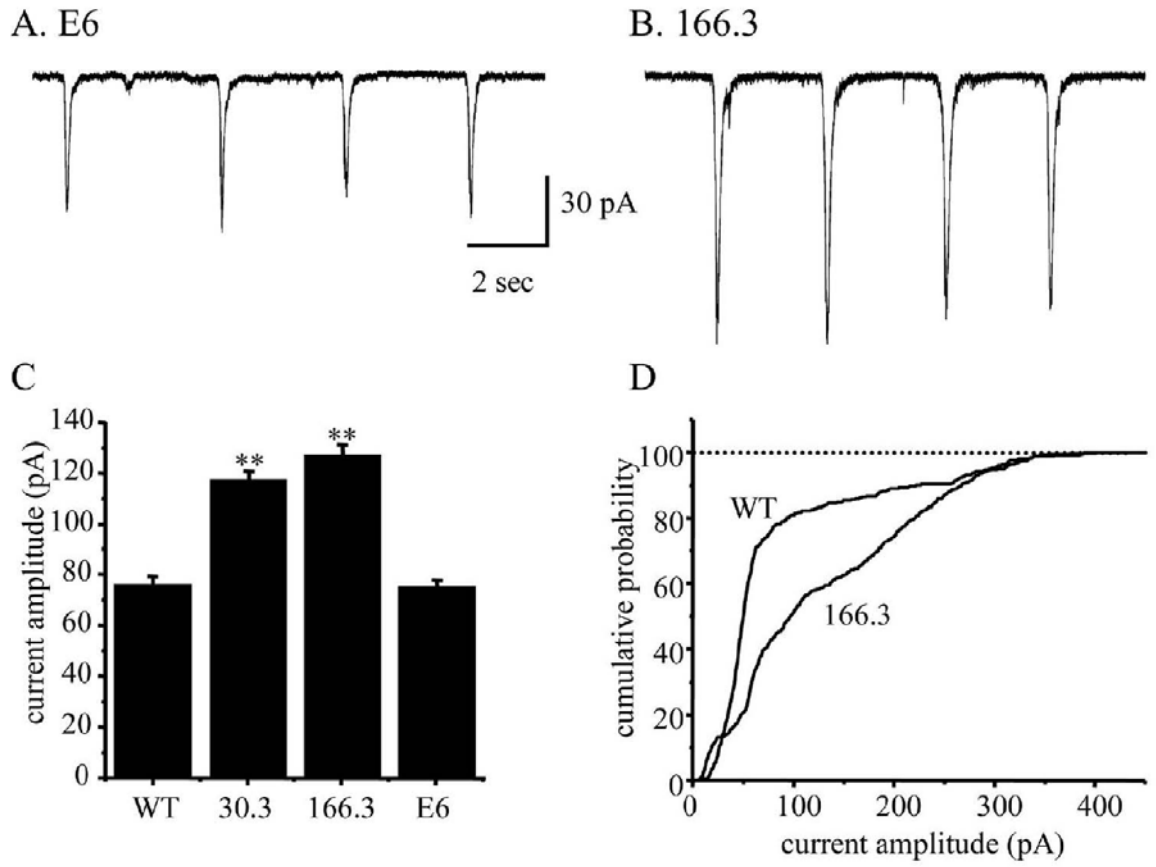


**Figure 3. Synaptic currents in motoneurons are increased in the absence of Dp186.**
(**A**) Whole cell voltage clamp recordings (Vh −60mV) from aCC/RP2 reveal large inward currents that are the result of action potential-dependent (evoked) release of presynaptic ACh (see Baines *et al.*, 1999; Baines *et al.*, 2001). (**B**) These currents are significantly increased in amplitude in Dp186 mutants ($dys^{Dp186\ 166.3}$ is shown). (**C**) The average amplitude of synaptic currents is significantly increased in both $dys^{Dp186\ 30.3}$ and $dys^{Dp186\ 166.3}$ compared to both Canton-S wild type (WT) and the homozygous DLP2 loss of function allele ($dys^{DLP2\ E6}$). Values shown are 76 ± 3.3, 117 ± 3.5, 127 ± 4.4 and 75 ± 2.5 pA respectively, ** $P \leq 0.01$ compared to both WT and $dys^{DLP2\ E6}$. Currents were recorded from at least 8 cells of each genotype. (**D**) Cumulative probability plots of individual excitatory currents reveals a clear increase in amplitude for the large majority of currents recorded in $dys^{Dp186\ 166.3}$ compared to WT.

There are a number of conceivable mechanisms that might underlie an increase in evoked synaptic transmission between presynaptic interneurons and aCC/RP2. Two likely possibilities are that there is an increase in evoked release of ACh from presynaptic terminals or increased postsynaptic sensitivity to this neurotransmitter. To distinguish between these two possibilities, recordings were repeated in the presence of TTX (0.1µM). All evoked transmitter release was blocked under these conditions (Figure 4A). Analysis of the TTX-insensitive miniature synaptic currents in aCC/RP2 showed no difference in amplitude between $dys^{Dp186\ 166.3}$, $dys^{Dp186\ 30.3}$ and wild type controls (9.3 ± 0.3, 8.9 ± 0.3 and 9.5 ± 0.4 pA, respectively, $P > 0.05$, Figure 4B, C). This result is consistent with there being no change in postsynaptic sensitivity to neurotransmitter in the mutant. The frequency of mepsc's was, however, significantly increased in both $dys^{Dp186\ 166.3}$and $dys^{Dp186\ 30.3}$ compared to control (14.6 ± 3.0, 16.3 ± 3.1 and 9.2 ± 1.7 events per min, respectively, $P \leq 0.05$, Figure 4D). An increase in mepsc frequency is consistent with, and indeed predictive of, an increase in the probability of presynaptic vesicle release.

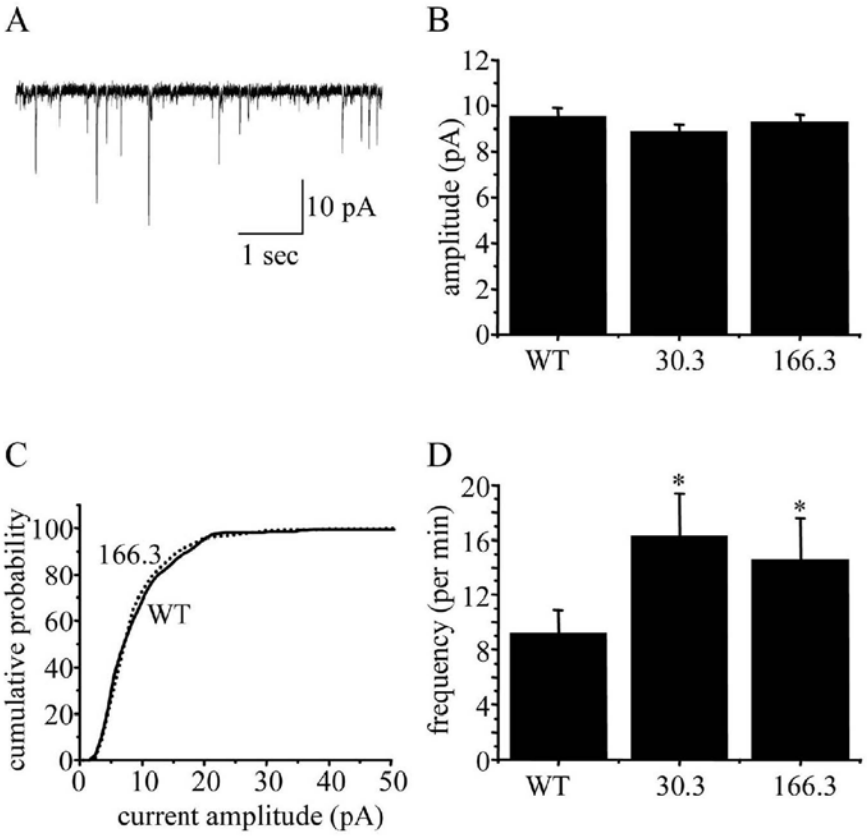


**Figure 4. Loss of Dp186 results in increased mepsc frequency but no change in amplitude.**
(**A**) In the presence of TTX (0.1 μM), evoked excitatory currents are abolished leaving only those currents that are elicited by the spontaneous release of vesicles from the presynaptic interneurons (mepsc's). Currents shown from Canton-S wild type. (**B**) Analysis of averaged mepsc amplitudes shows no change due to the absence of Dp186 ($dys^{Dp186\ 30.3}$ and $dys^{Dp186\ 166.3}$) compared to Canton-S wild type. Values shown are 9.5 ± 0.4, 8.9 ± 0.3 and 9.3 ± 0.3 pA, respectively, P > 0.05. (**C**). Cumulative probability plots of individual mepsc currents for both WT and $dys^{Dp186\ 166.3}$ show no differences in amplitude distribution. (**D**) The frequency of mepsc's is significantly greater in both $dys^{Dp186\ 30.3}$ and $dys^{Dp186\ 166.3}$ compared to wild type. Values shown are 9.2 ± 1.7, 16.3 ± 3.1 and 14.6 ± 3.0 events per min, *P ≤ 0.05. Currents were recorded from at least 8 cells of each genotype.

### 3.3 RNA interference and rescue experiments reveal a postsynaptic role for Dp186

Our data indicates that Dp186 is required for normal synaptic signaling between interneurons and motoneurons in the CNS. To better localize the site of action of Dp186, we adopted two approaches. First, we used targeted expression of an RNA interference (RNAi) transgene to reduce the endogenous levels of Dp186 protein specifically either pre- or postsynaptically. Second, we attempted to rescue the Dp186 mutant electrophysiological phenotype by tissue-specific expression of a wild type Dp186 transgene. Targeted expression of both RNAi and rescue constructs was achieved through use of two well-characterized Gal4 drivers. B19-Gal4 drives expression in all cholinergic neurons, including those interneurons that are presynaptic to aCC/RP2 (Baines, 2004). RN2-Gal4 is an even-skipped promoter-Gal4 transgene that drives expression in the aCC and RP2 motoneurons (Fujioka *et al.*, 2003; Baines, 2004). The efficiency of the RNA-interference was determined by quantitative RT-PCR and Dp186 expression levels were found to be approximately 3-fold reduced when the UAS-RNAi-Dp186 constructs were expressed throughout the larval neuropile by use of the Elav-Gal4 driver (Supplemental Figure).
Expression of the Dp186-RNAi transgene was sufficient to phenocopy the Dp186 mutant phenotype: synaptic currents were significantly increased, when expressed in either presynaptic cholinergic neurons or in the postsynaptic motoneurons aCC/RP2 (131 ± 4.0 and

164 ± 7.6 pA, respectively, P ≤ 0.01, Figure 5A). The increase in synaptic currents observed was, however, greater when the RNAi transgene was expressed in the postsynaptic aCC/RP2 motoneurons. Rescue of the Dp186 mutant phenotype by expression of a UAS-Dp186 transgene was only observed following expression in the postsynaptic aCC/RP2 motoneurons and not when expression was limited to the presynaptic cholinergic interneurons (104 ± 2.8 and 148± 2.4 pA, P ≤ 0.01 and P > 0.05, respectively, Figure 5B). Taken together, these data are consistent with a predominantly postsynaptic role for Dp186 in the regulation of the strength of central motoneuron synaptic excitation.

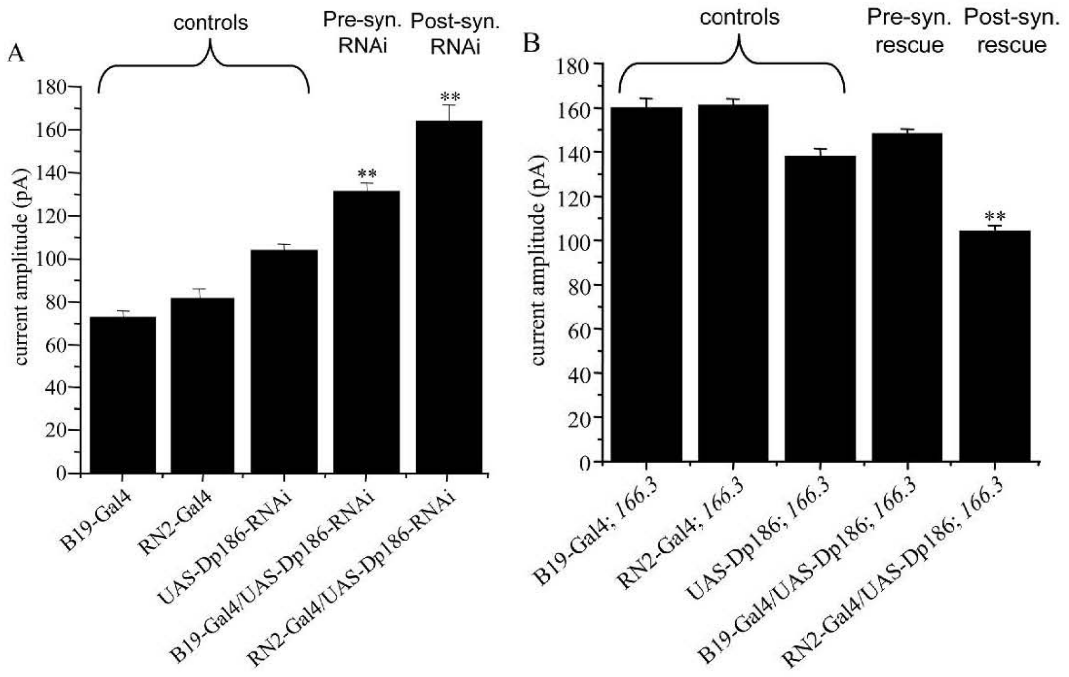


**Figure 5. Dp186 is required in postsynaptic motoneurons: tissue-specific RNA interference vs. rescue.** (**A**). Expression of an RNA interference transgene specifically targeting Dp186, is sufficient to increase action potential-dependent synaptic currents when expressed in postsynaptic motoneurons aCC/RP2 (RN2-Gal4) and to a lesser extent, in presynaptic cholinergic neurons (B19-Gal4). Averaged current amplitudes are plotted for parental control lines (B19- and RN2Gal4's and UAS-RNAi-Dp186) and expression lines (B19-Gal4/UAS-RNAi-Dp186 and RN2-Gal4/UAS-RNAi-Dp186). Values shown are 72 ± 3.1, 82 ± 4.2, 104 ± 3.4, 131 ± 4.0 and 164 ± 7.6 pA, respectively, ** P ≤ 0.01. (**B**) Rescue of the Dp186 ($dys^{Dp186\ 166.3}$) mutant phenotype requires postsynaptic expression of a UAS-Dp186 transgene (RN2-Gal4/UAS-Dp186). Values shown are 160 ± 4.2, 161 ± 3.1, 138 ± 3.4, 148 ± 2.4 and 104 ± 2.8 pA, ** P ≤ 0.01. Currents recorded from at least 8 cells in each genotype.

### 3.4 BMP signaling is required for increased synaptic transmission in the Dp186 mutant

The observation that Dp186 is required in postsynaptic motoneurons in order to regulate the efficacy of presynaptic neurotransmitter release is consistent with the existence of a retrograde signal. Recent studies have shown that such a signaling mechanism, based on the secretion of the BMP ligand Gbb, is active at both the NMJ and at motoneuron to interneuron synapses in the CNS (Aberle *et al.*, 2002; Marques *et al.*, 2002; McCabe *et al.*, 2003; Baines, 2004). To determine whether the increase in synaptic currents observed in aCC/RP2 in the Dp186 mutant is reliant on BMP retrograde signaling, we performed whole cell recordings in larvae lacking both Dp186 and Gbb ($gbb^{1}$;$dys^{Dp186\ 166.3}$) (Figure 6).

As previously reported (Baines, 2004), synaptic currents in aCC/RP2 were significantly reduced in homozygous $gbb^{1}$ mutants compared to heterozygous controls (54 ± 1.5 pA vs 79.5 ± 2.2, Figure 6A, see also Baines, 2004). In the presence of only a single copy of the wild type *gbb* gene, the absence of Dp186 ($gbb^{1}$/Cyo::GFP; $dys^{Dp186\ 166.3}$) was sufficient to increase synaptic currents to a level (128 ± 2.6 pA, Fig 7A) comparable to that observed in the Dp186

mutant with wild type levels of Gbb expression ($dys^{Dp186\ 166.3}$). In contrast, recordings from aCC/RP2 in the homozygous $gbb^{1}$;$dys^{Dp186\ 166.3}$ mutant did not exhibit increased synaptic currents to the same extent (82 ± 3.2 pA, P ≤ 0.01, Figure 6A). Cumulative probability plots show more clearly that the majority of currents recorded in $gbb^{1}$;$dys^{Dp186\ 166.3}$ resemble those recorded in the *gbb* null mutant, with the exception of a sub-population of larger currents still being present in $gbb^{1}$;$dys^{Dp186\ 166.3}$ (labeled by *, see Figure 6B). The observation that not all $dys^{Dp186\ 166.3}$-induced currents are reduced in the absence of Gbb indicates that Dp186 possibly regulates the efficacy of other retrograde signals, in addition to Gbb, at this synapse (see Discussion).

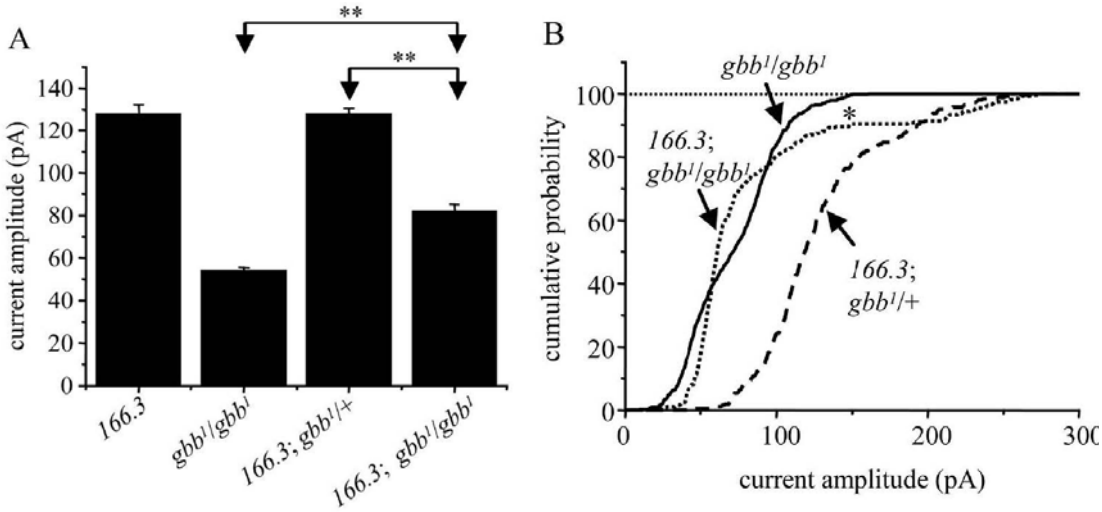


**Figure 6. Increased synaptic currents in aCC/RP2 are dependent on Gbb-signaling.**
**(A)** In the absence of Gbb, homozygosity for a Dp186 mutant allele ($gbb^{1}/gbb^{1}$; $dys^{Dp186\ 166.3}$) results in a significantly smaller increases in synaptic current amplitude recorded in aCC/RP2 than observed if either one ($gbb^{1}$/Cyo::GFP; $dys^{Dp186\ 166.3}$) or both ($dys^{Dp186\ 166.3}$) copies of *gbb* are present. The increase in average synaptic current amplitude observed in the absence of Gbb ($gbb^{1}/gbb^{1}$; $dys^{Dp186\ 166.3}$) is still, however, significantly larger than in *gbb* nulls ($gbb^{1}/gbb^{1}$). Values shown are 128 ± 4.4, 54 ± 1.5, 128 ± 2.6 and 82 ± 3.2 pA, **P ≤ 0.01. Currents recorded from at least 8 cells in each genotype. (**B**) Cumulative probability plots show that the majority of individual currents recorded in $gbb^{1}/gbb^{1}$; $dys^{Dp186\ 166.3}$ have the same amplitude distribution as those recorded in *gbb* nulls ($gbb^{1}/gbb^{1}$). However, this distribution deviates for larger amplitude currents in $gbb^{1}/gbb^{1}$; $dys^{Dp186\ 166.3}$ (marked by *).

## 4. Discussion

In addition to progressive muscle degeneration, many DMD patients display cognitive impairments (reviewed in Anderson *et al.*, 2002; Culligan and Ohlendieck, 2002), possibly reflecting alterations in CNS synaptic function. The localization of Dystrophin to synapse-rich regions (reviewed in Blake and Kroger, 2000) is also consistent with Dystrophin having a critical role in the CNS. While a mouse model lacking a brain-specific Dystrophin isoform exhibits memory deficits which likely reflect increased long-term potentiation in the hippocampus and decreased long-term depression in the cerebellum (Vaillend *et al.*, 2004), little is known about whether or not the physiology of Dystrophin-deficient central synapses is altered. We show in this study that the *Drosophila* Dp186 isoform is expressed in the CNS and is likely enriched in motoneuronal dendrites, the postsynaptic domains of contact between interneurons and the motoneuron. In the absence of Dp186, evoked presynaptic neurotransmitter release is considerably increased at an identified interneuron-motoneuron central synapse, while no apparent changes in postsynaptic receptor field sensitivity were observed. Transgenic RNA interference and rescue experiments indicate that Dp186 is required primarily in the postsynaptic motoneuron to maintain wild type levels of presynaptic release. The observed effects of the absence of the postsynaptic Dp186 isoform on

presynaptic neurotransmitter release at a central synapse and a requirement for the BMP, Gbb, in this phenotype are consistent with a role for Dp186 in retrograde signaling.

### 4.1 Dystrophin Dp186 accumulates at synaptic regions of the CNS

During embryogenesis, the Dp186 protein is found close to the longitudinal axon bundles of the ventral nerve cord. The colocalization of Dp186 and a presynaptic protein, Bruchpilot (Kittel *et al.*, 2006; Wagh *et al.*, 2006), indicates that this domain is rich in synaptic contacts and likely includes motoneuronal dendrites and their interneuronal inputs. In the third instar larval neuropile, Dp186 also colocalizes with the presynaptic Synapsin protein (Klagges *et al.*, 1996). The density of synaptic contacts in these regions precluded us from evaluating the precise degree of colocalization between Dp186 and these presynaptic markers, but these data support Dp186's synaptic localization. Double stainings performed with anti-Dp186 and membrane-associated GFP (mCD8-GFP, (Lee and Luo, 1999) expressed in motoneurons, reveal colocalization, suggesting that Dp186 is present at interneuron/motoneuron synapses, most likely at the motoneuron dendrites. This physical localization is supported by the transgenic rescue experiments that indicate a postsynaptic requirement for Dp186.

In addition to its expression in the larval neuropile, Dp186 is highly expressed in three lateral clusters, likely the thoracic neuromeres, at the anterior end of the neuropile. The thoracic neuromeres contain embryonic neuroblasts whose developmentally-arrested progeny differentiate into mature neurons during metamorphosis (Truman and Bate, 1988). As previously reported (Truman *et al.*, 2004), these structures were found to stain with fluorescently-tagged phalloidin, reflecting the presence of densely packed F-actin filaments. The significance of the localization of Dp186 to the thoracic neuromeres is at present unclear, however we note that, unlike the large Dystrophin isoforms, Dp186 does not bear an actin-binding domain and is therefore unlikely to localize to the neuromeres due to direct interactions with F-actin.

### 4.2 Dystrophin Dp186 is required for wild type synaptic physiology

In a previous study, we observed that the absence of the large postsynaptically-expressed Dystrophin DLP2 isoform resulted in increased presynaptic neurotransmitter release at the *Drosophila* NMJ (van der Plas *et al.*, 2006). To address whether Dp186 might play a similar role at interneuronal synapses, we generated mutant lines lacking Dp186 protein and evaluated the electrophysiology of a well-characterized synapse between the aCC/RP2 motoneurons and their presynaptic cholinergic interneurons (Baines *et al.*, 2001; Baines, 2003). Unlike the NMJ motoneurons, the presynaptic cholinergic neurons cannot be directly stimulated to allow evaluation of evoked responses. However, in the preparation used, endogenous evoked responses, that form part of the motor pattern generator, occur at defined frequencies from late embryogenesis onwards (Baines *et al.*, 2002), allowing the recording of endogenous evoked responses.

Recordings of such endogenous evoked currents in Dp186 mutants reveal that they are significantly increased in amplitude, but not frequency, relative to wild type controls and a mutant lacking DLP2. Recordings performed in the presence of TTX, which allows measurement of spontaneous mepsc events in the absence of evoked responses, indicate that the postsynaptic AChR field is apparently unaffected by the absence of Dp186. Together with the increased frequency of mepsc observed in the Dp186 mutant, these findings support the hypothesis that evoked presynaptic neurotransmitter release is significantly increased in the absence of Dp186.

We took advantage of a combination of the tissue-specific expression afforded by the UAS-Gal4 system and transgenic rescue and RNA interference to evaluate where Dp186 is required to maintain appropriate presynaptic release. Our results revealed that postsynaptic, but not presynaptic, expression of Dp186 in the $dys^{Dp186}$ mutant background rescued presynaptic

release to wild type levels. Therefore, in agreement with our localization of Dp186 to the motoneuron dendrites, Dp186 is apparently required postsynaptically.

Our results from the transgenic RNA interference approach are less unambiguous, but do show that the largest increase in presynaptic release occurred when post-synaptic Dp186 expression levels were decreased, supporting the rescue data. Presynaptic expression of Dp186-RNAi, however, also resulted in increased release, albeit to lower levels. A possible explanation is that suppression of Dp186 in first order interneurons that drive motoneurons might, in turn, elevate their own excitation from second order interneurons (to which they are postsynaptic). The resultant increased activity in these first order interneurons would likely manifest as increased synaptic excitation of downstream motoneurons. While many details of the circuitry that form the motor pattern generator are lacking, first order interneurons that synapse directly with motoneurons are indeed reliant on second order interneurons for synaptic excitation (Carhan *et al.*, 2004). At present, markers suitable for evaluating whether Dp186 is present at these upstream synapses are not available.

### 4.3 Dystrophin and the retrograde control of presynaptic release

The requirement for Dp186 in motoneurons for normal function of presynaptic cholinergic interneurons is consistent with the regulation of neurotransmitter release by a retrograde signal derived from the targets. Recent studies have shown that presynaptic release at these synapses is regulated, at least in part, by BMP signaling (Baines, 2004). Moreover, increasing expression of the BMP ortholog, Gbb, in postsynaptic motoneurons is sufficient to significantly increase synaptic current amplitudes, achieving levels similar to those observed in the Dp186 null mutants (Baines, 2004). We therefore examined the effects of eliminating Gbb signaling upon the Dp186 electrophysiological phenotype. In the absence of Gbb, the increase in synaptic currents caused by the lack of expression of Dp186 was significantly less than controls, suggesting Dp186 might regulate the release of Gbb or its efficacy of action on the presynaptic apparatus.

However, in the absence of both Dp186 and Gbb, the amplitude of evoked synaptic currents is still significantly higher compared to the *gbb* null control. While the precise reason for this remains unknown, one likely possibility is that Dp186 might regulate additional retrograde signals operating at these synapses. Indeed, preliminary evidence supports the existence of such additional signals; postsynaptic expression of CREB transgenes in aCC/RP2 is also sufficient to increase presynaptic release that does not depend on the presence of Gbb (R. Baines, unpublished data). The identity of this additional signal is unclear but it may be one or more of the six related BMP signaling molecules present in *Drosophila*, Dpp, Screw, Activin, Activin-like protein, Myoglianin and Maverick (Lo and Frasch, 1999; Keshishian and Kim, 2004).

Our previously reported observation that BMP signaling is required for the effects of reduced postsynaptic expression of DLP2 at the NMJ (van der Plas *et al.*, 2006) is likely due to the impaired responsiveness of the stunted synapse in the absence of BMP signaling. Reductions in BMP signaling at the interneuronal synapse studied here, did not significantly affect the morphology of the postsynaptic motoneuron cell bodies, dendrites and axons as assayed by visualization of retrograde dye-labeled neurons (RAB, unpublished data). Whether or not the presynaptic cholinergic interneurons that provide synaptic inputs to aCC/RP2 are affected by a lack of BMP signaling is unknown; these interneurons remain unidentified. However, the observation that only synaptic current amplitude, but not frequency, is affected in the Dp186 mutant is consistent with there being no change in the synaptic connectivity between interneurons and motoneurons. Thus, in contrast to the NMJ which is apparently completely reliant on BMP signaling for both morphological and functional development, the maturation of central synapses seem likely to be dependent on additional factors, other than BMP

signaling molecules. It remains to be determined what these additional signals are and, importantly, whether they are also active at the NMJ.

In summary, the *Drosophila* Dystrophin Dp186 isoform is required postsynaptically for wild type levels of neurotransmitter release at an identified cholinergic central synapse. Similarly, our previous study of the DLP2 isoform revealed that the absence of DLP2 from the muscle increased the probability of presynaptic release at the glutamatergic NMJ (van der Plas *et al.*, 2006). Therefore, these two postsynaptically-localized Dystrophin isoforms are required for the appropriate regulation of presynaptic release at two different types of synapses utilizing different neurotransmitters. At present, precisely how the absence of Dp186 or DLP2 results in increased presynaptic release and whether common downstream targets or mechanisms are present at both types of synapses is unclear. Furthering our understanding of the role of the Dystrophin isoforms in synaptic transmission in *Drosophila* should yield insights into evolutionarily-conserved roles of Dystrophin in the nervous system and perhaps shed light on the poorly understood mental retardation presented by a significant subset of DMD patients.

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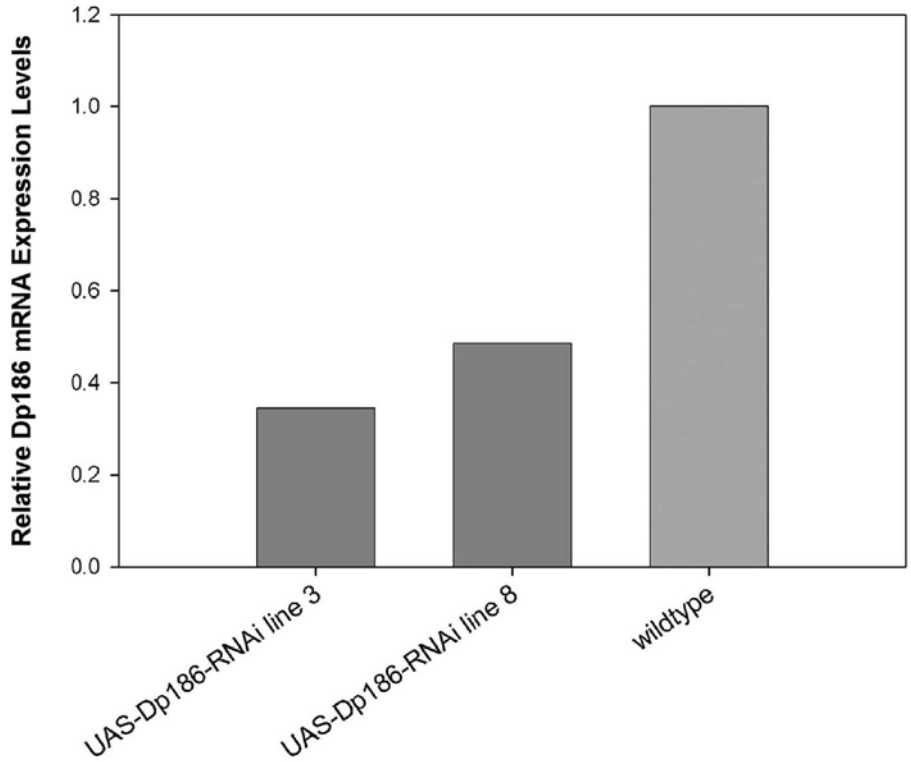
# 7. Supplemental data




**Supplemental Figure. Quantitative RT-PCR analysis of *dystrophin* expression in the RNAi-Dp186 lines and the mutant $dys^{Dp186\,166.3}$.**
Quantitative RT-PCR (qRT-PCR) analysis was performed to determine the levels of Dp186 transcript in UAS-Dp186-RNAi line 3 and UAS-Dp186-RNAi line 8 expressed under control of the pan-neuronal Elav-Gal4 driver. In brief, total RNA was prepared from dissected 3rd instar larval brain/imaginal disc complexes from the lines and wild type controls and reversed-transcribed into first-strand cDNA. qRT-PCR was performed on a 7900HT Fast Real-Time PCR System using Power Syber Green reagents (Applied Biosystems, Foster City, CA, USA). Dp186 levels were normalized between samples using contemporaneously amplified ribosomal subunit mRNA, RP49. The following primer sets were used: RTDp186F1: CCAGCTAACCCAGAACTCAAGC, RTDp186R1: TGAGTCGCTCAGCATGTTTTTC and RTRP49F1: TCTGAT GCCCAACATCGG T and RTRP49R1: AAACGCGGTTCTGCATGAG.