Dystrophin Is Required for Appropriate Retrograde Control of Neurotransmitter Release at the Drosophila Neuromuscular Junction

Mariska C. van der Plas,1,2* Gonneke S. K. Pilgram,1,2* Jaap J. Plomp,2,3 Anja de Jong,1 Lee G. Fradkin,1 and Jasprina N. Noordermeer1

1Laboratory of Developmental Neurobiology, 2Section of Neurophysiology, Department of Molecular and Cell Biology, and 3Department of Neurology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

Mutations in the human dystrophin gene cause the Duchenne and Becker muscular dystrophies. The Dystrophin protein provides a structural link between the muscle cytoskeleton and extracellular matrix to maintain muscle integrity. Recently, Dystrophin has also been found to act as a scaffold for several signaling molecules, but the roles of dystrophin-mediated signaling pathways remain unknown. To further our understanding of this aspect of the function of dystrophin, we have generated Drosophila mutants that lack the large dystrophin isoforms and analyzed their role in synapse function at the neuromuscular junction. In expression and rescue studies, we show that lack of the large dystrophin isoforms in the postsynaptic muscle cell leads to elevated evoked neurotransmitter release from the presynaptic apparatus. Overall synapse size, the size of the readily releasable vesicle pool as assessed with hypertonic shock, and the number of presynaptic neurotransmitter release sites (active zones) are not changed in the mutants. Short-term synaptic facilitation of evoked transmitter release is decreased in the mutants, suggesting that the absence of dystrophin results in increased probability of release. Absence of the large dystrophin isoforms does not lead to changes in muscle cell morphology or alterations in the postsynaptic electrical response to spontaneously released neurotransmitter. Therefore, postsynaptic glutamate receptor function does not appear to be affected. Our results indicate that the postsynaptically localized scaffolding protein Dystrophin is required for appropriate control of neuromuscular synaptic homeostasis.

Key words: Dystrophin; neuromuscular junction; neurotransmitter release; Drosophila; DGC; synaptic homeostasis

Introduction

Duchenne (DMD) and Becker muscular dystrophy are caused by mutations in the dystrophin (dys) gene (Hoffman et al., 1987). Dystrophin and its partially redundant homolog Utrophin are associated with a number of other proteins, members of the Dystrophin–glycoprotein complex (DGC) (Ervasti and Campbell, 1991). The DGC links the actin cytoskeleton to the extracellular basal lamina, providing tensile strength to muscle fibers. In addition to its structural role, the DGC acts to scaffold signaling molecules (for review, see Rando, 2001). How the DGC and associated signaling partners interact with other cellular pathways remains unclear.

Members of the mammalian DGC complex, including Dystrophin and Utrophin, are found in the CNS and the extrasynaptic and postsynaptic regions of muscle cells (for review, see Blake et al., 2002). Reduction of postsynaptic junctional folds and acetylcholine receptor (AChR) clustering are observed at the neuromuscular junction (NMJ) in the dystrophin/utrophin double knock-out mouse, but electrophysiological analyses revealed a normal postsynaptic response to spontaneous neurotransmitter release (Deconinck et al., 1997a; Grady et al., 1997). These and other data suggest that dystrophin and utrophin likely play partially redundant and subtle roles at the mammalian NMJ (Lyons and Slater, 1991; Deconinck et al., 1997b). A single dystrophin ortholog exists in Drosophila (Greener and Roberts, 2000); its isoforms are predominantly expressed in the muscle and nervous system (Neuman et al., 2001; Dekkers et al., 2004). In this study, we investigate the role of the postsynaptically localized dystrophin-like protein 2 (DLP2) Dystrophin isoform at the Drosophila NMJ.

During maturation and modification of synaptic contacts, homeostatic mechanisms match neurotransmitter release levels to changing postsynaptic requirements, keeping depolarization levels within a narrow range (for review, see Turrigiano, 1999; Davis and Bezprozvanny, 2001). Studies of myasthenia gravis patient...
NMJs (in which autoantibodies reduce AChR number) revealed that, in addition to anterograde signaling, the homeostatic machinery encompasses retrograde signals required for the appropriate regulation of presynaptic neurotransmitter release (Cull-Candy et al., 1980; Plomp et al., 1995). Similar compensatory neurotransmitter release upregulation was shown in rodsents with decreased postsynaptic AChR levels (Plomp et al., 1992; Sandrock et al., 1997) and in Drosophila with reduced DGLuRIIA glutamate receptor function (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999).

In Drosophila, several proteins have been implicated in NMJ retrograde signaling (Keshishian and Kim, 2004), including members of the bone morphogenetic protein (BMP) pathway, the presynaptic type II receptor wit (for wishful thinking), and its muscle-derived ligand gbb (for glass bottom boat) (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003). Postsynaptic calcium/calmodulin-dependent kinase (CaMKII) activity has also been reported to module retrograde signaling via wit (Haghighi et al., 2003).

Here, we take advantage of Drosophila genetics to study the role of dystrophin at the NMJ. We show that dystrophin is required for appropriate homeostatic control of neurotransmitter release. Absence of postsynaptically localized Dystrophin results in a wit-dependent increase in neurotransmitter release, leading to enhanced muscle depolarization. Furthermore, short-term synaptic facilitation is impaired in the dystrophin mutant, indicating an increase in the probability of release. These results reveal a novel role for dystrophin in the dynamics of neurotransmitter release.

Materials and Methods

Fly stocks. w1118, the genetic background in which the dystrophin mutations were generated, and dysEGH, a precise excision of the P-element used for the mutagenesis, served as the wild-type controls for stainings, electrophysiology, and EM analyses. The following Gal4 driver lines were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002). The GSI2472 insertion (Toba et al., 1999), 1.9 kb upstream of the DLP2 ATG initiator, was used to overexpress DLP2. Upstream activating sequence lines (UAS)-Ala and UAS-CaMKII (Griffith et al., 1993; Jin et al., 1993) were used: 24B-Gal4 (Brand and Perrimon, 1993), G14-Gal4 (Aberle et al., 2002), Elav-Gal4 (Luo et al., 1994), and OK6-Gal4 (Aberle et al., 2002).
mutants was performed at 1.5 mM Ca\(^{2+}\) to approximate physiological conditions. However, because the extent of increased transmitter release at dystrophin mutant NMJs did not show Ca\(^{2+}\) dependence (see Fig. 5A), we performed the remainder of the measurements at 0.6 mM Ca\(^{2+}\) to prevent occasional muscle contractions. Electrical input resistance of muscle fibers was similar for all genotypes (∼4.5 MΩ; data not shown). Synaptic signals were only measured when the resting membrane potential was below −50 mV (on average, the membrane potential was −60 mV in all samples, which is in accordance with the use of HL3 buffer) and recorded using a Geneclamp 500B amplifier (Axon Instruments, Union City, CA), low-pass filtered at 10 kHz, high-pass filtered at 0.1 Hz, and digitized using a Digidata 1322A and pClamp9 software (Axon Instruments). The miniature excitatory junction potentials (mEJPs) were recorded continuously for 1 min. During supramaximal stimulation of the ventral nerve cord via a suction electrode connected to a pulse generator, 30 EJPs were recorded at 0.3 Hz stimulation. Raw mEJP data were first filtered off-line in Clampfit 9.0 (Axon Instruments) using a boxcar filter with 19 smoothing points. Subsequently, the mean mEJP amplitude and frequency were determined using the peak detection feature of MiniAnalysis 6.0 (Synaptosoft, Decatur, GA); all events were verified by eye. mEJP amplitudes were analyzed using Clampfit 9.0. All mEJPs were normalized to a membrane potential of −60 mV, because all of the measurements were performed in the linear range of the EJP amplitude–V_m relationship (Jan and Jan, 1976). Quantal content (QC) was calculated per NMJ by dividing the mean EJP amplitude (calculated from 30 events) by the mean mEJP amplitude (calculated from ~100 events). The EJP amplitude was first corrected for nonlinear summation (Martin, 1955) using an f-factor of 0.4 (B. A. Stewart, personal communication) and a reversal potential of −10 mV. Failure analysis was performed in 0.25 mM Ca\(^{2+}\), and QC was calculated using the formula \( m = \frac{\ln(N_{f}/N_{0})}{N_{c}} \), where \( m \) is the QC, \( N_{f} \) is the number of stimuli, and \( N_{0} \) is the number of failures. QC estimated using the variance method was calculated using the formula \( m = \frac{(\text{mean EJP/SD})^{2}}{m} \). Paired-pulse facilitation of EJP amplitude was assessed using 50 ms interstimulus interval in 0.6 mM Ca\(^{2+}\). Hypertonic shock was induced by adding 50 mM sucrose to the HL3 medium as described previously (Suzuki et al., 2002), and the increased mEJP frequency was measured for 1 min after the start of the incubation. In some control experiments, we monitored the sucrose response for 5–20 min and observed that the elevated mEJP frequency declines only very slowly during the first 5–20 min after the start of the incubation (∼50% per 10 min). This contrast with the burst-like, only-seconds-lasting, response observed in Drosophila embryonic NMJs (Suzuki et al., 2002; Kidokoro et al., 2004). Third-instar larval Drosophila NMJs in this respect resemble frog and mouse NMJs, in which the sucrose response also remains active for many minutes (Grinnell et al., 2003) (J. J. Plomp, unpublished observations).

NMJs at muscles 6 of three separate segments were measured for each larva, and, for each parameter, the average larval value was calculated and taken into the calculation of the group mean values; the number of larvae (n value) per genotype is indicated in the figure legends. ANOVA statistics were performed (SPSS version 11; SPSS, Chicago, IL), and differences were considered significant when \( p < 0.05 \). Transmission electron microscopy and morphometric analysis. Previously described techniques for dissection, fixation, embedding, and sectioning were followed (Lin and Goodman, 1994). Complete semi-serial sections of muscles 6 and 7, segment A3–A5, were cut, and electron micrographs were made of type Ib boutons using a CM10 electron microscope (Philips, Amsterdam, The Netherlands). Within a bouton, the micrograph with the largest diameter of the bouton, corresponding to the bouton midline, was selected for analysis. Fifteen electron micrographs from three larvae of each genotype were scanned, and the morphometric and statistical analyses were performed using Scion (Frederick, MD) software and SPSS version 11, respectively.

Muscle area, terminal length, and bouton number were determined at muscles 6 and 7 of segment A3 from digital images captured by light and fluorescence microscopy and analyzed using Scion software. Anti-HRP (Jan and Jan, 1982) was used to visualize the boutons. Thirty segments from 15 individual larvae from each genotype were measured.

### Results

The large Dystrophin isoform DLP2 is expressed at the NMJ postsynaptic region

The Drosophila dystrophin gene encodes at least four protein isoforms bearing a number of highly conserved domains (Fig. 1) (Greenner and Roberts, 2000; Neuman et al., 2001). The three large isoforms DLP1, DLP2, and DLP3 have an N-terminal actin-binding domain, spectrin repeats, and a C-terminal cysteine-rich domain, which in mammals has been shown to interact with other DGC proteins. A shorter isoform, Dp186, has a unique N-terminal domain appended to the pan-Dystrophin C-terminal domain (Fig. 1).

DLP1, DLP2, and Dp186 are expressed during all stages of development, whereas DLP3 is only expressed in the adult fly (Neuman et al., 2001). During embryogenesis, the DLP1 mRNA is present in the visceral mesoderm, whereas DLP2 is expressed in the visceral mesoderm, in muscle attachment sites, throughout muscle fibers, in the mesectodermal cells at the midline, and the gut (supplemental Fig. 1, available online). The dysE6 2.7 kb deletion is indicated. The G20705 P-element insertion near the Dp186 initiator ATG reduces expression of both Dp186 and the large isoforms. Exons are indicated as bars and introns as horizontal lines. The conserved Dystrophin protein domains, an actin-binding domain, spectrin repeats, and a cysteine (cy)-rich C-terminal domain are indicated for DLP1, DLP2, DLP3, and Dp186. Dp186 has a unique N-terminal domain. Regions used to generate the anti-Dyslarge, anti-DysC02H, and anti-Dp186 antisera are indicated.
form DLP2 mRNA, but not DLP1, is found in all muscle fibers (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The large isoforms are not detectably expressed in the larval brain or neuropil (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Fig. 2H). In contrast, Dp186 mRNA is found most predominantly throughout the larval neuropil and brain and in the eye-antennal discs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

We have generated three region-specific antibodies that recognize (1) the known large Dystrophin isoforms DLP1, DLP2, and DLP3 (anti-Dyslarge), (2) the CNS-specific isoform (anti-Dp186), and (3) the C-terminal region common to all Dystrophin isoforms (anti-DysCO2H) (Fig. 1B). Western analyses of embryonic extracts using these antibodies show that the anti-Dyslarge antibodies recognize a protein of the expected size for the large isoforms (~400 kDa), whose level is strongly reduced in the dystrophin mutants dysGE20705 and increased when the large isoform DLP2 is overexpressed. The anti-Dp186 antibody recognizes a protein species of the appropriate size (~190 kDa), and the anti-DysCO2H antibody predominantly recognizes the large isoforms, but also Dp186, when overexpressed (Fig. 2F).

The region-specific antibodies were used to determine the protein expression domains of the Dystrophin isoforms. The anti-DysCO2H (Fig. 2A) and anti-Dyslarge (Fig. 2D) antibodies label synaptic and extrasynaptic sites of expression in third-instar larval body walls. To determine whether the Dystrophin protein is present at the presynaptic or the postsynaptic side of the NMJ, larval body walls were colabeled for the presynaptically localized HRP protein (Jan and Jan, 1982) and the Dystrophin protein. No overlap in staining was apparent (Fig. 2F), indicating that the Dystrophin protein is postsynaptically localized at the larval NMJ. This result is consistent with our finding that the Dystrophin protein is postsynaptically localized at the NMJ.

We used the region-specific antibodies to determine the localization of the Dystrophin isoforms. The anti-DysCO2H, which recognizes all Dystrophin isoforms (A–C), anti-Dyslarge, which recognizes only the large isoforms (D, E), double labeled with anti-HRP and anti-DysCO2H (F), or double labeled with anti-actin and anti-DysCO2H (G). A, Dystrophin protein is expressed at the wild-type third-instar larval NMJ at synaptic and extrasynaptic sites. B, Dystrophin protein is severely reduced in the dysGE20705 mutant. C, The DLP2 isoform protein accumulates highly at the NMJ after overexpression in the muscle (G14-Gal4/+; dysGE20705). D, Wild-type Dystrophin protein is recognized at the NMJ by the large isoform-specific antibody anti-Dyslarge. E, Overexpressed DLP2 (G14-Gal4/+; dysGE20705) can also be visualized using anti-Dyslarge antisera. F, Double labeling of a wild-type larval body wall with anti-HRP, staining presynaptic boutons (green), and anti-DysCO2H (red) reveals that the Dystrophin protein is postsynaptically localized at the NMJ. G, Double labeling of a wild-type larval body wall with anti-actin (green) and anti-DysCO2H (red) reveals that the Dystrophin colocalizes with actin at the NMJ extrasynaptic sites of expression. H, RNA in situ hybridization of wild-type larval neuropil (filled arrow), brain (arrowhead), and associated eye-antennal discs (open arrow) with an exon 4 antisense probe that labels all large dystrophin isoform mRNAs reveals no apparent expression of large dystrophin isoform mRNAs in the neuropil or brain. I, Western blot analysis of embryo extracts prepared from wild-type, dysGE20705, Elav-Gal4/GS12472 (overproducing DLP2), and Elav-Gal4/UAS-Dp186 (overproducing Dp186) using the indicated antibodies. The large Dystrophin isoforms (anti-Dyslarge panel) are absent from the mutant dysGE20705 and overexpressed in the Elav-Gal4/GS12472 embryos. Dp186 is expressed at decreased levels in dysGE20705 and is overexpressed in Elav-Gal4/UAS-Dp186 (anti-Dp186 panel). The pan-Dystrophin antibody (anti-dysCO2H, panel) confirms the overexpression of both the long isoforms and Dp186 in the overproducing embryos. The arrows indicate the large Dystrophin isoforms, and the short arrows indicate the Dp186 isoform. All lanes were loaded equally, as confirmed by the anti-ribosomal subunit PS3 antibody (anti-PS3 panel), except the last lane of the Dp186 blot, in which 5% of the protein was loaded to permit comparison of the extracts in a single exposure.

**Lack of postsynaptic Dystrophin results in an increase in neurotransmitter release**

To investigate the roles of dystrophin during Drosophila development, we have generated stocks that lack, or have severely reduced, expression of the large Dystrophin isoforms by P-element excision mutagenesis. The mutant dysE31 has a deletion of 2.7 kb (Fig. 1A); dysE31 is a precise excision control of the EP3397 P-element. We also used the independently derived P-element line dysGE20705 mentioned above, which is inserted 250 bp upstream of the Dp186 initiator codon. To determine whether, and to what extent, the mRNA levels of the specific Dystrophin isoforms in these mutants are affected, we performed semiquantitative RT-PCR on total RNA derived from mutant larval tissues using primers specific for each of the isoforms (see Material and Methods) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Dystrophin isoform proteins were proteolytically degraded during the preparation of larval extracts, thus precluding analysis of their expression levels by Western blotting. RT-PCR analyses revealed that dysE31 expresses DLP1 mRNA at wild-type levels but lacks DLP2 mRNA, whereas dysGE20705 shows an approximate threefold reduction in DLP1 and an approximate fourfold reduction in DLP2 mRNA levels.

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**Figure 2.** The large Dystrophin isoforms are localized postsynaptically. Third-instar larval body walls were stained with anti-DysCO2H, which recognizes all Dystrophin isoforms (A–C), anti-Dyslarge, which recognizes only the large isoforms (D, E), double labeled with anti-HRP and anti-DysCO2H (F), or double labeled with anti-actin and anti-DysCO2H (G). A, Dystrophin protein is expressed at the wild-type third-instar larval NMJ at synaptic and extrasynaptic sites. B, Dystrophin protein is severely reduced in the dysGE20705 mutant. C, The DLP2 isoform protein accumulates highly at the NMJ after overexpression in the muscle (G14-Gal4/+; dysGE20705). D, Wild-type Dystrophin protein is recognized at the NMJ by the large isoform-specific antibody anti-Dyslarge. E, Overexpressed DLP2 (G14-Gal4/+; dysGE20705) can also be visualized using anti-Dyslarge antisera. F, Double labeling of a wild-type larval body wall with anti-HRP, staining presynaptic boutons (green), and anti-DysCO2H (red) reveals that the Dystrophin protein is postsynaptically localized at the NMJ. G, Double labeling of a wild-type larval body wall with anti-actin (green) and anti-DysCO2H (red) reveals that the Dystrophin colocalizes with actin at the NMJ extrasynaptic sites of expression. H, RNA in situ hybridization of wild-type larval neuropil (filled arrow), brain (arrowhead), and associated eye-antennal discs (open arrow) with an exon 4 antisense probe that labels all large dystrophin isoform mRNAs reveals no apparent expression of large dystrophin isoform mRNAs in the neuropil or brain. I, Western blot analysis of embryo extracts prepared from wild-type, dysGE20705, Elav-Gal4/GS12472 (overproducing DLP2), and Elav-Gal4/UAS-Dp186 (overproducing Dp186) using the indicated antibodies. The large Dystrophin isoforms (anti-Dyslarge panel) are absent from the mutant dysGE20705 and overexpressed in the Elav-Gal4/GS12472 embryos. Dp186 is expressed at decreased levels in dysGE20705 and is overexpressed in Elav-Gal4/UAS-Dp186 (anti-Dp186 panel). The pan-Dystrophin antibody (anti-dysCO2H, panel) confirms the overexpression of both the long isoforms and Dp186 in the overproducing embryos. The arrows indicate the large Dystrophin isoforms, and the short arrows indicate the Dp186 isoform. All lanes were loaded equally, as confirmed by the anti-ribosomal subunit PS3 antibody (anti-PS3 panel), except the last lane of the Dp186 blot, in which 5% of the protein was loaded to permit comparison of the extracts in a single exposure.
Dp186 mRNA levels are wild type in dys\textsuperscript{E6} but are significantly reduced (~27-fold) in dys\textsuperscript{GE20705}. The precise excision control dys\textsuperscript{E1} shows wild-type expression levels of all isoforms. The lack of detectable large-isofom expression in the dys\textsuperscript{GE20705} mutant larval musculature was confirmed by staining mutant body walls with the anti-DysCO2H antibody (Fig. 2B) and by Western blot analysis of embryonic extracts using the three region-specific antibodies (Fig. 2I). The CG6255 gene, which encodes a putative testis-specific succinate–CoA ligase and is located in the intron between the DLP1 and DLP2 first exons, is deleted in dys\textsuperscript{E6} but not in dys\textsuperscript{GE20705}. RT-PCR analyses indicate that CG6255 is not expressed in dys\textsuperscript{E6} but is expressed in dys\textsuperscript{GE20705} at wild-type levels (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

We performed morphometric analyses of the muscle and motoneuron terminal at the well studied muscle 6/7 synapse (Atwood et al., 1993) to examine whether the dystrophin mutant NMJ or muscle displays any structural abnormalities. Wild-type and mutant body walls were labeled with anti–HRP, and muscle size, numbers of boutons, lengths of the synaptic termini, and the number of terminal branches were determined. No striking statistically significant differences were observed between dystrophin mutants and control larvae (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Furthermore, no apparent changes were seen in the distribution of the postsynaptically localized glutamate receptor subunits DGluRIIA and DGluRIIB, components of the major neurotransmitter receptors at the Drosophila NMJ (Fig. 3C–F). Because mammalian dystrophin isoforms have been reported to be found associated with T-tubules (Watkins et al., 1988), invaginations of the sarcolemma containing voltage-gated channels, we examined the distribution of the Discs-large protein, which labels the T-tubuli (Razzaq et al., 2001), in the dystrophin mutant. Because Dystrophin colocalizes extrasynaptically with actin, we also examined actin localization in the mutant. Both proteins exhibit wild-type expression in dys\textsuperscript{E6}, suggesting that general muscle morphology is not affected in the dystrophin mutants (Fig. 3).

The lack of overt muscle degeneration in the dystrophin mutants allowed us to examine whether the large Dystrophin isoforms play a role in synaptic transmission. We measured the depolarizations following from spontaneous and evoked neurotransmitter release with an intracellular microelectrode at NMJs of muscle 6 in wild-type and dystrophin mutant female third-instar larvae in medium containing 1.5 mM Ca\textsuperscript{2+} (Fig. 4A–E). EJP amplitudes, evoked by nerve stimulation at 0.3 Hz, were ~45% increased (p < 0.05) in dystrophin mutants compared with the control larvae (Fig. 4D). Spontaneous mEJP amplitudes were essentially unchanged in the mutants compared with the controls (Fig. 4C). QC, the number of neurotransmitter quanta released on a nerve impulse, was calculated by dividing the mean EJP amplitude, corrected for nonlinear summation (Martin, 1955), by the mean mEJP amplitude. QC in the mutants, dys\textsuperscript{E6} and dys\textsuperscript{GE20705}, was ~50–65% higher (p < 0.05) than at wild-type control synapses (Fig. 4E). Calculation of QC without correction for nonlinear summation also resulted in statistically significant differences between the dystrophin mutants and wild-type controls (data not shown). In addition, the frequency of spontaneous neurotransmitter release was elevated ~35–50% (p < 0.05) in the mutants (Fig. 4B). These results indicate that postsynaptic dystrophin is required to maintain wild-type levels of neurotransmitter release.

In addition, the inter-allelic dys\textsuperscript{E6}/dys\textsuperscript{GE20705} combination, the mutants each derived from an independent genetic background, showed a similar effect on EJPs, mEJPs, QC, and mEJP frequency as the homozygous mutants, further indicating that the phenotype observed was attributable to the mutation in dystrophin (Fig. 4). Interestingly, we saw a similar electrophysiological behavior in the heterozygotes, dys\textsuperscript{E6}/+ and dys\textsuperscript{GE20705}/+ (Fig. 4), indicating that dystrophin is haplo-insufficient for normal Drosophila NMJ physiology. Semiquantitative RT-PCR analysis shows that the DLP2 levels are reduced twofold to fourfold in the heterozygote animals (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

To determine whether the elevated neurotransmitter release in the mutant showed altered Ca\textsuperscript{2+} dependence, we compared the QCs of dystrophin mutant NMJs with controls measured at external Ca\textsuperscript{2+} concentrations ranging from 0.25 to 1.5 mM. Mutant NMJ QC was significantly higher than the controls at all concentrations tested (Fig. 5A). The slopes of the regression lines of the Ca\textsuperscript{2+} titration curves at the low Ca\textsuperscript{2+} range (0.25–0.6 mM) did not differ significantly, showing that the Ca\textsuperscript{2+} cooperativity at the mutant NMJ is apparently unchanged. Thus, the increased release observed in the dystrophin mutant is independent of the extracellular Ca\textsuperscript{2+} concentration and unlikely to be attributable to altered properties of the Ca\textsuperscript{2+} sensor that triggers presynaptic vesicle fusion.
We wanted to confirm the increase in QC using calculations independent of the mEJP amplitudes. Therefore, we applied failure analysis and the variance method (Boyd and Martin, 1956; Martin, 1966). Both failure and variance analyses performed on EJP data obtained at 0.25 mM Ca\(^{2+}\) demonstrated a similar increase in QC as calculated using the direct method (Fig. 5B). These results indicate that the increase in EJP amplitude is attributable to an increase in presynaptic glutamate release in the mutants.

Elevated QC can result from increased probability of release or increases in the size of the readily releasable vesicle pool. Because increased probability has been shown to be reflected by reduced paired-pulse facilitation (Rohrbough et al., 1999; Sandstrom, 2004), we determined this short-term dynamic behavior of neurotransmitter release in wild-type and dysE6 larvae (Fig. 5C). The protocol resulted in \(\approx 50\%\) facilitation in wild-type larvae but failed to do so in the dysE6 mutant (\(p < 0.05\)). The size of the readily releasable vesicle pool can be assessed experimentally by measuring asynchronous quantal release during incubation with hypertonic medium (Stevens and Tsujimoto, 1995). Application of 0.05 mM sucrose did not reveal significant differences in the elevated mEJP frequency level between the mutant and wild type (Fig. 5D). These analyses indicate that the elevation in evoked neurotransmitter release at the dystrophin-deficient NMJ resulted from increased probability of release rather than from an increased size of transmitter vesicle pool.

Transgenic RNA interference and rescue experiments show a postsynaptic requirement for Dystrophin

Our RT-PCR analyses indicated that the dysE6 mutation reduces the expression levels of DLP2 and CG6255 and dysGE207055 affects DLP2 and the CNS-specific isoform Dp186 but not CG6255. To further address potential roles for either CG6255 or Dp186 in the dystrophin mutant NMJ phenotype and to confirm that the postsynaptic absence of Dystrophin protein is responsible for the increased neurotransmitter release in the mutants, we used transgenic tissue-specific RNA interference to reduce Dystrophin levels specifically either presynaptically or postsynaptically. We generated transgenic flies that express double-stranded RNA (dsRNA) directed against dystrophin sequences present in the large dystrophin isoform mRNAs (UAS-RNAi-dysNH2) under Gal4 control (Brand and Perrimon, 1993). Semiquantitative RT-PCR analysis shows a more than eightfold decrease of DLP2 or Dp186 in the dystrophin mutant larvae (Fig. 6). However, when this construct is driven by the Elav-Gal4 transgene, no change in QC compared with the control was observed. The frequency of spontaneous neurotransmitter release was elevated in the dysE6 mutant (EJPs: 39.7 ± 0.6 mV (SEM) in dysE6 and 40.4 ± 0.9 mV in dysGE207055 vs 27.4 ± 1.9 mV in the control precise excision dysE31 larvae and 27.7 ± 1.3 mV in the wild-type (w1118/H11021) larvae; QCs, 147.4 ± 7.7 mV in dysE31 and 140.3 ± 7.7 mV in dysGE207055 vs 95.3 ± 8.0 in the control precise excision dysE31 larvae and 89.3 ± 4.7 in the wild-type larvae; mEJP frequencies, 3.6 ± 0.3/s in dysE6 and 3.3 ± 0.2/s in dysGE207055 vs 2.4 ± 0.4/s in the control precise excision dysE31 larvae and 2.4 ± 0.2/s in the wild-type larvae. *p < 0.05.

We then asked whether we could rescue the dystrophin mutant phenotype by increasing postsynaptic dystrophin expression. Lacking a rescue transgene, we took advantage of a P-element insertion, GS12472 (Toba et al., 1999), which lies 1.9 kb upstream of the DLP2 initiator codon and bears a UAS-dependent promoter appropriately oriented to express DLP2. Driving this P-element does not overexpress DLP1 or CG6255; the P-element insertion site is downstream of the unique first exon of DLP1, and CG6255 has the opposite orientation to the
The absence of postsynaptic Dystrophin results in an increase in the number of T-bars

To evaluate whether the electrophysiological changes in the mutant correlate with alterations in NMJ ultrastructure, we performed morphometric measurements on electron micrographs of mutant and control third-instar larval NMJs (Fig. 7) (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The bouton area and the surface area of the subsynaptic reticulum relative to the bouton area were similar for all genotypes, but the shape of the boutons in the mutants dysE20705 and dysE6 appeared slightly more elongated, as determined by the ratio of the longest and shortest diameter of a bouton section (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The area of the bouton occupied by vesicles was increased in the dysE6 (85.2 ± 1.6%; p < 0.05) and dysE20705 (80.5 ± 2.6%) mutants compared with wild type (73.2 ± 3.2%) and precise excision control dysE20705 (74.4 ± 2.8%). T-bars are electron-dense presynaptic structures possibly representing a subtype of neurotransmitter release site (Jia et al., 1993). The number of active zones with a T-bar relative to the total number of active zones was significantly increased in both the dysE6 and dysE20705 mutants by approximately two-fold (dysE6, 0.61 ± 0.05; dysE20705, 0.74 ± 0.05; wild type, 0.39 ± 0.03; p < 0.05) (Fig. 7), whereas the overall number of active zones did not increase. Although wild-type T-bar densities vary considerably between laboratories and their precise function is unclear, we report this increase in T-bars in the mutant relative to the isotonic control as an observed ultrastructural correlate of the increased neurotransmitter release in the dystrophin mutant.

BMP signaling at the NMJ is required for the increased neurotransmitter release in the Dystrophin mutant

The BMP pathway plays an important role in retrograde signaling at the larval NMJ (for review, see Keshishian and Kim, 2004) and in the Drosophila CNS (Baines, 2004). To evaluate the role of BMP signaling in the increase in neurotransmitter release induced by the absence of dystrophin, we performed electrophysiological recordings at NMJs lacking both dystrophin and wit (Fig. 8). We used the transgenic RNA interference approach described above to reduce postsynaptic dystrophin levels.

Homozygous wit mutant NMJs postsynaptically expressing dsRNA directed against the Dystrophin large isoforms (UAS-RNAi-dysNH2/+; G14-Gal4/+; wit) displayed mEJP amplitude, EJP amplitude, and QC values similar to the wit mutant alone. As shown previously (Marques et al., 2002), the homozygous wit
NMJ has very low EJP amplitudes (Fig. 8D) and low QC (Fig. 8E) but maintains wild-type level mEJP amplitudes. Thus, the absence of dystrophin failed to elicit an increase in QC in the wit background. Wit function and, by extension, BMP signaling is therefore required at the NMJ for the increase in neurotransmitter release elicited by the absence of dystrophin.

To evaluate whether the absence of postsynaptic dystrophin affects a known BMP target, we performed immunofluorescence analysis of mutant and wild-type embryonic ventral nerve cords, using an anti-phospho-Mad (PMad) antibody that recognizes the activated form of the Mad downstream effector of wit signaling (Tanimoto et al., 2000; Marques et al., 2002). We did not observe any differences in PMad expression levels or domains between the dystrophin mutants or individuals postsynaptically overexpressing DLP2 and controls, whereas homozygous wit mutants showed significantly decreased levels of the protein, as reported previously (supplemental Fig. 5, available at www.jneurosci.org as supplemental material) (Marques et al., 2002).

The dystrophin mutants show similar electrophysiological and morphological phenotypes as larvae postsynaptically expressing CaMKII inhibitors (Haghighi et al., 2003). Both display increased QC and an increase in the ratio of active zones with T-bars versus the total number of active zones, without additional significant changes in synaptic morphology. Furthermore, the increase in QC when CaMKII is reduced postsynaptically is dependent on wit function. Therefore, we examined embryonic ventral nerve cords that have reduced or elevated levels of CaMKII, UAS-Ala, and UAS-T287D, respectively, for PMad staining. No changes in the expression of PMad were observed (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). These results suggest that, although wit is required for increased QC at NMJs postsynaptically deficient for either Dystrophin or CaMKII, alteration of either Dystrophin or CaMKII levels does not result in changes in embryonic PMad expression. Thus, the interaction of dystrophin or CaMKII and wit in NMJ homeostasis is unlikely to involve regulation of PMad expression.

**Discussion**

This study provides evidence that postsynaptic dystrophin is required to maintain appropriate levels of presynaptic neurotransmitter release. The absence of dystrophin from the muscle results in abnor-

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**Figure 6.** RNA interference and rescue experiments show a postsynaptic requirement for the large Dystrophin isoforms at the larval NMJ. A, Representative traces of EJPs recorded from NMJs of wild-type (wt; w^{1118}) control, the mutant dys^{E6}, and the genotypes RNAi-dysNH2/G14 in which the large Dystrophin isoforms are downregulated and (Figure legend continues.)
Figure 7. The number of T-bars is increased in dystrophin mutants. Third-instar larval body walls were prepared by ultrathin sectioning, and the muscle 6 and 7 synapses were visualized by electron microscopy. A, A representative type I b bouton in a wild-type larva. B, A representative type I b bouton in a dystrophin mutant larva. T-bars are indicated by asterisks; M, mitochondria; SSR, subsynaptic reticulum. Scale bars, 1 μm. C, The graph shows the mean of the number of active zones with T-bars relative to the total number of active zones, based on the quantitative analysis of 84, 71, 107, and 58 active zones in at least 14 individual bouton sections at the midline for w^1118, dys^E6, dys^E31, and dys^E6/G14-Gal4 (n = 8), for the transgenic RNA interference genotypes, large-isoform-specific RNAi-dysNH2/+; Elav-Gal4/+; dys^E6 (n = 8)]#, for the rescue experiment, G14-Gal4/+, dys^E6 (n = 8); RNAi-dysNH2/G14-Gal4 (n = 8); RNAi-dysNH2/w^1118; dys^E6 (n = 8); OK6-Gal4/+, dys^E6 (n = 7); OK6-Gal4/+, dys^E6/G14-Gal4 (n = 8); dys^E6 (n = 8); dys^E6/G14-Gal4 (n = 8). A significant increase in T-bars is observed in the mutants, dys^E6 and dys^E6/G14-Gal4, but not when the large-isoform-specific dsRNA is expressed only in the muscle in the mutant background. *p < 0.05. D, The graph shows that neither the average length of active zones at the midline is significantly different between the genotypes nor the number of active zones normalized to the midline area of the bouton. The averaged numbers of active zones per bouton midline section are as follows: six for w^1118, five for dys^E6, seven for dys^E31, and five for dys^E6/G14-Gal4. AZ indicates active zones.

We performed morphometric analyses and stained with several muscle- and motoneuron-specific antibodies to examine the general morphological characteristics of the synapse and musculature in the mutants. These studies indicate that muscle and synapse size are not significantly altered by the lack of Dystrophin. Because we found that Dystrophin localizes extrasynaptically with actin, we examined actin distribution in the mutant muscle but did not observe differences relative to the controls. The morphology of the T-tubular network was also unaltered. That the absence of dystrophin does not grossly affect muscle morphology or glutamate receptor density/properties was reflected by our observation of unchanged electrical input resistance, mEJP amplitudes, and anti-glutamate receptor subunit antibody staining in the mutants.

Although DLP2 is expressed only postsynaptically and changes in synapse elaboration or muscle morphology were not observed in its absence, there are significant changes in the NMJ electrophysiology of the mutant. EJP amplitudes are increased with little or no change in the mEJPs, resulting in increased QC, i.e., the number of quanta released on stimulation, calculated by the direct, failure, or variance methods. We therefore examined presynaptic mechanisms whose alteration might account for the increase in QC at the mutant NMJ. The increase was correlated to an increase in the probability of release as indicated by reduced EJP facilitation at the mutant synapse (Schulz et al., 1994; Sandstrom, 2004).

In addition to increased release probability, an increased size of the readily releasable vesicle pool may account for the increased QC at the dystrophin mutant NMJ. However, this seems not the case in the dystrophin mutant because the increase in mEJP frequency at the mutant NMJ in response to hypertonic shock, a method used to assess the readily releasable pool (Stevens and Tsujimoto, 1995; Suzuki et al., 1996) studies indicate that, of the known dystrophin isoforms, only expression of DLP2 is affected by the mutation in dys^E6, (2) the mutant phenotype can be rescued by expressing DLP2 postsynaptically but not presynaptically, (3) expression of double-stranded RNA directed against sequences present in the DLP2 isoform results in increases in neurotransmitter release similar to those observed in the dys^E6 mutants, only when expressed postsynaptically, (4) RNA in situ analyses and RT-PCR assays show that DLP2 is highly expressed in larval muscle, DLP1 is not detectably expressed in the musculature, and all dystrophin large isoforms are absent from the neuropil in which the cell bodies of motoneurons are located, and (5) double labeling of larval body walls with anti-Dystrophin and anti-HRP, which labels the presynaptic membrane, reveals no overlapping expression domains.

We show in a number of experiments that the lack of the postsynaptically localized DLP2 isoform is responsible for the electrophysiological phenotype observed in the dystrophin mutants: (1) expression analyses and semiquantitative RT-PCR
al., 2002), did not differ from that at control NMJs. It should, however, be noted that it is not yet clear that only the readily releasable pool of vesicles is released after hypertonic shock of the Drosophila larval NMJ (Kidokoro et al., 2004).

Increased release probability may result from increased efficacy of the translation of presynaptic Ca\(^{2+}\) influx into transmitter release by the neuroexocytotic machinery, possibly attributable to increased sensitivity of Ca\(^{2+}\) sensors. However, our observation that both the mutant and wild-type NMJs have highly similar slope values for the log[Ca\(^{2+}\)] versus log[QC] argues against such an effect. An alternative explanation is that aberrant retrograde signaling in the absence of dystrophin results in altered modulation of presynaptic Ca\(^{2+}\) channel activity and thus leads to increased presynaptic Ca\(^{2+}\) influx.

Ultrastructural analysis of synaptic boutons showed a slight increase of the area occupied by vesicles in the mutants, which is unlikely to be sufficient to explain the increase in QC. Also, we did not observe significant changes in the number and size of active zones, whose increase could explain the increased neurotransmitter release. We did observe an increase in T-bars, the presumed docking sites for synaptic vesicles, in the mutant boutons. Although at present the precise function of these morphologically defined structures is unclear, an increase in T-bars has also been found correlated with increased neurotransmitter release in other studies (Haghighi et al., 2003).

How does the absence of dystrophin affect the retrograde control of release? The abnormally high EJP amplitudes observed at the dystrophin mutant NMJ suggests the possibility that the Dystrophin-deficient muscle inadequately signals to the motorneuron that it is inadequately depolarized. Thus, the absence of dystrophin may result in desensitization of an as yet unknown monitor of muscle function during depolarization. This raises the following questions: what is the homeostatic monitor and how does Dystrophin interact with it? Although a postsynaptic monitor regulating NMJ homeostatic pathways has been proposed (Davis and Bezprozvanny, 2001), its identity has proven elusive. The ligand-gated glutamate receptor channel, which conducts Ca\(^{2+}\), has been an attractive candidate (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999), and mammalian Dystrophin is known to scaffold a variety of postsynaptic Ca\(^{2+}\) channels (for review, see Carlson, 1998). However, we do not observe alterations in either glutamate receptor field sensitivity to spontaneous neurotransmitter release or localization of the DGlurRIA or DGlurRIB subunits in the dystrophin mutant, suggesting that glutamate receptors are unlikely to play a key role in the homeostatic pathways affected by the lack of dystrophin.

Glutamate receptor-independent monitors of muscle depolarization, possibly voltage-gated L-type channels, have been shown sufficient to trigger NMJ homeostatic mechanisms (Paradis et al., 2001). Alteration of L-type Ca\(^{2+}\) channel function or other changes in Ca\(^{2+}\) handling in the dystrophin-deficient larval muscle, which are well documented in mammals (for review, see Gaillly, 2002), might inappropriately trigger or prolong the action of the homeostatic machinery, resulting in aberrantly high neurotransmitter release and muscle hyperdepolarization. CaMKII and Ca\(^{2+}\)/calmodulin represent other attractive candidates to directly link Dystrophin to homeostatic pathways; Ca\(^{2+}\)/calmodulin has been shown to associate with the DGC (Madhavan et al., 1992), but its role in dystrophin function is not yet understood. Possibly, dystrophin indirectly affects cAMP-dependent mechanisms (Johnson et al., 2003) that have been shown to be involved in synaptic plasticity and short-term dynamics of release (Zucker and Regehr, 2002).

We find that the presynaptically localized type II BMP receptor wit is required for the increased QC observed in the dystrophin mutant, as also shown for increases in QC induced by the postsynaptic inhibition of CaMKII or glutamate receptor function (Haghighi et al., 2003). Dystrophin and CaMKII are unlikely, however, to signal through the PMad-dependent BMP signaling pathway, because we find that the expression levels and domains of PMad are unchanged when Dystrophin or CaMKII

Figure 8. Increases in quantal content in the absence of dystrophin require the wit BMP receptor. A, Representative traces of EJPs and mEJPs recorded at NMJs from wild-type (wt; w\(^{118B}\)) control and the genotypes RNAi-dysNH2/+; G14-Gal4/+; wt\(^{A12}/\)
wit\(^{A12}\) and RNAi-dysNH2/+; G14-Gal4/+; wit\(^{A12}/\). B–E, Bar graph representations of mean ± SEM values of mEJP frequency (B), mEJP amplitude (C), EJP amplitude (D), and QC (E) for the genotypes wild-type (n = 5), dys\(^{E6}\) (n = 8), RNAi-dysNH2/+; wt\(^{A12}/\)wit\(^{A12}\) (n = 5), RNAi-dysNH2/+; G14-Gal4/+; wt\(^{A12}/\)wit\(^{A12}\) (n = 6), and RNAi-dysNH2/+; G14-Gal4/+; wit\(^{A12}/\) (n = 6). All measurements were performed at 0.6 mM Ca\(^{2+}\). The homozygous wit NMJ displays a dramatic reduction in EJP amplitude and QC (EJP values, 3.9 ± 0.7 mV in the wit mutant vs 15.1 ± 1.3 mV in wild type; QC values, 8.9 ± 1.1 in the wit mutant vs 35.5 ± 3.2 in wild type, 0.6 mM Ca\(^{2+}\))². The UAS-RNAi-dysNH2/+; G14-Gal4/+; wit NMJs displayed mEJP, EJP, and QC values similar to the wit mutant. *p < 0.05.
levels are either decreased or increased. Our results address the previously posed question (Sanyal et al., 2004) as to whether the retrograde BMP signal directly participates in homeostatic signaling ("instructive") or is required for the overall development of the synapse ("permissive"). Our findings indicate that it is likely permissive, at least for the homeostatic mechanisms induced by perturbation of Dystrophin or CaMKII levels. BMP signaling may simply be required for the development of the presynaptic apparatus to a point at which it can respond to muscle-derived cues.

The degree of evolutionary conservation of the role of dystrophin in regulating neurotransmitter release at the NMJ and the potential role that defective NMJ homeostasis may play in the onset or progression of muscular dystrophy in humans is, at present, unclear. Differences between species in the severity of muscle wasting in the absence of dystrophin are observed (Megeney et al., 1996; Segalat, 2002). We do not observe muscle degeneration when DLP2 is absent from the musculature; other isoforms may be required for muscle integrity in Drosophila. The dystrophin-deficient cholinergic Caenorhabditis elegans NMJ also shows elevated levels of neurotransmitter, but this is attributable to decreased clearance of acetylcholine subsequent to deocalization of the SNF-6 acetylcholine transporter (Kim et al., 2004). Recent work on retinal synapses in the mdx mouse suggests that the large Dystrophin isoforms may also influence neurotransmitter release at other types of synapses (Green et al., 2004). Whether the CNS-specific Dp186 isoform plays roles at Drosophila inter-neuronal synapses, similar to those played by DLP2 at the NMJ, remains to be evaluated. Disrupted homeostasis at Dystrophin-deficient interneuronal brain synapses might contribute to the poorly understood mental impairments associated with DMD in humans.

References


