Synaptic transmission and plasticity are modulated by nonmuscle myosin II at the neuromuscular junction of Drosophila

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Seabrooke S, Stewart BA. Synaptic transmission and plasticity are modulated by nonmuscle myosin II at the neuromuscular junction of Drosophila. J Neurophysiol 105: 1966–1976, 2011. First published February 16, 2011; doi:10.1152/jn.00718.2010.—The synaptic vesicle population in a nerve terminal is traditionally divided into subpopulations according to physiological criteria; the readily releasable pool (RRP), the recycling pool, and the reserve pool. It is recognized that the RRP subserves synaptic transmission evoked by low-frequency neural activity and that the recycling and reserve populations are called on to supply vesicles as neural activity increases. Here we investigated the contribution of nonmuscle myosin II (NMMII) to synaptic transmission with emphasis on the role a motor protein could play in the supply of vesicles. We used Drosophila genetics to manipulate NMMII and assessed synaptic transmission at the larval neuromuscular junction. We observed a positive correlation between synaptic strength at low-frequency stimulation and NMMII expression: reducing NMMII reduced the evoked response, while increasing NMMII increased the evoked response. Further, we found that NMMII contributed to the spontaneous release of vesicles differentially from evoked release, suggesting differential contribution to these two release mechanisms. By measuring synaptic responses under conditions of differing external calcium concentration in saline, we found that NMMII is important for normal synaptic transmission under high-frequency stimulation. This research identifies diverse functions for NMMII in synaptic transmission and suggests that this motor protein is an active contributor to the physiology of synaptic vesicle recruitment.

electrophysiology; low-frequency depression; high frequency; vesicle pools

SYNAPTIC VESICLES ARE DESCRIBED as existing in three populations depending on how they participate in synaptic transmission (Rizzoli and Betz 2005). The readily releasable pool (RRP) of vesicles contains those vesicles that are released immediately upon action potential arrival at the nerve terminal. The RRP typically makes up 1–2% of the total vesicle pool and is rapidly depleted by repetitive stimulation. The recycling pool, comprising ~20% of all vesicles, contributes to synaptic release under modest or physiological levels of stimulation. This pool can be continuously refilled via the endocytotic machinery. The reserve pool is the largest pool of vesicles, ~80%, but these vesicles are only recruited under high demand stimulation frequencies, such as 10–30 Hz or greater.

From a variety of synaptic preparations, including the frog neuromuscular junction (NMJ), snake NMJ, and mammalian hippocampal and Calyx of Held synapses, it has been shown that the vesicle pools do not occupy different spatial domains within the nerve terminal (reviewed in Rizzoli and Betz 2005) but rather the vesicles intermingle and as such a biochemical tag likely distinguishes the morphologically uniform vesicles. Until recently, it was thought that the Drosophila larval NMJ provided an exception to this idea since work from Kuromi and Kidokoro (1998) generally supported the idea that reserve pool vesicles were maintained farther from the membrane than releasable vesicles of the RRP and recycling pool. However, that work was done at the light-microscope level. Recent studies at the level of the electron microscope showed that vesicles of the different pools also intermingle at the Drosophila larval NMJ (Akbergenova and Bykhovskaja 2009; Denker et al. 2009).

Despite mixing of the vesicle populations, it is generally recognized that vesicles are widely distributed within the nerve terminal and often at significant distance from the plasma membrane. The RRP vesicles are by necessity physically docked and primed to be released; upon RRP release the vesicles of the recycling and reserve pools must occupy release sites, or active zones, to participate in fusion. How does this transfer occur?

While it was thought that synaptic vesicles were relatively immobile [for example, Henkel et al. (1996) and Kraszewski et al. (1996)] more recent studies have found that vesicles are in fact dynamic within nerve terminal boutons (Gaffield et al. 2006; Jordan et al. 2005; Lemke and Klingauf 2005; Rea et al. 2004; Shtrahman et al. 2005) including our own work at the Drosophila NMJ (Nunes et al. 2006; Seabrooke et al. 2010). This suggests, that in addition to the biochemical signatures that must label vesicles of the different pools, dynamic vesicle transport exists within the bouton to ensure that vesicles can physically reach active zones.

Details of how this transport occurs, including molecular mechanisms and whether or not there is directional transport, remain to be determined. We have narrowed our studies of this problem to examine the actin cytoskeleton and myosin motors based on prior genetic work (Laviolette et al. 2005; Nunes et al. 2006; Peyre et al. 2006).

There have been numerous studies on the actin cytoskeleton and its role in synaptic transmission, but no clear picture has emerged on its role. FM dye loading experiments have implicated actin in potentiating (Cole et al. 2000; Kuromi and Kidokoro 1998; Richards et al. 2004), depressing (Sankararanayanan et al. 2003), and not altering the exo/endocytic (Holt et al. 2003; Job and Lagnado 1998) processes of synaptic transmission. Electrophysiological techniques have also indicated presynaptic actin in potentiation (Cole et al. 2000; Sakaba and Neher 2003) and depression (Morales et al. 2000) of synaptic transmission. While these results differ, they suggest a com-
plex function for presynaptic actin in synaptic transmission that may involve the activity of actin binding partners.

There have been only a few studies on the potential role of myosin in synaptic transmission, but some have implicated nonmuscle myosin II (NMMII). Inhibition of myosin II using antimyosin II antibody (Mochida et al. 1994) or introduction of myosin IIB heavy chain fragments into rat neurons (Takagishi et al. 2005) inhibited synaptic transmission. There is also some evidence that myosin II is involved in the delivery of synaptic vesicles to active zones (Polo-Parada et al. 2005) and NMMII contributes to vesicle fusion in adrenal chromaffin cells (Doreian et al. 2008).

Myosin family members have also been implicated in short-term plasticity of synaptic transmission. For example, at the Calyx of Held synapse, inhibition of myosin light chain kinase (MLCK) gradually increased EPSCs during low-frequency (0.1 Hz) stimulation, increased the RRP size, and did not affect the release probability of neurotransmitter (Srinivasan et al. 2008). MLCK inhibition in the Calyx of Held of mice and rats also potentiated early excitatory postsynaptic currents (Lee et al. 2008; Srinivasan et al. 2008). Alternatively, in hippocampal cells MLCK inhibition prevented mobilization of the reserve pool (Ryan 1999) and did not affect transmitter release (Tokuoka and Goda 2006). In superior cervical ganglion cells from rats, Mochida et al. (1994) also observed increased postsynaptic depression following inhibition of MLCK with wortmannin over the long term (60 min). Wortmannin is a fungal metabolite that acts at or near the catalytic site of MLCK. This inhibition is irreversible but can be prevented by a high concentration of ATP (Nakanishi et al. 1992).

In recent work from our laboratory, we found that NMMII was important in maintaining vesicle mobility at the unstimulated Drosophila NMJ (Seabrooke et al. 2010). Thus the present experiments set out to identify whether those same mutants also exhibited changes in synaptic transmission and in particular whether NMMII is important for maintenance of high-frequency synaptic transmission and the expression of synaptic plasticity.

MATERIALS AND METHODS

Drosophila stocks. Drosophila melanogaster were crossed and maintained at 22°C on Bloomington fly media. zipper1/CyO (Het) is a heterozygous loss-of-function NMMII allele obtained from the Bloomington Stock Center (FlyBase ID: FBal0018862) and was rebalanced over CyO-GFP to detect the balancer. UASzipperRNAi (K/D) is a NMMII RNAi construct obtained from the Vienna Drosophila RNAi Center (FlyBase ID: FBal0210938). The gain-of-function NMMII construct zipperGS50077(O/E) is a unidirectional UAS construct inserted upstream of NMMII and was obtained from the Drosophila Gene Search Project (Toba et al. 1999). OregonR (OreR) was used as a control and was crossed to zipper1/CyO-GFP, elav14/Gal4 (ct) was used to drive expression of the UASzipperRNAi and zipperGS50077 NMII alleles in the nervous system using the Gal4/UAS system of expression (Brand and Perrimon 1993).

Electrophysiology. Third instar larvae were dissected and bathed in HL3 physiological solution as described previously (Stewart et al. 1994), with the calcium concentrations adjusted as described in the text. The stimulating electrode was backfilled with the corresponding HL3 solution, and the recording electrode was backfilled with 3 mM KCl and had a resistance of ~25 MΩ. In every case, muscle resistance was recorded to determine if there were any differences between the genotypes and no more than two recordings were obtained from any individual larvae. All data were obtained from muscle 6 of segments 3 or 4.

Recordings were made using an AxoClamp 2B amplifier (Axon Instruments, Burlingame, CA) and recorded using Clampfit 10.0. From these recordings, miniature excitatory junctional potentials (mEJPs) were recorded for 1–2 min. mEJPs with slow rise times, assumed to originate in electrically coupled neighboring cells, were excluded, and only single mEJPs were used in the calculation of amplitude average to avoid errors resulting from doublet mEJPs.

Evoked EJPs were produced by stimulating the segmental nerve at strength sufficient to recruit both axons that innervate muscle 6. For high-frequency experiments, a baseline recording of 16 stimuli at 1 Hz was obtained and immediately followed by 10-Hz stimulation for 10 min and concluded with 0.1-Hz stimulation for 5 min. Recordings were not used if one of the two axons failed during the stimulation protocol. This was visualized as two distinct EJP amplitudes intermixed during the depression, one being small and the other being much larger, as opposed to a gradual consistent decrease in EJP amplitude.

For paired-pulse recordings, the nerve was stimulated by two pulses 20 ms apart every 10 s. For each sampling, the paired pulse was repeated for 16 cycles and averaged. To estimate the amplitude of the EJP of the pulses, the repolarization phase of the first EJP was fit and extrapolated under the second EJP. The height of the second EJP was then calculated by measuring the EJP amplitude from the extrapolated line to the highest peak of the second EJP. The ratio of the second EJP amplitude to first EJP amplitude was calculated to determine the extent of amplitude increase during a paired pulse.

Drug administration. To access the affects of NMMII inhibition on synaptic transmission, a 13-mM solution of (−)-blebbistatin (Sigma-Aldrich Canada, Oakville, Canada) dissolved in DMSO was diluted to a 50-μM solution in HL3 (Stewart et al. 1994). This solution was incubated with the preparation in the dark for 20 min before electrophysiological recordings. During recordings, the microscope light was turned off to minimize light exposure to the drug.

Statistical analysis. All analysis was completed in GraphPad Prism 4. One-way ANOVA was used to test for statistical significance with a P < 0.05 accepted as statistically significant. A Neuman-Keuls or Dunnett posttest was used for comparisons between genotypes, with a significance taken at P < 0.05. Data are expressed as means ± SE, where n represents the number of electrophysiological recordings measured. All error bars represent means ± SE.

RESULTS

NMMII plays a role in basal synaptic transmission. To determine if NMMII has a physiological function, our strategy was to perturb the levels of NMMII using Drosophila genetics and assess the effects on synaptic transmission with electrophysiological recordings at the NMJ. In Drosophila, NMMII is encoded by the zipper (zip) gene and several genetic reagents are available to manipulate its expression. Here we have used the zip loss of function allele (Young et al. 1993) generated by methyl methanesulfonate mutagenesis, as well as zip overexpression and RNAi-mediated zip knockdown. Overexpression and knockdown were targeted specifically to the nervous system using the UAS/Gal4 system while zip is ubiquitous. We previously characterized the effect of these strains on zipper-encoded protein in the larval brain (Seabrooke et al. 2010) and showed that the overexpression results in a 95% increase in protein above control levels while zip+/− reduces zipper protein levels to ~50% of control levels. RNAi mediated knockdown reduces NMMII to 10% of control. We complemented this genetic approach with a pharmacological one and applied 50 μM (−)-blebbistatin to dissected preparations. This com-
pound is believed to be a NMMII-specific antagonist that stabilizes the ADP-Pi intermediate of the myosin ATPase activity, thus reducing the molecule’s ability to completely cycle through ATP hydrolysis (Kovacs et al. 2004).

We first quantified spontaneous vesicle fusion by determining the frequency and amplitude of mEJP over a 1- to 2-min period (Fig. 1, B, D, and E). The average amplitude of the mEJPs was ~1 mV, which was not significantly different among the genotypes tested (P > 0.05). These results indicate that there have not been changes in vesicle size, transmitter concentration, or postsynaptic sensitivity to glutamate. However, 50 μM (−)-blebbistatin applied to OreR reduced mEJP amplitude to 0.82 ± 0.02 mV (n = 11, P < 0.05) compared with untreated OreR controls (1.02 ± 0.05 mV, n = 11; Fig. 1D), suggesting a mild postsynaptic effect of (−)-blebbistatin.

The frequency of mEJPs was affected in NMMII mutants (Fig. 1, B and E). The frequency for overexpression of NMMII using zipGS50077/+;elav3αGal4/+ was 2.08 ± 0.17 Hz (O/E, n = 11) and was significantly less than the elav3αGal4/elav3αGal4 (ct, 5.38 ± 0.48 Hz, n = 11, P < 0.05), OreR (4.61 ± 0.60 Hz, n = 11), and zipGS50077/zipGS50077 (O/E ct, 4.86 ± 0.47 Hz, n = 12; P < 0.05) controls. Knockdown of NMMII using UASzipRNAi/+;elav3αGal4/+ also exhibited a reduced frequency, 1.59 ± 0.13 Hz (K/D, n = 12), compared with elav3αGal4/elav3αGal4 and OreR (P < 0.05) but not compared with the UASzipRNAi/UASzipRNAi control (K/D ct, 2.21 ± 0.28 Hz, n = 12, P > 0.05). Fifty micromolars of (−)-blebbistatin (1.80 ± 0.12 Hz, n = 11) and the heterozygous loss-of-function zip+/+ of NMMII (Het, 2.20 ± 0.17 Hz, n = 12) also exhibited a reduced frequency compared with elav3αGal4/elav3αGal4 and OreR (P < 0.05; Fig. 1E).

Thus the presynaptic overexpression or knockdown of NMMII does not affect mEJP amplitude, but both overexpression and loss of NMMII is associated with reduced mEJP frequency suggesting that NMMII is important for spontaneous release of vesicles.

Nerve-evoked responses were first measured in 1 mM external calcium saline at a stimulation frequency of 1-Hz to recruit only the recycling pool of vesicles, and the amplitude of EJPs was measured (Fig. 1, A and C). Calcium saline (1 mM) was selected to mimic physiological calcium levels. These experiments indicated that EJPs in larvae overexpressing NMMII (O/E, 41.39 ± 1.53 mV, n = 12) were slightly bigger than those found in controls (33.87 ± 1.39 mV, n = 12; P < 0.05 for O/E ct and 37.42 ± 1.01 mV, n = 10, P > 0.05 for ct). A modest reduction in NMMII did not significantly alter the EJP (Het, 34.50 ± 1.31 mV, n = 12, P > 0.05), whereas knockdown (K/D) of NMMII and 50 μM (−)-blebbistatin exhibited a reduced EJP (27.37 ± 1.10 mV, n = 12, P < 0.05 and 26.79 ± 1.79 mV, n = 12, P < 0.05, respectively; Fig. 1C). Therefore, overexpression of NMMII increased EJP amplitude, whereas EJPs were smaller when NMMII expression or activity was reduced.

NMMII affects short-term synaptic potentiation. We next investigated whether alterations in NMMII affected the ability of the nerve to maintain synaptic transmission with a more challenging level of stimulation and chose a stimulus paradigm in which a baseline recording of synaptic transmission was taken by measuring EJP responses at 1 Hz for 16 s that was immediately followed by 10-Hz stimulation for 10 min followed by a further 5 min of low-frequency stimulation (0.1 Hz). Recordings were first conducted in 1 mM calcium saline, which is similar to physiological calcium levels.

Under these conditions (Figs. 2 and 3, A and B), control genotypes showed a small immediate depression at the onset of high-frequency stimulation but then the EJP returned to near-

![Fig. 1. Electrophysiological recordings made from the neuromuscular junction (NMJ) of larvae in an altered nonmuscle myosin II (NMMII) background. Representative traces of evoked junctional potentials (EJP, A) and miniature (m)EJP (B) from overexpression \( [zip^{GS50077}]^{+/+};elav3αGal4^{+/+} \) (O/E), OreR, heterozygous loss-of-function \( [zip^{+/+}]^{(Het)} \), and RNAi knockdown \( [UASzipRNAi^{+/+};elav3αGal4^{+/+}] \) (K/D) of NMMII. Arrow represents a mEJP fusion event. C: overexpression (O/E) of NMMII resulted in slightly larger EJP amplitudes. Heterozygous loss-of-function of NMMII (Het) did not alter the EJP amplitude while knockdown (K/D) of NMMII and application of 50 μM (−)-blebbistatin significantly reduced the EJP amplitude. D: amplitude of the mEJP is unchanged across the genotypes tested. Application of 50 μM (−)-blebbistatin to OreR significantly reduced the amplitude of the mEJP suggesting a postsynaptic response from the drug. E: frequency of the mEJP is significantly reduced when NMMII expression is altered. UASzipRNAi/UASzipRNAi control (K/D ct) exhibited an abnormally low mEJP frequency suggesting some basal expression of the construct. Additional genotypes listed are as follows: ct \( (elav3αGal4^{+/+};elav3αGal4^{+/+}) \) and O/E ct \( (zip^{GS50077})^{(Het)} \). Error bars represent SE. Similar letters indicate peaks that are statistically the same determined with one-way ANOVA (Newman-Keuls posttest). *P < 0.05. Samples sizes were between 10 and 12 recordings.](http://jn.physiology.org/content/jn/105/5/1968/F1.large.jpg)
baseline levels and was maintained throughout the 10-Hz challenge. The initial depression at the onset of 10-Hz stimulation is thought to represent depletion of the RRP while the recovery corresponds to a time delayed mobilization of the recycling and reserve pools. The full mobilization of the reserve pool can take 2–3 min (Delgado et al. 2000) at the Drosophila neuromuscular junction. Interestingly, none of the genotypes, or blebbistatin treatment, showed a significant difference in the extent of the initial depression, which occurred at the onset of 10-Hz stimulation (Fig. 4A). Following the initial depression, there is a recovery phase in which EJP amplitudes recover towards or exceed the baseline value. OreR and elav\textsuperscript{A5}-gal4 (ct) controls along with zip\textsuperscript{+/+} all behaved similarly in this phase with EJPs returning to about the baseline level. In contrast however, in larva that were overexpressing (O/E) NMII or larva with NMII activity inhibited using 50 \textmu M (−)-blebbistatin (Blebb) significantly increased EJP amplitude above baseline and peaked at a significantly greater amplitude than OreR during the first 4 min of 10-Hz stimulation (Fig. 4B); the percent increase of these genotypes from their initial depression was 63.85% ± 10.35% (n = 7, P < 0.05) for O/E and 60.74% ± 17.47% (n = 6, P < 0.05) for blebbistatin than OreR (27.35% ± 4.25%, n = 8). The elav\textsuperscript{A5}Gal4/elav\textsuperscript{A5}Gal4 control and a moderate reduction in the expression of NMII using zip\textsuperscript{+/+} (Het) did not affect short-term synaptic plasticity (percent increase: 39.65% ± 3.97%, n = 6, P > 0.05 and 31.69% ± 7.34%, n = 6, P > 0.05, respectively) relative to OreR. While the RNAi knockdown (K/D) did not exhibit a significant increase in EJP amplitude following the initial depression at 10 Hz, O/E, 50 \textmu M (−)-blebbistatin, and K/D did exhibit a significant fold increase in EJP amplitudes relative to the initial EJP measured at 1 Hz (1.22 ± 0.06, n = 7, 1.26 ± 0.08, n = 6, and 1.26 ± 0.08, n = 6, respectively, P < 0.05) compared with OreR (1.01 ± 0.03, n = 8; Fig. 4C). Fold change represents the change in amplitude relative to the control amplitude, which is calculated as 1 and may be written as 100%. It is calculated as a ratio of the experimental value/control value, thus making, for example, 1.22, 22% larger than 1.00. This increase in synaptic potentiation for K/D thus likely stems from the slight potentiation of EJP amplitude occurring early during 1-Hz stimulation. The average baseline EJP amplitude of K/D displayed a significant fold increase relative to the first EJP of the baseline recording (1.09 ± 0.04, n = 6, 0.05) compared with ct (0.99 ± 0.02, n = 6). The average baseline EJP amplitude relative to the first EJP in the other groups was ~0.99 and not significantly different from ct (P > 0.05). elav\textsuperscript{A5}Gal4/elav\textsuperscript{A5}Gal4 (ct, 1.00 ± 0.07, n = 6, P > 0.05) and zip\textsuperscript{+/+} (Het, 1.02 ± 0.04, n = 6, P > 0.05) did not exhibit a significant fold increase from OreR.

Overexpression, RNAi knockdown and (−)-blebbistatin resulted in a more significant degradation of EJP amplitude following maximal potentiation. At the end of 10 min, the
changes in EJP amplitude were no longer significantly different than controls (−88% of average baseline EJP, P > 0.05), suggesting there may be a stronger depletion of the reserve pool in these larvae. The extent of synaptic potentiation is affected by the initial amplitude of the EJP. Therefore, EJPs of similar amplitude were used in the evaluation of synaptic potentiation and did not differ significantly (−32 mV, P > 0.05) among the genotypes. These results suggest that either increasing NMMII expression or reducing NMMII expression and activity results in synaptic potentiation in 1 mM Ca²⁺ saline during 10-Hz stimulation for 10 min.

NMMII affects posttetanic potentiation. Posttetanic potentiation (PTP) is a characteristic feature observed following high-frequency stimulation. PTP is characterized by an increase in EJP amplitude followed by a gradual reduction of EJP amplitude back towards baseline. The increase in EJP amplitude results from release of calcium that had been stored in the endoplasmic reticulum or mitochondria during high-frequency stimulation (Bardo et al. 2006; Xu et al. 2007; Zucker 1999; Zucker et al. 1991). As those calcium reserves are depleted, EJP amplitude returns towards baseline. No significant differences in the maximal amplitude of PTP in NMMII alleles, or with application of 50 μM (−)-blebbistatin (−1.3 fold increase, P > 0.05), were observed (Fig. 4D). As EJP amplitude returned towards baseline following PTP, overexpression (O/E) of NMMII and inhibition of NMMII with 50 μM (−)-blebbistatin (0.56 ± 0.04, n = 7 and 0.53 ± 0.05, n = 6, respectively, P < 0.05) exhibited a significantly greater decrease in EJP amplitude compared with OreR (0.73 ± 0.05, n = 8; Fig. 4E). Neither the RNAi knockdown, elav<sup>U2Gal4/</sup>elav<sup>U2Gal4</sup>, nor the heterozygous loss-of-function of NMMII (Het) resulted in significant differences in EJP amplitude following PTP (0.71 ± 0.03, n = 6, 0.73 ± 0.03, n = 6 and 0.79 ± 0.05, n = 6, respectively, P > 0.05; Figs. 3 and 4E). However, overexpression of NMMII, inhibition with 50 μM (−)-blebbistatin and RNAi knockdown displayed a significantly greater decrease in EJP amplitude relative to their average initial amplitude compared with OreR (0.71 ± 0.05, n = 5, 0.71 ± 0.12, n = 6, 0.78 ± 0.03, n = 6 and 0.88 ± 0.08, n = 8, respectively; Fig. 4F).

NMMII does not substantially affect synaptic depression. The <i>Drosophila</i> NMJ has been shown to have a maximal recycling rate of 200 vesicles/s at 10-Hz stimulation (Dickman et al. 2005). Thus conditions that elicit synaptic release >200 vesicle/s should result in synaptic depression. To achieve these conditions, the <i>Drosophila</i> NMJ was challenged with 10 Hz for 10 min with 10 mM calcium in modified HL3 (Dickman et al. 2005). During a baseline recording at 1 Hz for 16 s, large evoked responses were observed that did not vary significantly among recordings (−53 mV, P > 0.05). The uncorrected EJP amplitudes in these conditions indicate that ~530 vesicles are released per second, which surpasses the maximal recycling rate of 200 vesicles/s and resulted in synaptic depression of the EJP (Figs. 5 and 6) [for review of synaptic depression, see Zucker and Regehr (2002)]. Following high-frequency stimulation (10 Hz) for 10 min, the nerve was allowed to recover during 5 min of low-frequency stimulation (0.1 Hz). The onset of 10-Hz stimulation in 10 mM calcium led to rapid depression of the EJP in all genotypes, but this reduction was especially prominent in larvae overexpressing NMMII (O/E), which resulted in an increased initial depression of EJP amplitude (0.41 ± 0.01, n = 5, P < 0.05) compared with ct (0.58 ± 0.04, n = 5; Fig. 7A). None of the other alleles exhibited significant differences from each
In the subsequent 6–8 min of stimulation, the EJP amplitude slowly depressed following the rebound. The extent of depression was evaluated using linear regression between 230–615 s (Fig. 7D). Overexpression of NMMII displayed a significantly decrease in amplitude compared with ct (slope: $-0.00303 \pm 0.00003$ for ct and $-0.00043 \pm 0.00001$ for O/E, $P < 0.05$). None of OreR, Het, or K/D displayed slopes that were significantly different from ct (approximately $-0.0003$, $P > 0.05$). (--)Blebbistatin application resulted in a slope that was significantly different from OreR and K/D ($-0.00038 \pm 0.00002$, $P < 0.05$, $-0.00029 \pm 0.00002$, and $-0.00031 \pm 0.00001$, respectively).

Following 10-Hz stimulation, the depressed EJP recovered towards baseline during 5 min of low-frequency stimulation (0.1 Hz). There was a very rapid phase lasting about three impulses in which EJP amplitude returned to about 80–90% of the baseline value and then a slower phase during which EJPs recovered slightly further. To calculate the recovery, the fold increase from the last point at 10 Hz to the last point at 0.1 Hz was calculated and normalized to OreR. The overexpression of NMMII resulted in a significantly greater rebound of EJP amplitude (O/E, $1.50 \pm 0.26$-fold increase, $n = 5$, $P < 0.05$) than the OreR control ($1.00 \pm 0.05$-fold increase, $n = 5$ for OreR, $0.89$-fold increase for the rest of the groups, which did not vary significantly from OreR). Thus although overexpression of NMMII experienced greater initial synaptic depression, these larvae also showed greater recovery following 10-Hz stimulation (Figs. 6 and 7C).

Paired-pulse stimulation indicates that release probability is not changed in NMMII alleles. To determine whether changes observed with NMMII alleles in short-term synaptic plasticity were due to changes in vesicle release probability $P(r)$, paired-pulse experiments were conducted. If the initial probability of release is high, then facilitation of the second pulse is low. Alternatively, if the initial probability of release is low, then the facilitation of the second pulse is high. This experiment was completed in low calcium (0.5 mM Ca$^{2+}$) to enhance the possibility of observing a facilitating second response. If NMMII affected the $P(r)$, then during the paired-pulse experiment, any changes in $P(r)$ would be observable as changes in the EJP amplitude of the second stimulus relative to the first. However, this was not observed for the NMMII alleles. Neither overexpression, RNAi knockdown nor 50 mM (--)blebbistatin resulted in any significant changes in the EJP amplitude of the second pulse of the paired-pulse experiment ($-1.3$-fold increase for the second EJP, $n = 8–9$, $P > 0.05$; Fig. 8). This suggests the short-term synaptic plasticity is not a result of changes in $P(r)$ as a result of altering NMMII. Instead the high-frequency conditioning over a 4-min period was required to elicit synaptic potentiation, suggesting NMMII affects mobilization of the reserve pool.
Low-frequency synaptic depression with altered NMMII activity requires high-frequency conditioning. As shown in Figs. 3 and 4, evidence was found for synaptic depression in the period following 10-Hz stimulation when recordings were made in 1.0 mM calcium saline. In that experiment, we expected the EJP amplitude to return to baseline levels following PTP but were somewhat surprised to observe that EJP amplitudes went well below baseline levels. Thus we investigated whether this synaptic depression, observed most prominently in the overexpressing strain and with blebbistatin application, is attributable to low-frequency depression (LFD). To investigate this, experiments were conducted with the same low-frequency stimulation (0.1 Hz) for 5 min applied in larvae that had no prior exposure to high-frequency stimulation. This experiment was conducted in 1 mM Ca\(^{2+}\)/H11001, and no substantial LFD was observed (Fig. 9, A–F). Neither overexpression (O/E) nor 50 \(\mu\)M (−)-blebbistatin caused EJP amplitude to become significantly smaller with this stimulus protocol (Fig. 9, D and F). Linear regression analysis of the EJP amplitudes failed to demonstrate a nonzero slope (\(n = 6–7, P > 0.05\)). Therefore, these results indicate that the synaptic depression we observed during low-frequency stimulation for overexpression (O/E) and
Fig. 9. Low-frequency stimulation of larvae with altered NMMII activity. A–F: 5 min of a 0.1-Hz stimulation was completed in 1 mM calcium saline using muscle 6 on larval preparations with no previous exposure to high-frequency stimulation. D and F: overexpressing NMMII (O/E) and inhibiting NMMII with 50 μM (−)-blebbistatin did not result in significant changes in EJP amplitude over the 5-min period. In addition, a nonzero slope was not detected during low-frequency stimulation. OreR and elavA-Gal80 (ct) are the controls. KD indicates the RNAi knockdown of NMMII, and Het indicates the heterozygous loss-of-function of NMMII. Sample size is 6–7 muscles per condition. Linear regression was used to evaluate slopes.

50 μM (−)-blebbistatin is not simply due to LFD but requires the prior high-frequency conditioning.

DISCUSSION

Our research indicates that NMMII contributes to the physiology of synaptic transmission at the Drosophila NMJ. We previously demonstrated the presence of NMMII at the Drosophila NMJ, consistent with its localization at the mouse NMJ (Vega-Riveroll et al. 2005), and showed that NMMII plays a role in vesicle dynamics (Seabrooke et al. 2010). Here we manipulated NMMII using genetic techniques to both overexpress and reduce NMMII levels and employed a variety of nerve stimulation protocols to explore the role of NMMII in synaptic physiology.

**Basal synaptic transmission is modulated by NMMII.** A positive correlation was found between NMMII expression and the amplitude of evoked responses under low-frequency stimulation. Reducing NMMII using zipRNAi reduced the EJP amplitude, consistent with interference of NMMII function by antibody (Mochida 1995; Mochida et al. 1994) or peptides (Takagishi et al. 2005) in rat superior cervical ganglion neurons. We can add that overexpression of NMMII led to a moderately increased EJP amplitude at this low frequency of stimulation. Since this low frequency of stimulation should only recruit vesicles from the RRP, our data support the idea that there is a relationship between the level of NMMII expression and synaptic transmission. A moderate reduction of NMMII using the heterozygous loss-of-function allele did not affect the EJP amplitude, suggesting that reduction in protein levels >50% is required to observe effects on evoked synaptic transmission.

We attempted to determine whether these changes in synaptic transmission were due to alterations in vesicular release probability with a paired-pulse experiment. The general rationale for this approach is that if the initial release probability is high, there will be less facilitation of the second synaptic release, and correspondingly if initial release probability is low, then there will be more facilitation observed. Our results showed that there was no major difference in paired-pulse facilitation among the genotypes tested or with blebbistatin treatment. The mean value of the overexpression strain tended to a higher level of facilitation, but the difference did not achieve significance with an ANOVA test. Therefore, it does not seem a change in release probability underlies the observations made for this level of transmission but rather the availability of vesicles within the RRP may be increased.

We interestingly found that the frequency of mEJPs was reduced in both RNAi and overexpression strains, suggesting NMMII also affects spontaneous release of vesicles but that NMMII manipulation differentially affects spontaneous and evoked release. Although it has long been held that the vesicles contributing to spontaneous and nerve-evoked synaptic transmission come from the same pool (Del Castillo and Katz 1954; Groemer and Klingauf 2007), this is giving way to the notion that spontaneous release arises from a separate population of vesicles (Fred and Burrone 2009; Sera et al. 2005). Indeed, prior genetic work on the Drosophila neuronal-synaptobrevin gene contributed to this idea (Deitcher et al. 1998).

The amplitude of mEJPs was not significantly different among the genotypes tested, which supports a presynaptic effect of NMMII. However, application of (−)-blebbistatin, showed a slightly reduced mEJP amplitude. Blebbistatin applied to the bath will affect both pre- and postsynaptic NMMII, whereas our transgenic RNAi or overexpression affects only the presynaptic NMMII. This observation suggests that inhibiting NMMII postsynaptically reduced the ability of the muscle to respond to stimulus. Liebl and Featherstone (2005) conducted a genetic screen to identify genes involved in clustering of glutamate receptors in Drosophila. Within their screen, a number of actin cytoskeletal genes were identified. It is possible that inhibiting NMMII may also affect the clustering of glutamate receptors on the postsynaptic surface, although we did not specifically test for this possibility.
NMMII contributes to short-term synaptic plasticity. Because of our prior work on vesicle dynamics, the involvement of actin and myosin in vesicle trafficking, and the likely requirement of vesicle transport when synaptic demand is high, we wished to determine the affect of altering NMMII levels on synaptic transmission under higher frequencies of neural stimulation. We used a stimulus protocol of 10-Hz stimulation for 10 min, preceded by a short period of 1-Hz stimulation to collect baseline data and followed by a short period of 0.1 Hz stimulation to monitor recovery from the bout of 10-Hz stimulation. Prior work on this terminal has showed that there are ~50,000–80,000 vesicles available to be released and that when endocytosis is blocked a few thousand stimuli completely exhaust the supply of vesicles (Delgado et al. 2000; Stewart et al. 2002). Since our protocol delivers 6,000 stimuli, releasing ~35 vesicles per stimulus per terminal at 1 mM calcium, we expect to engage all the vesicle pools available at this nerve terminal.

Our studies conducted at 1 mM external calcium in control strains are generally consistent with similar published studies (Verstreken et al. 2009; Yao et al. 2009). Following a short-lived reduction in EJP amplitude, due to depletion of the RRP, EJP amplitudes recover and are maintained at about baseline levels throughout the stimulus protocol. However, at this calcium level there was a clear difference between controls and the NMMII-manipulated strains; the latter strains showed enhanced EJP amplitudes during the 10-Hz stimulation. Interestingly, the same observation was made whether NMMII levels were reduced by RNAi knockdown, NMMII levels were increased by overexpression, or NMMII activity was reduced by blebbistatin.

At present, it is difficult to reconcile how both increasing and decreasing NMMII enhances synaptic transmission at 10-Hz stimulation, but it does indicate the more active engagement of the recycling and reserve pools of vesicles. Similar effects for increasing and decreasing NMMII have been previously shown. In our previous work on vesicle mobility, we (Seabrooke et al. 2010) likewise found similar phenotypic effects when NMMII was up- or downregulated. Additionally, a study of macrophage motility reported that inhibition or enhancement of Myosin II phosphorylation both had the same effect on cell migration (Wilson et al. 1991). Furthermore, phosphorylation of the regulatory light chain of NMMII is crucial for cytokinesis (Matsumura 2005), but overexpression of myosin II in fission yeast inhibits cytokinesis (May et al. 1997). However, Franke (2006) showed that association of the regulatory light chain with the heavy chain is required for solubility of the protein. If expression of the regulatory light chain is not increased with overexpression of the heavy chain, insoluble aggregates may form. This may be functionally equivalent to a knockdown of the protein. Overexpression of the regulatory and essential light chains in conjunction with overexpression of the heavy chain may rescue the overexpression phenotype. In addition, NMMII binds directly to the actin cytoskeleton. NMMII also cross-links actin filaments by homotypic interactions in the tail of the protein. Future studies to investigate how disrupting NMMII affects actin dynamics will be important to clarify how the interactions of actin and myosin are involved in regulating synaptic transmission. Ideally mutations that knock out individual functions of NMMII, such as binding to actin, cross-linking actin filaments, or associating with vesicles, will resolve how individual functions of NMMII contribute to synaptic transmission.

We did not find any evidence for changes in endocytosis when NMMII was altered. Bona fide mutants of endocytosis genes typically cannot maintain synaptic transmission under high-frequency stimulation (Dickman et al. 2005; Koenig and Ikeda 1999; Koh et al. 2007) and recover from such bouts of activity at much slower rates than normal (Dickman et al. 2005). In contrast, we found that NMMII overexpression or knockdown larvae performed better at 10-Hz stimulation and recovered just as quickly as controls. Therefore, it seems unlikely that endocytosis is affected. This is consistent with MLCK inhibition in mice Calyx of Held synapses (Srinivasan et al. 2008). Overexpression of NMMII resulted in a significantly greater recovery during low-frequency stimulation. This may be a result of a larger recycling pool that displays rapid clathrin coat endocytosis.

We further challenged the synaptic response using 10 mM calcium and observed results similar to previously published studies (Dickman et al. 2005; Verstreken et al. 2002). The main observations were that overexpression of NMMII leads to greater synaptic depression during the initial phase and slightly better posttetanic recovery, while knockdown led to greater recovery following the initial depression than the other genotypes. Overall, with 10 mM calcium there were not many substantial differences between the genotypes.

Low-frequency synaptic depression in NMMII mutants required high-frequency conditioning. In 1 mM calcium saline, PTP was observed upon changing from 10-Hz to 1-Hz stimulation (Bardo et al. 2006; Xu et al. 2007; Zucker 1999; Zucker et al. 1991); however, this was soon followed by a depression of the EJP below baseline levels, reminiscent of LFD. In particular, larvae overexpressing NMMII or larvae with inhibited NMMII activity displayed a significantly greater decrease in EJP amplitude following PTP. This finding is of significance as little is known about mechanisms of LFD. In the mammalian neonatal cortex and hippocampus, prolonged low-frequency stimulation induced long-term synaptic depression and may be dependent on increases in phosphatase activity (Mulkey et al. 1993; Yasuda et al. 2003). In some crayfish neuromuscular synapses, a LFD occurred at 0.5 to 1 Hz, which was not likely related to depletion of synaptic vesicles (Bryan and Atwood 1981). In invertebrates, LFD has also been suggested to involve a shift in balance between phosphorylated and unphosphorylated substrates (Silverman-Gavrila and Charlton 2009; Silverman-Gavrila et al. 2005). Our findings suggest that NMMII is a contributor to a form of LFD, which requires high-frequency conditioning. When low-frequency stimulation without prior high-frequency conditioning was conducted, there were no significant differences in NMMII alleles compared with the controls.

In summary, our research has shown that NMMII contributes to basal synaptic transmission and displays a positive correlation with EJP amplitude and NMMII expression. Additionally, electrophysiological analysis indicates that NMMII is involved in short-term synaptic plasticity at the Drosophila NMJ and our data are consistent with the idea that NMMII is important for the ability of vesicles to transition from the reserve pool to the recycling pool. However, the complexity of our results likely also indicates...
that NMII plays several important, but nonexclusive roles in synaptic transmission. Given our previous work investigating how NMII contributes to vesicle mobility (Seabrooke et al. 2010), NMII may be important in mobilizing vesicles for synaptic release. Further experiments aimed at understanding how NMII modulates and influences synaptic function will be important to determine the mechanisms by which NMII regulates synaptic transmission.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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