RESEARCH ARTICLE

Postsynaptic glutamate receptors regulate local BMP signaling at the *Drosophila* neuromuscular junction

Mikolaj Sulkowski, Young-Jun Kim and Mihaela Serpe*

ABSTRACT

Effective communication between pre- and postsynaptic compartments is required for proper synapse development and function. At the *Drosophila* neuromuscular junction (NMJ), a retrograde BMP signal functions to promote synapse growth, stability and homeostasis and coordinates the growth of synaptic structures. Retrograde BMP signaling triggers accumulation of the pathway effector pMad in motoneuron nuclei and at synaptic termini. Nuclear pMad, in conjunction with transcription factors, modulates the expression of target genes and instructs synaptic growth; a role for synaptic pMad remains to be determined. Here, we report that pMad signals are selectively lost at NMJ synapses with reduced postsynaptic sensitivities. Despite this loss of synaptic pMad, nuclear pMad persisted in motoneuron nuclei, and expression of BMP target genes was unaffected, indicating a specific impairment in pMad production/maintenance at synaptic termini. During development, synaptic pMad accumulation followed the arrival and clustering of ionotropic glutamate receptors (iGluRs) at NMJ synapses. Synaptic pMad was lost at NMJ synapses developing at suboptimal levels of iGluRs and Neto, an auxiliary subunit required for functional iGluRs. Genetic manipulations of non-essential iGluR subunits revealed that synaptic pMad signals specifically correlated with the postsynaptic type-A glutamate receptors. Altering type-A receptor activities via protein kinase A (PKA) revealed that synaptic pMad depends on the activity and not the net levels of postsynaptic type-A receptors. Thus, synaptic pMad functions as a local sensor for NMJ synapse activity and has the potential to coordinate synaptic activity with a BMP retrograde signal required for synapse growth and homeostasis.

KEY WORDS: BMP signaling, Glutamatergic synapses, Glutamate receptor, *Drosophila*, Neuromuscular junction

INTRODUCTION

Synapse development is initiated by genetic programs, but is coordinated by intercellular communications between the pre- and postsynaptic compartments, and by neuronal activity itself. Neurons exert both instantaneous and long-lasting effects on the postsynaptic cell through synaptic transmission (reviewed by Malenka and Nicoll, 1999), and postsynaptic cells influence the growth, maturation and function of the presynaptic neurons through retrograde signals (Tao and Poo, 2001; Marqués and Zhang, 2006). Retrograde signals have been identified at both neuromuscular junctions (NMJs) and central synapses (Sanes and Lichtman, 1999; Tao and Poo, 2001; Davis, 2006; Turrigiano, 2007; Turrigiano et al., 2012), but little is known about how such signals detect the status of synaptic activity and relay this information to presynaptic neurons.

The *Drosophila* NMJ is an extremely useful model to study synapse development and plasticity. *Drosophila* NMJ synapses are glutamatergic, similar in composition and function to the mammalian central AMPA/kainate synapses (Littleton and Ganetzky, 2000). The fly NMJ ionotropic glutamate receptors (iGluRs) are heterotetrameric complexes composed of three essential subunits – GluRIIC, GluRIID and GluRIIE – and either GluRIIA or GluRIIB (DiAntonio, 2006). Mutations that delete any of the shared subunits, or GluRIIA and GluRIIB together, abolish the NMJ synaptic transmission and limit the localization of iGluRs at synaptic locations (DiAntonio et al., 1999; Marrus et al., 2004; Featherstone et al., 2005; Qin et al., 2005). Type-A and type-B receptors differ in their single-channel properties, synaptic currents and regulation by second messengers (DiAntonio, 2006). Mechanisms that differentially regulate the synaptic levels and activity of these two channels have profound effects on synapse strength and plasticity. Manipulations that decrease the activity of type-A receptors produce large decreases in quantal size (Petersen et al., 1997; Davis et al., 1998), yet the evoked transmission remains normal due to a compensatory increase in presynaptic release. Several factors have been shown to trigger the retrograde signal and control synaptic homeostasis (Haghighi et al., 2003; Frank et al., 2006; Goold and Davis, 2007; Dickman and Davis, 2009; Frank et al., 2009; Marie et al., 2010; Müller et al., 2011; Müller and Davis, 2012). However, the molecular nature of the retrograde signal remains a mystery.

At the *Drosophila* NMJ, Glass bottom boat (Gbb), a bone morphogenetic protein (BMP)-type ligand secreted by the muscle, provides a retrograde signal that promotes synaptic growth and confers synaptic homeostasis (Aberle et al., 2002; Marqués et al., 2002; Sweeney and Davis, 2002; McCabe et al., 2003; Goold and Davis, 2007). Gbb signals by binding to presynaptic heterotetrameric complex of type-I [Thickveins (Tkv) and Saxophone (Sax)] and type-II [Wishful thinking (Wit)] receptors. Activated receptors recruit and phosphorylate the BMP pathway effector Mad. Phosphorylated Mad (pMad) accumulates at two locations: in the motoneuron nuclei (nuclear pMad) and at the NMJ synapses (synaptic pMad) (McCabe et al., 2003; Dudu et al., 2006). Nuclear pMad in conjunction with other factors modulates expression of BMP target genes, including trio, which encodes for a Rac-activating protein important for cytoskeletal remodeling, and target of wit (twi), which encodes for a Ly-6 related molecule important for NMJ synapse activity (Ball et al., 2010; Kim and Marqués, 2012). The function of synaptic pMad remains less well understood. Recent evidence suggests that synaptic pMad does not translocate to the motoneuron nuclei and instead may engage in a local, unknown activity (Smith et al., 2012). Selective loss of synaptic pMad in *importin-β11* mutants causes developmental and functional defects at NMJ synapses (Higashi-Kovtun et al., 2014).
 RESULTS
Synaptic but not nuclear pMad is diminished at suboptimal Neto levels
As Neto-deprived NMJs have phenotypes reminiscent of BMP signaling pathway mutants, we hypothesized that Neto influences the retrograde BMP signaling. We tested this possibility by examining the levels and distribution of pMad, the effector of the BMP signaling pathway. Whereas control animals had clear pMad puncta decorating synaptic boutons, the pMad signals were drastically reduced at synaptic locations in neto109 third instar larvae (Fig. 1A,B). This difference was not caused by genetic background, as control and neto109 animals are excisions of the same transposable element (Kim et al., 2012). The loss of synaptic pMad at suboptimal Neto levels was confirmed using several anti-pMad antibodies (supplementary material Fig. S1) and resembled the loss of pMad in mutants of the BMP pathway, such as wit (Fig. 1C). Downregulation of neto via RNAi uncovered a similar decrease of synaptic pMad following the levels of postsynaptic Neto (Fig. 1D,E). A duplication covering the neto locus effectively rescued the loss of pMad in neto109 larvae (Fig. 1F), indicating that synaptic pMad specifically depends on Neto. To test if Neto affects the levels or synaptic localization of BMP pathway components we compared the distribution of Mad and BMP receptors, Tkv and Wit, and the transcriptional response to the retrograde BMP signaling in neto109 larvae and controls (supplementary material Fig. S2; and see below). The loss of pMad signals at neto109 NMJs did not appear to be a consequence of reduced synaptic BMP pathway components. Also, the loss of synaptic pMad at Neto-deprived NMJs could not be attributed to defective PSD structures, as detailed below.

In contrast to synaptic terminals, nuclear pMad signals appeared normal in neto109 third instar ventral ganglia (Fig. 2A,B). Quantification revealed only mild reduction of nuclear pMad signals in neto109 compared with controls, whereas nuclear pMad was not detectable in wit mutants (Fig. 2C). A similar specific reduction in synaptic but not nuclear pMad was reported for importin-β11 mutants (Higashi-Kovtun et al., 2010). Furthermore, suboptimal Neto levels did not appear to impact transcription of BMP target genes in the motoneurons. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) from third instar ventral ganglia indicated that twist expression was reduced by 16% in neto109 and by 83% in wit mutants compared with controls (Fig. 2D).

Importantly, suboptimal Neto levels did not affect gbb expression in either ventral ganglia or muscle tissue (qPCR from larval muscle cDNA shown in Fig. 2E). At the Drosophila NMJ, multiple pathways control Gbb transcription and secretion and therefore

Fig. 1. Suboptimal Neto prevents pMad accumulation at the NMJ. (A-F) Synaptic localization of Bruchpilot (green) and pMad (red) at third instar larvae NMJ (muscle 4, abdominal segment 3). The anti-horseradish peroxidase (HRP) antibody (blue) labels motoneuron arbors. pMad localizes at NMJ synapses in control animals (A), but is diminished in neto hypomorphs, neto109 (B), similar to wit null mutants (C). Synaptic pMad signals correlate with Neto levels (D,E). A duplication containing the neto gene restores synaptic pMad at neto109 NMJs (F). Scale bars: 10 μm. Genotypes: (A) control (precise excision); (B) neto109; (C) wit112/wit112; (D) UAS-Dicer/UAS-neto109-6D; 24B-Gal4/+; (E) UAS-neto109-6D/+; 24B-Gal4/+; (F) neto109; Dp(1;3)DC270.
However, animals with single copies of both are indistinguishable from controls (McCabe et al., 2003) (Fig. 3). Heterozygous larvae have normal numbers of synaptic boutons and components have smaller NMJs, but this phenotype is recessive; components. Larvae with mutations in combinations, but larvae heterozygous for neto, neto and appearance of synaptic pMad signals, which remained organized and appeared a cumulative effect on synaptic pMad. To rule out such a combined effect we performed developmental timecourse analyses and compared pMad in control and neto109 animals starting from late embryo stages.

In embryos 21 hours after egg laying (AEL), Neto forms distinct puncta that mark iGluR clusters at neuronal arbors (Kim et al., 2012) (Fig. 4A). Some of these puncta colocalized with a few pMad immunoreactivities present at this stage. The synaptic pMad puncta were very rare in control embryos and were never found in neto109 null larvae, which also lacked Neto-positive signals (Fig. 4A’). First and second instar larvae had well defined synapses with boutons decorated by Neto puncta (Fig. 4B,C). By second instar all Neto-positive puncta were accompanied by pMad signals in control larvae, but pMad immunoreactivities remained very small and scattered along the axonal arbor in neto109, mirroring the dim Neto signals (Fig. 4B,C’, arrows). By contrast, nuclear pMad levels were similar in neto109 and control animals at all stages of development (Fig. 4D-F). Furthermore, although they die paralyzed, neto109 null embryos had normal nuclear pMad (not shown). The selective loss of pMad observed throughout development suggests that the loss of synaptic pMad is not a consequence of impaired development at Neto-deprived synapses; instead, neto is directly required for the accumulation of local pMad.

As synaptic pMad followed the temporal and spatial accumulation of Neto at developing synapses, we examined whether increased Neto levels affect pMad accumulation. Synthetic Neto showed punctate as well as diffuse distribution when neto109 hemizygotes were tested whether further reduction of BMP signaling pathway components affected the lethality. We found that neto109 lethality was increased from 50% to >80% when one copy of gbb or mad was removed (supplementary material Fig. S3). This synthetic lethality assay also revealed a modest interaction between neto and wit, but not put. Together, these genetic interactions indicated that neto and gbb formed trans-heterozygous combinations for which phenotypic threshold effects could be consistently observed.

If neto is upstream of gbb in the BMP signaling pathway, then lowering Neto levels should not aggravate gbb null mutant phenotypes. Indeed, third instar gbb null larvae (gbb1/gbb2) showed a ~50% reduction in synaptic boutons with either one or two copies of neto (Fig. 3B). However, overexpression of gbb did not rescue the synaptic arbors or local pMad in neto109 (Fig. 3C; supplementary material Fig. S4). In control experiments, overexpression of gbb in the muscle significantly rescued the NMJ growth phenotype of gbb null mutants (supplementary material Fig. S5). If neto is upstream gbb but cannot be rescued by genetic manipulations of Gbb levels, then how does Neto impact BMP signaling? One possibility could be that Neto controls localization of Gbb activities: in this scenario, overexpression of Gbb cannot suppress neto loss-of-function phenotypes, even though neto is upstream of gbb.

Synaptic pMad does not accumulate at Neto-deprived synapses throughout development
As reduced Neto levels efficiently disrupted synaptic pMad without affecting nuclear pMad levels, we wondered if our observations captured a cumulative effect on synaptic pMad. To rule out such a combined effect we performed developmental timecourse analyses and compared pMad in control and neto109 animals starting from late embryo stages.

In embryos 21 hours after egg laying (AEL), Neto forms distinct puncta that mark iGluR clusters at neuronal arbors (Kim et al., 2012) (Fig. 4A). Some of these puncta colocalized with a few pMad immunoreactivities present at this stage. The synaptic pMad puncta were very rare in control embryos and were never found in neto109 null larvae, which also lacked Neto-positive signals (Fig. 4A’). First and second instar larvae had well defined synapses with boutons decorated by Neto puncta (Fig. 4B,C). By second instar all Neto-positive puncta were accompanied by pMad signals in control larvae, but pMad immunoreactivities remained very small and scattered along the axonal arbor in neto109, mirroring the dim Neto signals (Fig. 4B,C’, arrows). By contrast, nuclear pMad levels were similar in neto109 and control animals at all stages of development (Fig. 4D-F). Furthermore, although they die paralyzed, neto109 null embryos had normal nuclear pMad (not shown). The selective loss of pMad observed throughout development suggests that the loss of synaptic pMad is not a consequence of impaired development at Neto-deprived synapses; instead, neto is directly required for the accumulation of local pMad.

As synaptic pMad followed the temporal and spatial accumulation of Neto at developing synapses, we examined whether increased Neto levels affect pMad accumulation. Synthetic Neto showed punctate as well as diffuse distribution when neto transgenes were overexpressed in the striated muscles (Fig. 4G). Neto puncta colocalized with the iGluR subunits, whereas diffuse Neto exceeded the receptor field. However, excess Neto did not affect the intensity and appearance of synaptic pMad signals, which remained organized in distinct puncta. The similarities between iGluRs and synaptic

...
pMad labeling at various Neto levels raised the possibility that synaptic pMad correlates with clustered Neto/iGluRs.

**Synaptic pMad correlates with glutamate receptor levels**

To test whether synaptic pMad follows the Neto/iGluRs clusters we examined pMad at various iGluRs levels. We first targeted the essential subunits of iGluR complexes, GluRIIC, GluRIID and GluRIIE, via muscle specific RNAi. Staining and imaging conditions were kept identical to facilitate comparison of various genotypes. Mild reduction of iGluR levels, such as in GluRIIE RNAi, induced attenuation of Neto and GluRIIC signals and a corresponding decrease in synaptic pMad (Fig. S6; supplementary material Fig. 5F). Strong reduction of iGluR levels (i.e. GluRIID RNAi, GluRIIC RNAi) disrupted larval development and reduced Neto and GluRIIC synaptic levels in a concentration-dependent manner (supplementary material Fig. S6). The levels of pMad closely followed the synaptic properties of Neto/iGluR clusters (Fig. 5C-E). A similar phenotype was found in GluRIIC ‘weakly’ rescued flies, carrying a leaky UAS-GluRIIC cDNA transgene (Marrus et al., 2004) (Fig. 5F). Similar to neto109, iGluR-deprived larvae had normal levels of nuclear pMad in ventral ganglia (Fig. 5G-I). Thus, synaptic pMad mirrors the levels of postsynaptic Neto/iGluRs, whereas nuclear pMad appears insensitive to the glutamate receptors status.

The *Drosophila* NMJ utilizes two types of iGluRs, type-A and type-B, which differ in their composition, channel properties and subcellular localization (DiAntonio, 2006). To test whether synaptic pMad differentiated between these channels, we examined the synapses at various GluRIIA and GluRIIB levels. Consistent with the previous demonstration that GluRIIA and GluRIIB compete for the essential subunits for synaptic targeting (Sigrist et al., 2002; Marrus et al., 2004), we found that GluRIIA overexpression produced strong reduction of GluRIIB synaptic levels (Fig. 6A,B). Also, lowering GluRIIA (or GluRIIB) levels produced a significant increase in synaptic accumulation of GluRIIB (or respectively, GluRIIA) (Fig. 6C-E). None of these genetic manipulations induced changes in the GluRIIC and Neto synaptic signals (not shown; Fig. 6). We found that synaptic pMad specifically followed synaptic GluRIIA levels over a wide range of concentrations, in RNAi experiments or heteroallelic combinations (Fig. 6F-J). These striking variations in synaptic pMad levels cannot be due to structural disruptions at postsynaptic specializations. Unlike neto109, GluRIIA mutant NMJs have normal distribution of postsynaptic scaffolds such as Discs-large (Dlg) (supplementary material Fig. S7) (Kim et al., 2012). Furthermore, nuclear pMad remained relatively constant over a wide range of GluRIIA levels (Fig. 6K-N). Thus, postsynaptic type-A receptors selectively modulate the accumulation of synaptic pMad.

**Activity-dependent accumulation of synaptic pMad**

The relative ratio of synaptic type-A versus type-B glutamate receptors is also a key determinant of quantal size. Overexpression of GluRIIA induces a dose-dependent increase in quantal size, whereas overexpression of GluRIIB induces a dose-dependent decrease (Petersen et al., 1997; DiAntonio et al., 1999). Therefore, synaptic pMad may correlate with the net levels of type-A receptors or may reflect a change in synapse activity/quantal size. To distinguish between these two possibilities, we next manipulated the activity of postsynaptic glutamate receptors. Postsynaptic protein kinase A (PKA; PKA-C1 – FlyBase) controls quantal size through a mechanism that requires the presence of GluRIIA subunit; the current model is that PKA may phosphorylate and inhibit the activity of type-A receptors without changing the levels of synaptic receptors (Davis et al., 1998). If synaptic pMad depends on the activity of type-A receptors, then PKA activity should have profound effects on synaptic pMad accumulation. We tested this possibility by genetic manipulation of PKA activity in postsynaptic muscles using transgenes that express either a constitutively active catalytic subunit (PKA-act) or a mutant regulatory subunit of PKA with reduced affinity for cAMP that inhibits the release of catalytic subunits (PKA-inh) (Davis et al., 1998). Expression of these transgenic lines in selected muscles using BG487-Gal4 promoter produced mosaic animals suitable for comparative analyses within the same muscles. We followed the synaptic levels of Neto/iGluR clusters (Fig. 5C-E). A decrease in synaptic pMad (Fig. 5B; supplementary material Fig. S6). Strong reduction of iGluR levels (i.e. GluRIID RNAi, GluRIIC RNAi) disrupted larval development and reduced Neto and GluRIIC synaptic levels in a concentration-dependent manner (supplementary material Fig. S6). The levels of pMad closely followed the synaptic properties of Neto/iGluR clusters (Fig. 5C-E). A similar phenotype was found in GluRIIC ‘weakly’ rescued flies, carrying a leaky UAS-GluRIIC cDNA transgene (Marrus et al., 2004) (Fig. 5F). Similar to neto109, iGluR-deprived larvae had normal levels of nuclear pMad in ventral ganglia (Fig. 5G-I). Thus, synaptic pMad mirrors the levels of postsynaptic Neto/iGluRs, whereas nuclear pMad appears insensitive to the glutamate receptors status.

The *Drosophila* NMJ utilizes two types of iGluRs, type-A and type-B, which differ in their composition, channel properties and subcellular localization (DiAntonio, 2006). To test whether synaptic pMad differentiated between these channels, we examined the synapses at various GluRIIA and GluRIIB levels. Consistent with the previous demonstration that GluRIIA and GluRIIB compete for the essential subunits for synaptic targeting (Sigrist et al., 2002; Marrus et al., 2004), we found that GluRIIA overexpression produced strong reduction of GluRIIB synaptic levels (Fig. 6A,B). Also, lowering GluRIIA (or GluRIIB) levels produced a significant increase in synaptic accumulation of GluRIIB (or respectively, GluRIIA) (Fig. 6C-E). None of these genetic manipulations induced changes in the GluRIIC and Neto synaptic signals (not shown; Fig. 6). We found that synaptic pMad specifically followed synaptic GluRIIA levels over a wide range of concentrations, in RNAi experiments or heteroallelic combinations (Fig. 6F-J). These striking variations in synaptic pMad levels cannot be due to structural disruptions at postsynaptic specializations. Unlike neto109, GluRIIA mutant NMJs have normal distribution of postsynaptic scaffolds such as Discs-large (Dlg) (supplementary material Fig. S7) (Kim et al., 2012). Furthermore, nuclear pMad remained relatively constant over a wide range of GluRIIA levels (Fig. 6K-N). Thus, postsynaptic type-A receptors selectively modulate the accumulation of synaptic pMad.

**Activity-dependent accumulation of synaptic pMad**

The relative ratio of synaptic type-A versus type-B glutamate receptors is also a key determinant of quantal size. Overexpression of GluRIIA induces a dose-dependent increase in quantal size, whereas overexpression of GluRIIB induces a dose-dependent decrease (Petersen et al., 1997; DiAntonio et al., 1999). Therefore, synaptic pMad may correlate with the net levels of type-A receptors or may reflect a change in synapse activity/quantal size. To distinguish between these two possibilities, we next manipulated the activity of postsynaptic glutamate receptors. Postsynaptic protein kinase A (PKA; PKA-C1 – FlyBase) controls quantal size through a mechanism that requires the presence of GluRIIA subunit; the current model is that PKA may phosphorylate and inhibit the activity of type-A receptors without changing the levels of synaptic receptors (Davis et al., 1998). If synaptic pMad depends on the activity of type-A receptors, then PKA activity should have profound effects on synaptic pMad accumulation. We tested this possibility by genetic manipulation of PKA activity in postsynaptic muscles using transgenes that express either a constitutively active catalytic subunit (PKA-act) or a mutant regulatory subunit of PKA with reduced affinity for cAMP that inhibits the release of catalytic subunits (PKA-inh) (Davis et al., 1998). Expression of these transgenic lines in selected muscles using BG487-Gal4 promoter produced mosaic animals suitable for comparative analyses within the same muscles.

segments had mild expression of PKA-act and reduced change in expression and showed decreased synaptic pMad, whereas posterior including its anteroposterior gradient. For example, muscle 6/7 of GluRIIA (supplementary material Fig. S8). We found that increased PKA activity in postsynaptic muscles produced a significant decrease in the levels of synaptic pMad, but not GluRIIA levels, compared with the control (Fig. 7A-E). We quantified this difference relative to HRP and found that pMad levels were reduced at synapses with normal or even slightly increased GluRIIA levels (Fig. 7E,F). The synaptic pMad levels were inversely proportional to the BG487-Gal4 expression pattern, including its anteroposterior gradient. For example, muscle 6/7 synapses in anterior segments had greatly increased PKA-act expression and showed decreased synaptic pMad, whereas posterior segments had mild expression of PKA-act and reduced change in synaptic pMad (supplementary material Fig. S8). By contrast, decreased PKA activity induced no significant change in synaptic pMad levels (Fig. 7C,D). Furthermore, high levels of PKA activity throughout development, such as driven by G14-Gal4, resulted in complete loss of synaptic pMad signals and larval lethality (Fig. 7E,F). In this case, escaper third instar larvae also showed a severe reduction of GluRIIA levels and diminished Neto signals. In addition, overexpression of a dominant-negative GluRIIA receptor with a mutation in the putative ion conduction pore (DiAntonio et al., 1999) induced a significant reduction in synaptic pMad indicating that synaptic pMad does not correlate with the net levels of GluRIIA (supplementary material Fig. S9). In spite of these drastic reductions in synaptic pMad, we found no changes in nuclear pMad levels in motoneurons (Fig. 7G-J). Together, these findings indicate that accumulation of synaptic pMad depends on the activity of type-A receptors and not the net receptor levels. Thus, synaptic pMad reflects the activity status of postsynaptic glutamate receptors.

DISCUSSION

We have previously described Neto as the first nonchannel subunit required for the clustering of iGluRs and formation of functional synapses at the Drosophila NMJ. Neto and iGluR complexes associate in the striated muscle and depend on each other for targeting and clustering at postsynaptic specializations. Here, we show that Neto/iGluR synaptic complexes induce accumulation of pMad at synaptic termini in an activity-dependent manner. The effect of Neto/iGluR clusters on BMP signaling is selective, and limited to synaptic pMad; nuclear accumulation of pMad appears largely independent of postsynaptic glutamate receptors. We demonstrate that synaptic pMad mirrors the activity of postsynaptic type-A receptors. As such, synaptic pMad may function as an acute sensor for postsynaptic sensitivity. Local fluctuations in synaptic pMad may provide a versatile means to relay changes in synapse activity to presynaptic neurons and coordinate synapse activity status with synapse growth and homeostasis.

**Synaptic pMad as a sensor for synapse activity**

Drosophila NMJs maintain their evoked potentials remarkably constant during development, from late embryo to the third instar larval stages (Keshisian and Kim, 2004). This coordination between motoneuron and muscle properties requires active trans-synaptic signaling, including a retrograde BMP signal, which promotes synaptic growth and confers synaptic homeostasis (Marqués, 2005; Goold and Davis, 2007). Nuclear pMad accumulates in motoneurons during late embryogenesis. However, embryos mutant for BMP pathway components hatch into the larval stages, indicating that BMP signaling is not required for the initial assembly of NMJ synapses and instead modulates NMJ growth and development (Aberle et al., 2002; Marqués et al., 2002). Here we demonstrate that synaptic accumulation of pMad follows GluRIIA arrival at nascent NMJs (Fig. 4) and depends on optimal levels of synaptic Neto and iGluRs (Figs 1, 5). As type-A receptors have been associated with nascent synapses, and type-B receptors mark mature NMJs, accumulation of synaptic pMad appears to correlate with a growing phase at NMJ synapses. Furthermore, synaptic pMad
secreted Mav, which regulates gbb expression and indirectly modulates the Gbb-mediated retrograde signaling (Fuentes-Medel et al., 2012). RNAi experiments revealed that knockdown of mad in muscle induces a decrease in synaptic pMad, albeit much reduced in amplitude compared with knockdown of mad in motoneurons (supplementary material Fig. S10) (Fuentes-Medel et al., 2012). Also, knockdown of wit in motoneurons, but not in muscle, and knockdown of put in muscle, but not in motoneurons, triggers reduction of synaptic pMad (data not shown) (Fuentes-Medel et al., 2012). Intriguingly, the synaptic pMad is practically abolished in GluRIIA and neto109 mutants and cannot be further reduced by additional decrease in Mad levels (supplementary material Fig. S10). Whereas loss of postsynaptic pMad could be due to a Mav-dependent feedback mechanism that controls Gbb secretion from the muscle, the absence of presynaptic pMad demonstrates a role for GluRIIA and Neto in modulation of BMP retrograde signaling.

As BMP signals are generally short lived (O’Connor et al., 2006; Tucker et al., 2008; Ramel and Hill, 2012), synaptic pMad probably reflects accumulation of active BMP/receptor complexes at synaptic termini. Recent evidence suggests that BMP receptors traffic along the motoneuron axons, with Gbb/receptors complexes moving preferentially in a retrograde direction. By contrast, Mad does not appear to traffic (Smith et al., 2012). Thus, Mad is likely to be phosphorylated and maintained locally by a pool of active Gbb/BMP receptor complexes that remain at synaptic termini for the time postsynaptic type-A receptors are active.

How does postsynaptic glutamate receptor activity modulate local pMad?

The activity of type-A glutamate receptors may control synaptic pMad accumulation (1) indirectly via activity-dependent changes that are relayed to both pre- and postsynaptic cells, or (2) directly by influencing the production and signaling of varied Gbb ligand forms or by localizing Gbb activities. For example, inhibition of postsynaptic receptor activity induces trans-synaptic modulation of presynaptic Ca\(^{2+}\) influx (Müller and Davis, 2012). Such Ca\(^{2+}\) influx changes may trigger events that induce a local change in synaptic pMad accumulation. One possibility is that changes in Ca\(^{2+}\) influx may recruit Importin-β11 at presynaptic termini, which in turn mediate synaptic pMad accumulation.

At the Drosophila NMJ, Gbb is secreted in the synaptic cleft from both pre- and postsynaptic compartments. The secretion of Gbb is regulated at multiple levels, transcriptionally and post-translationally (Ellis et al., 2010; Nahm et al., 2010; James and Brohier, 2011; Fuentes-Medel et al., 2012; Nahm et al., 2013). Furthermore, the Gbb prodomain could be processed at several cleavage sites to generate Gbb ligands with varying activities (Akiyama et al., 2012). The longer, more active Gbb ligand retains a portion of the prodomain that could influence the formation of Gbb/BMP receptor complexes (Sengle et al., 2011). Synaptic pMad may result from signaling by selective forms of Gbb. Or type-A receptors could modulate secretion and processing of Gbb in an activity-dependent manner. Understanding the function of different pools and active forms of Gbb within the synaptic cleft will help explain the multiple roles for Gbb at Drosophila NMJs.

Alternatively, active postsynaptic type-A receptor complexes may directly engage and stabilize presynaptic Gbb/BMP receptor signaling complexes via trans-synaptic interactions. CUB domains can directly bind BMPs (Lee et al., 2009); thus Neto may utilize its extracellular CUB domains to engage Gbb and/or presynaptic BMP receptors. As synaptic pMad mirrors active type-A receptors, such...
trans-synaptic complexes will depend on Neto in complexes with active type-A receptors. We have been unable to capture a direct interaction between Gbb and Neto CUB domains in co-immunoprecipitation experiments (data not shown). Nonetheless, a trans-synaptic complex that depends on the activity of type-A receptors could offer a versatile means for relaying synapse activity status to the presynaptic neuron via fast assembly and disassembly.

Irrespective of the strategy that correlates synaptic pMad pool with the active type-A receptor/Neto complexes, further mechanisms must act to maintain the Gbb/BMP receptor complexes at synapses and protect them from endocytosis and retrograde transport. Such mechanisms must be specific, as general modulators of BMP receptors endocytosis impact both synaptic and nuclear pMad (Sweeney and Davis, 2002; Wang et al., 2007; O’Connor-Giles and Ganetzky, 2008; O’Connor-Giles et al., 2008; Nahm et al., 2013). A candidate for differential control of BMP/receptor complexes is Importin-β11. Loss of synaptic pMad in importin-β11 is rescued by neuronal expression of activated BMP receptors, by blocking retrograde transport, but not by neuronal expression of Mad (Higashi-Kovtun et al., 2010). As Mad does not appear to traffic (Smith et al., 2012), presynaptic Importin-β11 must act upstream of the BMP receptors, perhaps to stabilize active Gbb/BMP receptor complexes at the neuron membrane. By contrast, local pMad cannot be restored at Neto-deprived NMJs by overactivation of presynaptic BMP receptors or by blocking retrograde transport (supplementary material Fig. S4). As neto and gbb interact genetically, it is tempting to speculate that postsynaptic Neto/type-A complexes localize Gbb activities and stabilize Gbb/BMP receptor complexes from the extracellular side. Additional extracellular factors, for example heparan proteoglycans (Dani et al., 2012), or intracellular
modulators, such as Nemo kinase (Merino et al., 2009), may control the distribution of sticky Gbb molecules within the synaptic cleft and their binding to BMP receptors, or may stabilize Gbb/BMP receptor complexes at synaptic termini.

Possible functions for synaptic pMad

Synaptic pMad may act locally and/or in coordination with the transcriptional control of BMP target genes to ensure proper growth and development of the synaptic structures. A presynaptic pool of pMad maintained by Importin-β11 neuronal activities ensures normal NMJ structure and function (Higashi-Kovtun et al., 2010). Like importin-β11, GluRIIA and Neto-deprived synapses show a significantly reduced number of boutons (Table 1) (Petersen et al., 1997; Higashi-Kovtun et al., 2010; Kim et al., 2012). Intriguingly, the absence of GluRIIA induces up to 20% reduction in bouton numbers, whereas knockdown of GluRIIB does not appear to affect NMJ growth (Table 1) (Petersen et al., 1997; Reiff et al., 2002). Although the amplitude of the growth phenotypes we observed in normal culturing conditions (25°C) was modest, this phenomenon may explain the requirement for GluRIIA reported for activity-dependent NMJ development (at 29°C) (Sigrist et al., 2002; Sigrist et al., 2003). Furthermore, knockdown of Neto or any iGluR essential subunit affect synaptic pMad and NMJ growth in a dose-dependent manner (Figs 1, 5; Table 1) (Kim et al., 2012). We could not find significant changes in nuclear pMad or expression of BMP target genes in GluRIIA or Neto-deprived animals, but the restoration of synaptic pMad by presynaptic constitutively active BMP receptors rescues the morphology and physiology of importin-β11 mutant NMJs (Higashi-Kovtun et al., 2010). The smaller NMJs observed in the absence of local pMad may reflect a direct contribution of synaptic pMad to retrograde BMP signaling, a pathway that provides an instructive signal for NMJ growth (O’Connor-Giles et al., 2008). Thus, BMP signaling may integrate synapse activity status with the control of synapse growth.

Synaptic pMad may also contribute to synapse stability. Mutants in BMP signaling pathway have an increased number of ‘synaptic footprints’: regions of the NMJ where the terminal nerve once resided and has retracted (Eaton and Davis, 2005). It has been proposed that GluR binding to its receptors activates the Williams Syndrome-associated Kinase LIMK1 to stabilize the NMJ (Eaton and Davis, 2005). Synaptic pMad may further contribute to the stabilization of synapse contacts by engaging in interactions that anchor the Gbb/BMP receptor complexes at synaptic termini. During neural tube closure, local pSmad1/5/8 mediates stabilization of BMP signaling complexes at tight junction via binding to apical polarity complexes (Eom et al., 2011). Flies may utilize a similar anchor mechanism that relies on pMad-mediated interactions for stabilizing BMP signaling complexes and other components at synaptic junctions. Local active

BMP signaling complexes are thought to function in this manner in the maintenance of stemness (Michel et al., 2011) and in epithelial-to-mesenchymal transition (Zeisberg et al., 2003).

Separate from its role in synapse growth and stability, BMP signaling is required presynaptically to maintain the competence of motoneurons to express homeostatic plasticity (Goold and Davis, 2007). The requirements for BMP signaling components for the rapid induction of presynaptic response may include a role for synaptic pMad in relaying acute perturbations of postsynaptic receptor function to the presynaptic compartment. At the very least, attenuation of local pMad in relaying acute perturbations of postsynaptic receptor function may include a role for synaptic pMad-dependent complexes that are thought to function in this manner in the Drosophila NMJ, the use of synaptic pMad as a sensor for synaptic activity may enable the BMP signaling pathway to monitor synaptic activity then function to adjust synaptic growth and stability during development and homeostasis.

### MATERIALS AND METHODS

#### Fly stocks

The neto<sup>P454</sup> lines were generated by insertion of a neto cDNA PCR fragment in pUAST-R57 followed by germline transformation using standard protocols. The PCR primers utilized were: RNAi-F (5'-AAGGGCTTACATGGCGACCCGTGGTTGTTGACGATGACATGTC-3') and RNAi-Rev (5'-AACTCAGAGGATCCTTTTGTGGTTTGAGGTTTGGACGC3'). Precise excisions of transposomal elements Mi(ET1)Neto[MB05569] and Mi(ET1)Neto[MB04917] were utilized to generate the neto<sup>109</sup> and neto<sup>66</sup> alleles were used as controls (Bellen et al., 2011; Kim et al., 2012). The duplication covering neto locus is Dp(1;3)DC270 (Venken et al., 2010).

Other stocks used in this study are as follows: wit<sup>A12</sup> and wit<sup>B11</sup> (Marqués et al., 2002); gbb<sup>1</sup> (Khalsa et al., 1998); gbb<sup>2</sup> (Wharton et al., 1999); UAS-gbb<sup>6,9</sup> (McCabe et al., 2003); UAS-tdTomato<sup>Q253D</sup> (Nellen et al., 1996) and UAS-staurosporine<sup>Q253D</sup> (Haery et al., 1998) (from M. O’Connor, University of Minnesota); mad<sup>mutant</sup> (Takanasu et al., 2005); mad deficiency Df(2L)C28 (Adachi-Yamada et al., 1999); GluRIIC<sup>Q454</sup> (DiAntonio et al., 1999); GluRIIC<sup>Q253D</sup> (DiAntonio et al., 1999); GluRIIA<sup>Q253D</sup>; Df(2L)ec1<sup>64</sup>; and UAS-GluRII<sup>A</sup> (Petersen et al., 1997) (all from A. DiAntonio, Washington University; impß1124 (from C. Goodman, University of California at Berkeley). For RNAi-mediated knockdown we used the following TRIP lines generated by the Transgenic RNAi Project, Harvard Medical School: GluRII

---

**Table 1. Genotypes and quantification of bouton numbers**

**Muscles 6/7 NMJ**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bouton number</th>
<th>Boutons/µm²×10⁴</th>
<th>Segments counted</th>
<th>P value versus control</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precise excision</td>
<td>109.9±6.8</td>
<td>19.5±1.2</td>
<td>10</td>
<td>1</td>
<td>(Petersen et al., 1997)</td>
</tr>
<tr>
<td>G14+/−</td>
<td>120.3±8.7</td>
<td>19.3±1.4</td>
<td>11</td>
<td>1</td>
<td>(Reiff et al., 2002)</td>
</tr>
<tr>
<td>G14&gt;GluRIIA&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>87.2±2.8</td>
<td>15.4±0.5</td>
<td>8</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIE&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>87.5±3.2</td>
<td>13.6±1</td>
<td>8</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIC&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>59.4±2.5</td>
<td>12.0±0.5</td>
<td>10</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIID&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>45.6±5.3</td>
<td>9.5±1</td>
<td>12</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>GluRIIA&lt;sup&gt;Q16Df&lt;/sup&gt;</td>
<td>86±5.2</td>
<td>16.8±1.1</td>
<td>10</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>neto&lt;sup&gt;109&lt;/sup&gt;</td>
<td>64.2±12.9</td>
<td>9±0.6</td>
<td>10</td>
<td>&lt;0.001</td>
<td>(Kim et al., 2012)</td>
</tr>
<tr>
<td>GluRIIC&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>59.3±2.5</td>
<td>11.7±0.5</td>
<td>9</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIB&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>101.8±9.5</td>
<td>15.8±1.4</td>
<td>8</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>MHC&gt;PKA&lt;sup&gt;Q16CT&lt;/sup&gt;</td>
<td>121.5±9.3</td>
<td>20.8±1.6</td>
<td>7</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>mad&lt;sup&gt;Q16Df&lt;/sup&gt;</td>
<td>32.7±4.4</td>
<td>11.4±1.7</td>
<td>10</td>
<td>&lt;0.001</td>
<td>(McCabe et al., 2004)</td>
</tr>
<tr>
<td>wi1&lt;sup&gt;A12Df&lt;/sup&gt;</td>
<td>37.2±5.9</td>
<td>9.3±1.4</td>
<td>6</td>
<td>&lt;0.001</td>
<td>(Ball et al., 2010)</td>
</tr>
</tbody>
</table>

**Muscle 4 NMJ**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bouton number</th>
<th>Boutons/µm²×10⁴</th>
<th>Segments counted</th>
<th>P value versus control</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precise excision</td>
<td>35.3±1.1</td>
<td>11±0.3</td>
<td>14</td>
<td>1</td>
<td>(Petersen et al., 1997)</td>
</tr>
<tr>
<td>G14+/−</td>
<td>36.1±3.2</td>
<td>11.2±1</td>
<td>8</td>
<td>1</td>
<td>(Reiff et al., 2002)</td>
</tr>
<tr>
<td>G14&gt;GluRIIA&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>30.3±1</td>
<td>11.5±0.5</td>
<td>8</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIE&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>30.3±1.6</td>
<td>9.8±0.5</td>
<td>8</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIC&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>18.7±1.1</td>
<td>7.7±0.5</td>
<td>10</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIID&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>15.5±0.7</td>
<td>6.4±0.4</td>
<td>12</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>GluRIIA&lt;sup&gt;P16Df&lt;/sup&gt;</td>
<td>24.4±1.3</td>
<td>9.7±0.5</td>
<td>10</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td>neto&lt;sup&gt;109&lt;/sup&gt;</td>
<td>18.9±1.5</td>
<td>6.5±0.5</td>
<td>8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>GluRIIC&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>19.9±0.8</td>
<td>7.3±0.3</td>
<td>10</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>G14&gt;GluRIIB&lt;sup&gt;Q454&lt;/sup&gt;</td>
<td>27.6±4.6</td>
<td>10.9±1.8</td>
<td>8</td>
<td>0.805</td>
<td></td>
</tr>
<tr>
<td>MHC&gt;PKA&lt;sup&gt;B11CT&lt;/sup&gt;</td>
<td>27.6±3</td>
<td>9.3±1</td>
<td>10</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>mad&lt;sup&gt;Q16Df&lt;/sup&gt;</td>
<td>10±1.2</td>
<td>6.2±1.1</td>
<td>8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>wit&lt;sup&gt;A12Df&lt;/sup&gt;</td>
<td>13.1±6.2</td>
<td>6.7±1</td>
<td>6</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Bouton numbers (ss.e.m.) at muscle 4 and muscle 6/7 NMJs, and in abdominal segment A3 are shown.

Protein purification and antibody generation
A rabbit polyclonal anti-GluRIIC antibody was generated against the N-terminal domain of GluRIIC subunit. Drosophila S2 cells were used for producing recombinant proteins as described previously (Serpe and O’Connor, 2006). An AcPA-based construct containing the N-terminal domain of GluRIIC subunit (residues Q48-M446) C-tagged with RGS-Hx6 was transiently transfected into S2 cells, and the secreted fragment was purified from conditioned supernatant using a His-Trap column (Pharmacia). The antigen was further purified by SDS-PAGE and the gel pieces were used for immunization (Open Biosystems). Positive sera were cleaned by protein A/G affinity, eluted using 0.1 M Gly-HCl (pH 2.8) and quenched with 0.1 vol of 1 M Tris-HCl (pH 9.0).

Immunohistology
Embryos 18 hours AEL were dechorionated, genotyped and, after an additional incubation (2 hours at room temperature), dissected as described previously (Budnik et al., 2006). Larvae were dissected as described previously in ice-cooled Ca2+-free HL-3 solution (Stewart et al., 1994; Budnik et al., 2006). The samples were fixed in either 4% formaldehyde (Polysciences) for 30 minutes or in Bouin’s fixative (Bio-Rad) for 3 minutes and washed in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. Primary antibodies from Developmental Studies Hybridoma Bank were used at the following dilutions: mouse anti-GluRIIA (M12H2), 1:200; mouse anti-Disc-large (Dlg) (4F3), 1:1000; mouse anti-Bruchpilot (Brp) (Nc82), 1:200; mouse anti-Wishful thinking (Wit) (23C7), 1:10. Other primary antibodies were as follows: rabbit anti-phosphorylated Mothers against decapentaplegic (pMad), 1:500 (a gift from Carl Heldin) (Persson et al., 1998); rabbit anti-p-Mad, 1:100 (Peluso et al., 2011); rabbit anti-pSmad3, 1:500 (Epitomics) (Smith et al., 2012); rabbit anti-GluRIIB, 1:2000 (a gift from Aaron DiAntonio) (Marrus et al., 2004); goat anti-Mad, 1:100 (Santa Cruz Biotechnology); FITC-, rhodamine- and Cy5-conjugated goat anti-HRP, 1:1000 (Jackson ImmunoResearch Laboratories); rabbit anti-GFP, 1:250 (Acbam); rat anti-neto, 1:100 (Kim et al., 2012). Alexa Fluor 488-, Alexa Fluor 568- and Alexa Fluor 647-conjugated secondary antibodies (Molecular Probes) were used at 1:400. All samples were mounted in ProLong Gold (Invitrogen). Samples of different genotypes were processed simultaneously and imaged under identical confocal settings using laser scanning confocal microscopes (Carl Zeiss 510 and LSM710). Boutons were counted using anti-HRP and anti-Dlg immunoreactivities. All quantifications were performed while blinded to genotype. The numbers of samples analyzed are indicated inside the bars.

Fluorescence intensity measurements
For nuclear pMad, confocal regions of interest (ROIs) were determined while blinded to genotype. The numbers of samples analyzed are indicated inside the bars.

Glutamate receptor expression regulates quantal size and quantal content at the Drosophila neuromuscular junction. J. Neurosci. 326, 1127-1130.

The authors declare no competing financial interests.

Acknowledgements
We thank Bing Zhang, Ed Ginzinger, Mark Mayer, Chi-Hon Lee and Alan Hinnebusch for helpful discussions and suggestions. We are grateful to Aaron DiAntonio, Carl Heldin, Mike O’Connor and Ben White for antibodies and Drosophila stocks. We thank Peter Nguyen for technical assistance. We also thank the Bloomington Stock Center at the University of Indiana for fly stocks and the Developmental Studies Hybridoma Bank at the University of Iowa for antibodies.

Competing interests
The authors declare no competing financial interests.

Author contributions
M. Sulkowski, Y.-J.K. and M. Serpe designed and performed the experiments; M. Sulkowski and M. Serpe wrote the paper.

Funding
This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Child Health and Human Development [grants Z01 HD008869 and Z01 HD008914]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242/dev.097758/DC1

References


