Neuron, Vol. 39, 255–267, July 17, 2003, Copyright ©2003 by Cell Press

# Retrograde Control of Synaptic Transmission by Postsynaptic CaMKII at the *Drosophila* Neuromuscular Junction

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## Summary

**Retrograde signaling plays an important role in synaptic homeostasis, growth, and plasticity. A retrograde signal at the neuromuscular junction (NMJ) of *Drosophila* controls the homeostasis of neurotransmitter release. Here, we show that this retrograde signal is regulated by the postsynaptic activity of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII). Reducing CaMKII activity in muscles enhances the signal and increases neurotransmitter release, while constitutive activation of CaMKII in muscles inhibits the signal and decreases neurotransmitter release. Postsynaptic inhibition of CaMKII increases the number of presynaptic, vesicle-associated T bars at the active zones. Consistently, we show that glutamate receptor mutants also have a higher number of T bars; this increase is suppressed by postsynaptic activation of CaMKII. Furthermore, we demonstrate that presynaptic BMP receptor *wishful thinking* is required for the retrograde signal to function. Our results indicate that CaMKII plays a key role in the retrograde control of homeostasis of synaptic transmission at the NMJ of *Drosophila*.**

## Introduction

Dynamic modification of synaptic structure and function occurs during the development of intricate neuronal networks and underlies the process of learning and memory throughout the adult life of the organism. Precise control of synaptic structure and function can only be achieved when synaptic modification is combined with homeostatic mechanisms. Synaptic modification and homeostasis depend on presynaptic neurotransmitter release and the activity of neurotransmitter receptors as well as voltage-gated ion channels. Many lines of evidence suggest that in order to maintain the homeostasis of synaptic activity, presynaptic neurotransmitter release and both the activity and number of ligand-gated and voltage-gated ion channels are dynamically altered and controlled (Davis and Bezprozvanny, 2001; Davis and Goodman, 1998; Petersen et al., 1997; Sandrock et al., 1997; Turrigiano et al., 1994, 1998; Wu et al., 1996). In addition to anterograde signaling from the presynaptic neuron to the postsynaptic target cell, retrograde signaling from the postsynaptic target cell to the presynaptic neuron plays an important role in the control of this process (Constantine-Paton and Cline, 1998; Davis and Bezprozvanny, 2001; Tao and Poo, 2001). Although many retrograde signals have been identified both at the neuromuscular junction (NMJ) and central synapses (Fitzsimonds and Poo, 1998; Sanes and Lichtman, 1999; Tao and Poo, 2001), we know little about how such signals are triggered, maintained, or regulated by synaptic activity.

We have previously demonstrated that a retrograde signal controls homeostasis of synaptic transmission at the NMJ of *Drosophila* (Davis et al., 1998; DiAntonio et al., 1999; Petersen et al., 1997). The *Drosophila* NMJ is a glutamergic synapse that shares many functional and structural similarities with the glutamergic central synapses in vertebrates (Atwood et al., 1993; Schuster et al., 1996). So far, two subtypes of glutamate receptors have been characterized in *Drosophila*, designated DGluRIIA and DGluRIIB (Petersen et al., 1997; Schuster et al., 1991). These receptor subtypes are non-NMDA, but, despite their homology to AMPA and kainate receptors, they have not yet been classified as either. When the activity of postsynaptic glutamate receptors is reduced, either by the genetic removal of one of the glutamate receptor subunits (DGluRIIA) (Petersen et al., 1997) or by transgenic expression of a constitutively active PKA catalytic subunit in muscles (Davis et al., 1998), the amplitude of miniature excitatory postsynaptic potential (mEPSP, quantal size) is significantly decreased. One would predict that muscle depolarization upon stimulation of the presynaptic nerve would also be reduced accordingly. However, muscle depolarization upon nerve stimulation (defined by the amplitude of excitatory postsynaptic potential [EPSP]) in these animals remains at wild-type levels, indicating a significant increase in presynaptic neurotransmitter release (quantal content). This increase in quantal content is caused by a retrograde signal of unknown nature from the muscle to the motor neuron.

Since changes in the postsynaptic activity of glutamate receptors (i.e., in *DGluRIIA*$^{-/-}$ mutants) are accompanied by a drastic decrease in the single-channel mean open time (DiAntonio et al., 1999) and glutamate receptors are highly $Ca^{2+}$ permeable (Chang et al., 1994), these changes can alter $Ca^{2+}$ influx into the muscle per evoked or spontaneous release. Therefore, it is conceivable that changes in $Ca^{2+}$ levels in the postsynaptic muscle might be responsible for the induction of this retrograde signal. $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) is an attractive candidate to monitor synaptic levels of $Ca^{2+}$ (Lisman et al., 2002; Soderling et al., 2001); CaMKII has been shown to respond to $Ca^{2+}$ signals and modulate a wide range of target proteins in the nervous system, many of which are thought to be involved in mechanisms associated with learning and memory

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(Rongo, 2002; Soderling et al., 2001). Depending on the magnitude and the frequency of $Ca^{2+}$ signals, initial activation of CaMKII can be followed by autophosphorylation of a threonine residue at the position 286 (Thr 287 in *Drosophila*) leading to persistent kinase activity (Lisman et al., 2002). When several CaMKII subunits are phosphorylated, kinase activity can then persist even after $Ca^{2+}$ levels have fallen to baseline levels. Because of this unique response, CaMKII can act as a frequency detector of calcium oscillations and a potential sensor for synaptic activity (De Koninck and Schulman, 1998; Lisman et al., 2002).

How CaMKII transduces synaptic activity has been the subject of extensive studies in vertebrates. For example, in CA1 hippocampal synapses, the NMDA receptor-dependent increase in intracellular $Ca^{2+}$ (Malenka and Nicoll, 1999) leads to activation of CaMKII, which in turn causes an enhancement of AMPA receptor current or membrane trafficking, leading to induction of long-term potentiation (Barria et al., 1997; Derkach et al., 1999). Similarly, CaMKII controls both physiological and morphological maturation of tectal synapses in the *Xenopus* retinotectal system (Cline, 2001; Wu et al., 1996). Postsynaptic activation of CaMKII in response to NMDA-dependent $Ca^{2+}$ influx leads to enhancement of AMPA currents in these synapses (Wu et al., 1996). CaMKII is also highly concentrated at the NMJ in *Drosophila* and has been implicated in synaptic protein localization, synaptic structure, and function and behavioral plasticity (Griffith et al., 1993; Jin et al., 1998; Kazama et al., 2003; Koh et al., 1999; Wang et al., 1994).

We hypothesized that postsynaptic CaMKII at the *Drosophila* NMJ could regulate the retrograde control of neurotransmitter release. To test this hypothesis, we have taken advantage of the molecular and genetic tools available in *Drosophila* to examine the role of postsynaptic activity of CaMKII in controlling the retrograde signal from the muscle to the motor neuron. We show here that inhibition of CaMKII only in postsynaptic muscles is sufficient to trigger a retrograde signal to motor neurons, leading to an increase in quantal content without affecting the kinetics of mEPSPs or EPSPs. We further demonstrate that constitutive activation of CaMKII in muscles can inhibit the retrograde signal in glutamate receptor mutant larvae and lower quantal content. While the number of boutons per muscle surface area appears unchanged, ultrastructural analysis of synaptic boutons indicates a significant increase in the number of electron-dense T bars at presynaptic active zones in response to inhibition of CaMKII in muscles. Consistent with this observation, we show that the number of T bars per active zone is almost doubled in glutamate receptor mutants; this increase is significantly suppressed by postsynaptic activation of CaMKII in these mutants. Finally, we demonstrate that presynaptic bone morphogenic protein (BMP) signaling is required for the retrograde increase in quantal content in response to postsynaptic CaMKII inhibition as well as the enhancement of quantal content in glutamate receptor mutants. Our results identify CaMKII as a key regulator of retrograde signaling and homeostasis of neurotransmission at the NMJ of *Drosophila*.

## Results

### Inhibition of CaMKII in Muscles Causes an Increase in Quantal Content

In order to examine the role of postsynaptic activity of CaMKII, we first examined the effect of transgenic muscle expression of either a CaMKII inhibitory peptide (Ala) or a constitutively active form of CaMKII ($CaMKII^{T287D}$) (Griffith et al., 1993; Koh et al., 1999; Wang et al., 1994) on neurotransmitter release. Expression of either transgene did not affect the size of mEPSPs (wild-type, 0.86mV $\pm$ 0.04mV; Mef2-Gal4 $\times$ UAS-Ala, 0.85mV $\pm$ 0.03mV; Mef2-Gal4 $\times$ UAS-$CaMKII^{T287D}$, 0.85mV $\pm$ 0.03mV) (Figures 1A and 1B). However, EPSP amplitudes recorded from muscles expressing Ala (Mef2-Gal4 $\times$ UAS-Ala, 34.70mV $\pm$ 1.63mV, $n = 12$) were 20% larger ($p < 0.001$) than those recorded from wild-type muscles (29.16mV $\pm$ 1.08mV, $n = 20$), resulting in a 20% increase ($p < 0.005$) in quantal content, from 34.15 $\pm$ 1.22 to 41.18 $\pm$ 1.95 (Figure 1B). When corrected for nonlinear summation (Martin, 1955), the increase in quantal content was 28% in larvae expressing Ala in postsynaptic muscles when compared to wild-type. These results are consistent with the observation by Wang et al. (1994) that ubiquitous heat-shock induction of Ala can enhance synaptic activity at the *Drosophila* NMJ. In contrast, EPSPs recorded from muscles expressing constitutively active $CaMKII^{T287D}$ (Mef2-Gal4 $\times$ UAS-$CaMKII^{T287D}$, 28.03mV $\pm$ 1.14mV, $n = 18$) were not significantly different from wild-type (Figures 1A and 1B). We also examined the effect of presynaptic expression of Ala, using *elav*-Gal4 on quantal size and quantal content; quantal size and quantal content were not statistically different from wild-type (Figure 1B).

CaMKII has been shown to phosphorylate and enhance the activity of AMPA receptors during long-term potentiation (Barria et al., 1997; Derkach et al., 1999). If CaMKII exerted similar effects on glutamate receptors in *Drosophila*, one would predict an increase in the postsynaptic response to glutamate release as a result of increased CaMKII activity. Instead, as described above, we observed an increase in EPSP size upon inhibition of CaMKII and no change in mEPSP size. We further examined whether CaMKII activity has any direct effect on the kinetics of postsynaptic glutamate receptors. We analyzed the kinetics of mEPSPs by measuring the duration of mEPSPs at half-maximal amplitude (see Experimental Procedures). This index was not significantly different in larvae expressing Ala in muscles compared to other genotypes (wild-type, 28.72 $\pm$ 0.95 ms; Mef2-Gal4 $\times$ UAS-Ala, 27.95 $\pm$ 0.59 ms; *elav*-Gal4 $\times$ UAS-Ala, 28.95 $\pm$ 1.38 ms; Mef2-Gal4 $\times$ UAS-$CaMKII^{T287D}$, 27.68 $\pm$ 0.85 ms). Similarly, the decay time constants for mEPSPs and EPSPs were not significantly different among genotypes. Together with the observation that mEPSP amplitude is not affected by changing the postsynaptic activity of CaMKII, these results indicate that the change in EPSP amplitude caused by postsynaptic expression of Ala cannot be due to a direct effect on glutamate receptor kinetic properties.

Although the Ala peptide is commonly used as an inhibitor of CaMKII, we wished to confirm our data using another inhibitor of CaMKII (CaMKIINtide) that has been shown to specifically inhibit CaMKII from *Drosophila*

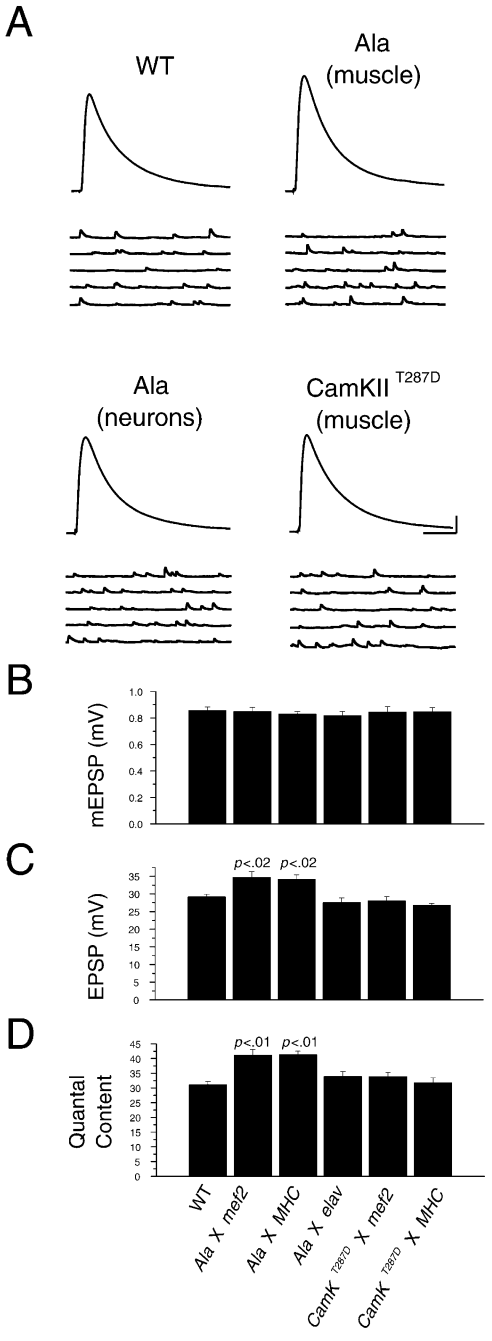


Figure 1. Muscle Expression of a CaMKII Inhibitory Peptide Enhances Presynaptic Neurotransmitter Release

(A) Representative traces of evoked and spontaneous potentials from wild-type, UAS-Ala × Mef2-Gal4, UAS-Ala × *elav*-Gal4, and UAS-CaMKII$^{T287D}$ × Mef2-Gal4. The upper trace in each panel shows an average of ten consecutive EPSPs (at 0.5 Hz) for each genotype. The lower traces in each panel show continuous recordings of mEPSPs in the absence of stimulation. Calibration: 5mV/40 ms in upper traces; 5mV/400 ms in lower traces. (B–D) Bar graph representations of mean ± SEM for mEPSP amplitude (B), EPSP amplitude (C), and quantal content (D) for the indicated genotypes. All recordings were performed at 0.6 mM $Ca^{2+}$. Muscle expression of Ala increases EPSP amplitude and quantal content. Where there is statistical significance, the p value is indicated.

brain extracts (Chang et al., 2001). CaMKIINtide is a 28 amino acid peptide corresponding to the inhibitory region of CaMKIIN$\alpha$, an endogenous CaMKII inhibitor in vertebrates (Chang et al., 2001). Our search using the published *Drosophila* genome did not identify a candidate homolog of CaMKIIN. We generated transgenic flies carrying one or two copies of CaMKIINtide (see Experimental Procedures). Expression of CaMKIINtide in either muscles or neurons did not affect the amplitude of mEPSPs (control, 0.89mV ± 0.03mV, n = 16; MHC-Gal4 × UAS-CaMKIINtide, 0.89mV ± 0.07mV, n = 10; *elav*-Gal4 × UAS-CaMKIINtide, 0.88mV ± 0.05mV, n = 10) (Figures 2A and 2B). Expression of one copy of CaMKIINtide in muscles caused an increase in EPSPs; however, this increase did not show statistical significance, based on the p value of less than 0.05 (wild-type, 16.28mV ± 0.9mV; MHC-Gal4 × UAS-CaMKIINtide, 20.74mV ± 2.67mV, p = 0.067). Quantal content, on the other hand, was significantly greater that wild-type (wild-type, 18.44 ± 1.18; MHC-Gal4 × UAS-CaMKIINtide, 23.17 ± 2.49, $p < 0.05$). Expressing two copies of CaMKIINtide in muscles further increased the amplitude of EPSPs and quantal content without affecting the size of mEPSPs (mEPSP, 0.83mV ± 0.05mV; EPSP, 22.58mV ± 2.09mV, $p < 0.01$; quantal content, 27.13 ± 2.47, n = 10, $p < 0.001$). We also examined the frequency of mEPSPs in all different genetic combinations in Figure 2B; only, when we expressed two copies of CaMKIINtide, we observed a significant increase in this measure compared with wild-type (wild-type, 2.42 ± 0.21 Hz; MHC-Gal4 × UAS-CaMKIINtide, 3.36 ± 0.31 Hz, $p < 0.02$) (Figure 2B). According to our measurements, both membrane potential and input resistance of muscles expressing either inhibitory CaMKIINtide, Ala, or constitutively active CaMKII$^{T287D}$ were indistinguishable from control muscles. And we did not observe any spontaneous discharges following nerve stimulation, such as those seen with heat-shock Ala (Griffith et al., 1994). These results suggest that membrane properties, including muscle excitability, could not have been significantly affected. To lower the effect of nonlinear summation that is seen at high concentrations of $Ca^{2+}$, we repeated our quantal analysis of expression of Ala and CaMKII$^{T287D}$ at lower concentrations of $Ca^{2+}$ (Figure 2B). These results were consistent with those described above. None of the genetic combinations caused a change in the size of mEPSPs (Figure 2B). Only expression of Ala in muscles caused a significant increase in both EPSP amplitude and quantal content without affecting mEPSP amplitude (Figure 2B).

To further verify the effectiveness of Ala in inhibiting CaMKII, we simultaneously coexpressed UAS-Ala and UAS-CaMKII$^{T287D}$ in muscles with MHC-Gal4 driver. Coexpression of CaMKII$^{T287D}$ together with Ala suppressed the increase in quantal content seen when Ala was expressed alone (wild-type, 18.44 ± 1.18, n = 16; MHC-Gal4 × UAS-Ala, 22.50 ± 0.98, $p < 0.01$, n = 12; MHC-Gal4 × UAS-Ala;UAS-CaMKII$^{T287D}$, 17.95 ± 1.95, n = 10) (Figure 2B), further showing specificity for the action of Ala in muscles. We also performed failure analysis as an alternative way to measure quantal content (Petersen et al., 1997). Expression of both Ala and CaMKIINtide in muscles caused a significant increase in quantal content based on the natural logarithm of the

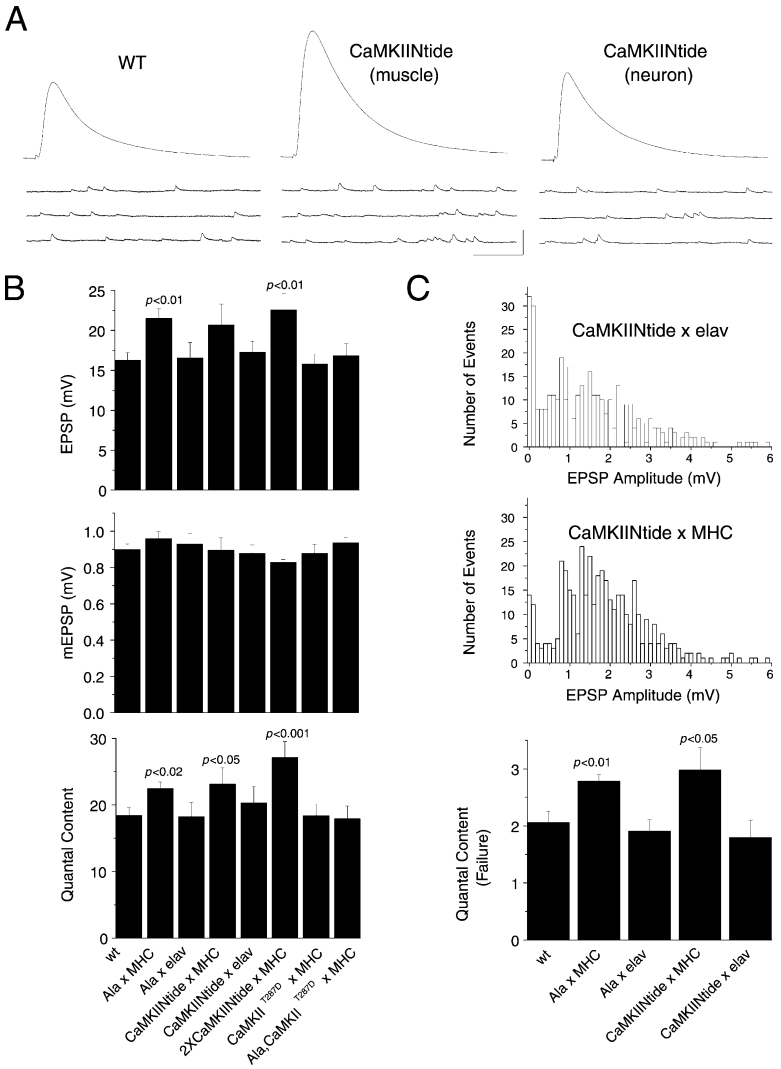


Figure 2. Muscle Expression of CaMKIINtide, a CaMKII Inhibitory Peptide, Enhances Presynaptic Neurotransmitter Release

(A) Representative traces of evoked and spontaneous potentials from wild-type, UAS-CaMKIINtide × MHC-Gal4, and UAS-CaMKIINtide × *elav*-Gal4. The upper trace in each panel shows an average of seven consecutive EPSPs (at 0.5 Hz) for each genotype. The lower traces in each panel show continuous recordings of mEPSPs in the absence of stimulation. Calibration: 5mV/40 ms in upper traces; 5mV/400 ms in lower traces.

(B) Bar graph representations of mean ± SEM values for mEPSP amplitude, EPSP amplitude, and quantal content for the indicated genotypes. All recordings were performed at 0.4 mM $Ca^{2+}$. Muscle expression of Ala increases EPSP amplitude and quantal content.

(C) The upper two panels show frequency histograms of 400 evoked responses recorded at 0.25 mM $Ca^{2+}$ for the indicated genotypes. The lower panel is a bar graph representation of quantal content measurements for the indicated genotypes. Quantal content was calculated as the natural logarithm of the number of events divided by the number of failures. Where there is statistical significance, the p value is indicated.

number of failures divided by the number of trials (wild-type, 2.06 ± 0.20, n = 8; MHC-Gal4 × UAS-Ala, 2.80 ± 0.11, n = 8, $p < 0.006$; *elav*-Gal4 × UAS-Ala, 1.91 ± 0.11, n = 6; MHC-Gal4 × UAS-CaMKIINtide, 2.98 ± 0.40, n = 8, $p < 0.05$; *elav*-Gal4 × UAS-CaMKIINtide, 1.86 ± 0.30, n = 6) (Figure 2C). These results further indicate that inhibition of CaMKII in postsynaptic muscles is sufficient to increase neurotransmitter release in a retrograde manner.

### Constitutive Activation of CaMKII in Muscles Causes a Decrease in Quantal Content

Our results indicated that inhibiting the postsynaptic activity of CaMKII was sufficient to trigger a retrograde signal from the muscle to the motor neuron, causing an increase in neurotransmitter release. If the retrograde signal present in *DGluRIIA* mutants (*DGluRIIA*$^{-/-}$) is also regulated by the postsynaptic CaMKII activity, then increasing the postsynaptic activity of CaMKII in these mutants should lead to a decrease in neurotransmitter release. *DGluRIIA*$^{-/-}$ mutants show a significant reduction in quantal size but have EPSPs similar to those of wild-type larvae, accompanied by a significant increase in quantal content (Petersen et al., 1997) (Figure 3A). Postsynaptic expression of constitutively active CaMKII$^{T287D}$ in *DGluRIIA*$^{-/-}$ mutants caused a 33% decrease ($p < 0.001$) in the EPSP amplitude (*DGluRIIA*$^{-/-}$, 29.47mV ± 1.88mV, n = 16; *DGluRIIA*$^{-/-}$/UAS-CaMKII$^{T238D}$ × Mef2-Gal4, 20.54mV ± 1.51mV, n = 22) without affecting quantal size (Figures 3A–3C). This resulted in a significant decrease ($p < 0.001$) in quantal content (*DGluRIIA*$^{-/-}$, 88.46 ± 6.89, n = 16; *DGluRIIA*$^{-/-}$/UAS-CaMKII$^{T287D}$ × Mef2-Gal4, 58.40 ± 4.87, n = 22) (Figure 3D). When we corrected for nonlinear summation, the decrease in quantal content as a result of muscle expression of CaMKII$^{T287D}$ in *DGluRIIA* mutants was 57%.

To further verify these key results, we repeated these measurements at lower concentrations of $Ca^{2+}$ using a second muscle Gal-4 driver, MHC-Gal4. We found that the results were consistent with those described above (Figures 3B and 3C). At 0.4 mM extracellular $Ca^{2+}$, expression of CaMKII$^{T287D}$ caused a significant decrease ($p < 0.001$) in the amplitude of EPSPs and quantal content in *DGluRIIA*$^{-/-}$ mutant larvae (EPSP: *DGluRIIA*$^{-/-}$, 14.30mV ± 1.07mV, n = 12; *DGluRIIA*$^{-/-}$/UAS-

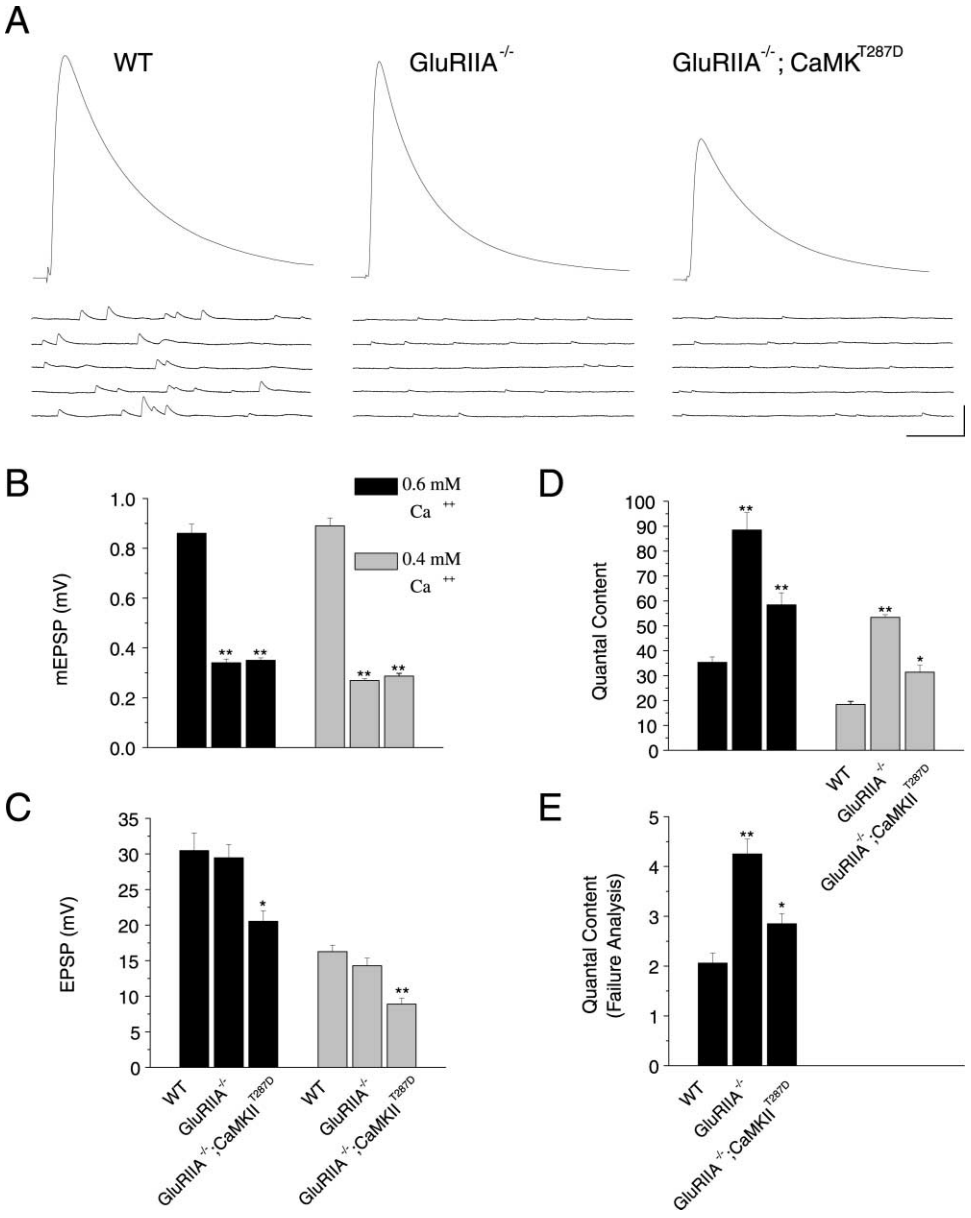


Figure 3. Muscle Expression of Constitutively Active CaMKII$^{T287D}$ Inhibits the Retrograde Signal and Synaptic Transmission

(A) Representative traces of evoked EPSPs and spontaneous mEPSPs from the indicated genotypes at 0.6 mm $Ca^{2+}$. *DGluRIIA*$^{-/-}$ mutants have much smaller mEPSPs but similar EPSPs compared to those of wild-type. The EPSP amplitude is significantly reduced when CaMKII$^{T287D}$ is expressed postsynaptically in these mutants. Calibration: 5mV/40 ms in upper traces; 5mV/400 ms in lower traces. Bar graph representations of mean ± SEM values for mEPSP amplitude (B), EPSP amplitude (C), and quantal content (D) for the indicated genotypes. Black bar graphs correspond to measurement at 0.6 mM $Ca^{2+}$, and gray bar graphs correspond to measurement at 0.4 mM $Ca^{2+}$. (E) shows quantal content measurements through failure analysis performed at 0.25 mM $Ca^{2+}$. Statistical significance is indicated as follows: *$p < 0.01$ and **$p < 0.001$.

CaMKII$^{T287D}$ × MHC-Gal4, 8.9mV ± 0.86mV, n = 12; quantal content: *DGluRIIA*$^{-/-}$, 53.39 ± 3.99; *DGluRIIA*$^{-/-}$/UAS-CaMKII$^{T287D}$ × MHC-Gal4, 31.39 ± 2.78 compared to wild-type, 18.44 ± 1.18, n = 16). We also performed failure analysis on these genetic combinations as an alternative way to measure quantal content (Figure 3E). Quantal content was significantly smaller when CaMKII$^{T287D}$ was expressed postsynaptically in *GluRIIA* mutants (wild-type, 2.06 ± 0.20, n = 8; *DGluRIIA*$^{-/-}$, 4.56 ± 0.28, n = 10; *DGluRIIA*$^{-/-}$/UAS-CaMKII$^{T287D}$ × MHC-Gal4, 2.85 ± 0.18, n = 10, $p < 0.001$). These results indicate that increasing the activity of CaMKII in muscles can oppose the retrograde signal and significantly reduce neurotransmitter release once the retrograde signal is triggered by the genetic removal of DGluRIIA.

To test the role of CaMKII in controlling the retrograde signal further, we examined whether CaMKII could modulate the retrograde signal triggered upon the expression of a dominant-negative DGluRIIA transgene, DGluRIIA$^{M/R}$, where a methionine (M) residue in the pore has been replaced with an arginine (R) (DiAntonio et al., 1999). Postsynaptic expression of DGluRIIA$^{M/R}$ causes a

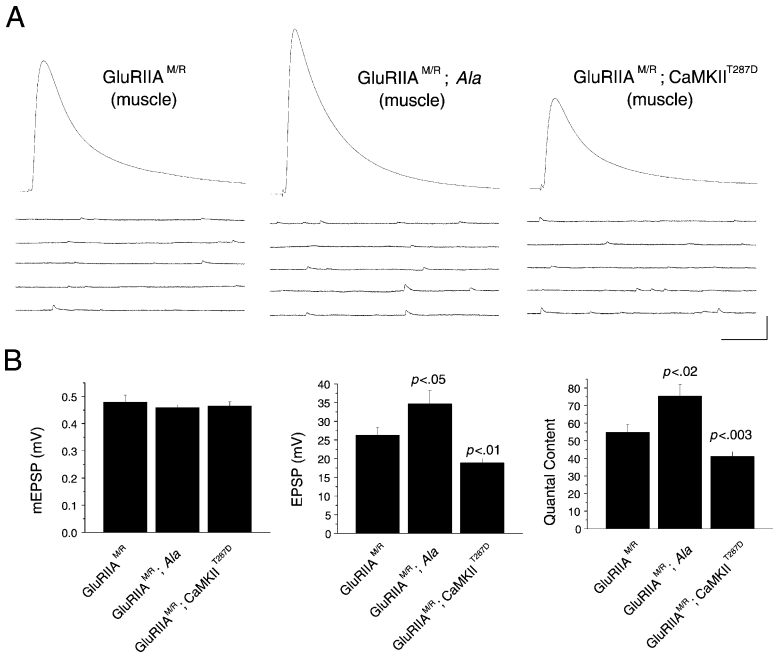


Figure 4. Postsynaptic CaMKII Modulates the Strength of the Retrograde Signal and Neurotransmitter Release

(A) Representative traces of evoked EPSPs and spontaneous mEPSPs from UAS-DGluRIIA$^{M/R}$ × C142-Gal4; UAS-DGluRIIA$^{M/R}$; UAS-Ala × C142-Gal4, and UAS-DGluRIIA$^{M/R}$; UAS-CaMKII$^{T287D}$ × C142-GAL4. Muscle expression of a DGluRIIA$^{M/R}$ reduces the amplitude of mEPSPs but does not affect the EPSP amplitude compared with wild-type (see Figure 1A). Inhibition of CaMKII in muscles in the presence of DGluRIIA$^{M/R}$ leads to an increase in the EPSP amplitude, while activation of CaMKII in muscles causes a decrease in the EPSP amplitude. Calibration: 5mV/40 ms in upper traces; 5mV/400 ms in lower traces. (B) Bar graph representation of mean ± SEM values for mEPSP amplitude, EPSP amplitude, and quantal content for the indicated genotypes. In all three genotypes, the amplitude of mEPSPs remains unchanged. Where there is statistical significance, the p value is indicated. All measurements were performed at 0.6 mM $Ca^{2+}$.

decrease in quantal size (UAS-DGluRIIA$^{M/R}$, 0.88mV ± 0.04mV, n = 8; UAS-DGluRIIA$^{M/R}$ × C142-Gal4, 0.48mV ± 0.08mV, n = 8), while increasing quantal content (UAS-DGluRIIA$^{M/R}$, 30.56 ± 1.24; UAS-DGluRIIA$^{M/R}$ × C142-Gal4, 54.90 ± 4.37, $p < 0.01$) (Figures 4A and 4B). Expression of CaMKII$^{T287D}$ together with DGluRIIA$^{M/R}$ in postsynaptic muscles caused more than a 25% decrease ($p < 0.01$) (55% decrease when corrected for nonlinear summation) in quantal content as compared with muscles expressing only DGluRIIA$^{M/R}$ (UAS-DGluRIIA$^{M/R}$ × C142-Gal4, 54.90 ± 4.37, n = 8; UAS-DGluRIIA$^{M/R}$;UAS-CaMKII$^{T238D}$ × C142-Gal4, 41.11 ± 2.68, n = 10) (Figures 4A and 4B). This decrease was due to a significant reduction ($p < 0.01$) in the amplitude of EPSPs (UAS-DGluRIIA$^{M/R}$ × C142-Gal4, 26.34mV ± 2.00mV, n = 8; UAS-DGluRIIA$^{M/R}$;UAS-CaMKII$^{T238D}$ × C142-Gal4, 18.91mV ± 1.07mV, n = 10) (Figures 4A and 4B). Expression of CaMKII$^{T287D}$ in muscles did not affect the amplitude of mEPSPs (UAS-DGluRIIA$^{M/R}$ × C142-Gal4, 0.48mV ± 0.08mV, n = 8; UAS-DGluRIIA$^{M/R}$;UAS-CaMKII$^{T238D}$ × C142-Gal4, 0.46mV ± 0.02mV, n = 10). In contrast, when Ala was expressed in muscles together with DGluRIIA$^{M/R}$, quantal content was increased more than 35% ($p < 0.01$) compared to DGluRIIA$^{M/R}$ expression alone, without affecting quantal size (Figures 4A and 4B). These results indicate that the postsynaptic activity of CaMKII plays a key role in regulating the strength of the retrograde signal triggered by the reduction of glutamate receptor activity.

### Postsynaptic Inhibition of CaMKII Does Not Affect the Number of Synaptic Boutons but Increases the Number of T Bars per Active Zone

To assess the effect of activation or inhibition of CaMKII on synaptic structure, we examined NMJs by light and electron microscopy. Heat-shock induction of Ala has been reported to alter nerve terminal projections at the *Drosophila* NMJ (Wang et al., 1994). We quantified the number of boutons per muscle surface area in larvae expressing Ala or CaMKII$^{T287D}$ either pre- or postsynaptically (Figure 5) and found no significant differences compared to wild-type ($10^{-3}/\mu m^2$): MHC-Gal4 × UAS-Ala, 1.82 ± 0.08, n = 28; *elav*-Gal4 × UAS-Ala, 1.72 ± 0.08, n = 17; UAS-CaMKII$^{T287D}$ × MHC-Gal4, 2.01 ± 0.07 (n = 22); and UAS-CaMKII$^{T287D}$ × *elav*-Gal4, 2.02 ± 0.12 (n = 21); compared to UAS-Ala × UAS-CaMKII$^{T287D}$, 1.94 ± 0.08 (n = 30). The number of boutons were not significantly different between control larvae and other genetic combinations: MHC-Gal4 × UAS-Ala, 114.6 ± 4.6 (n = 28); *elav*-Gal4 × UAS-Ala, 115.7 ± 3.49 (n = 17); UAS-CaMKII$^{T287D}$ × MHC-Gal4, 123.1 ± 3.14 (n = 22); and UAS-CaMKII$^{T287D}$ × *elav*-Gal4, 115.2 ± 4.4 (n = 21); compared to UAS-Ala × UAS-CaMKII$^{T287D}$, 125.3 ± 4.08 (n = 30).

We also examined the expression of glutamate receptors in muscles expressing either Ala or CaMKII$^{T287D}$; we did not observe any qualitative differences in the expression or localization of glutamate receptors in these larvae compared to wild-type (data not shown). Moreover, inhibition or activation of CaMKII in muscles did not have any effect on localization of a CD8-GFP-Shaker chimeric protein (Zito et al., 1999) that we use routinely to visualize synapses, suggesting the lack of any significant influence on the ability of Shaker K channels to localize at the synapse (data not shown).

We also examined the effect of pre- or postsynaptic inhibition of CaMKII in type Ib boutons on muscles 6 and 7 by electron microscopy. Expression of Ala either pre- or postsynaptically did not affect the overall ultrastructure of boutons (wild-type, n = 18; MHC-Gal4 × UAS-Ala, n = 6; *elav*-Gal4 × UAS-Ala, n = 4) (Figures 6A–6C). The subsynaptic reticulum (SSR) structure and the number of clear synaptic vesicles appeared to be qualitatively wild-type. Boutons in larvae expressing Ala presynaptically displayed an unusual invagination of the presynaptic membrane at active zones (Figure 6C) that

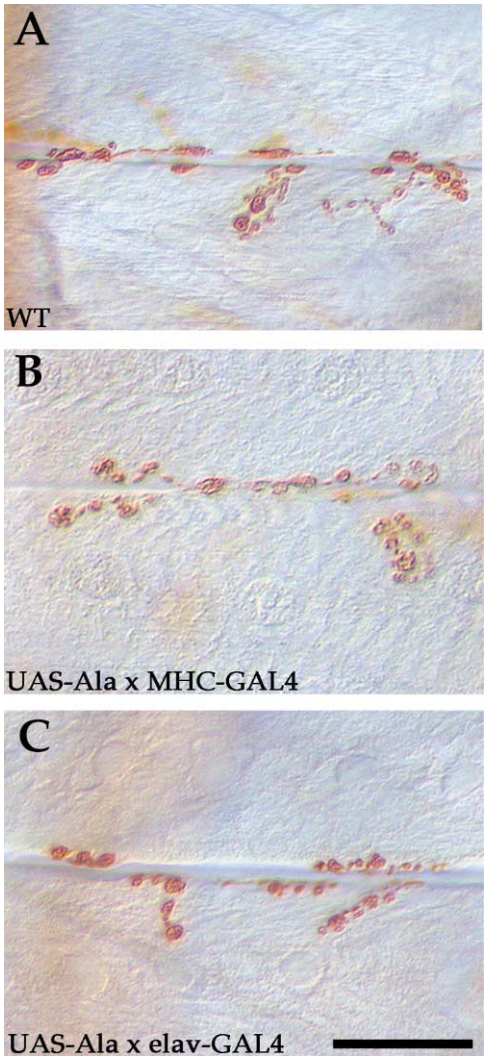


Figure 5. Postsynaptic Inhibition of CaMKII Does Not Alter the Number of Boutons

(A–C) Photographs of NMJ synapses on muscles 6 and 7 in segment A3 of third instar larvae stained with anti-synaptotagmin for the indicated genotypes. The number of boutons and muscle surface area were not significantly different when comparing each genotype with the control. Scale bar, 50 μm.

was not seen in wild-type larvae or larvae expressing Ala postsynaptically. More importantly, our morphometric analysis revealed a 60% increase in the number of T bars per active zone ($p < 0.001$) (Figure 6F) as a result of postsynaptic expression of Ala (MHC-Gal4 × UAS-Ala, 1.03 ± 0.07; *elav*-Gal4 × UAS-Ala, 0.68 ± 0.08; compared to wild-type, 0.61 ± 0.03). T bars are electron-dense structures at the active zone where synaptic vesicles cluster (Atwood et al., 1993; Jia et al., 1993; Figures 5A and 5B). Since T bars have been suggested to play a role in synaptic transmission (Prokop, 1999; Atwood and Wojtowicz, 1999), these results suggested to us that the number of T bars could correlate with the observed retrograde increase in quantal release. If this hypothesis is true, then *DGluRIIA*$^{-/-}$ mutants should have a higher number of T bars per active zone. To test this hypothesis, we examined boutons of *DGluRIIA*$^{-/-}$ third instar larvae at the ultrastructural level. Indeed, *DGluRIIA*$^{-/-}$ larvae showed a major increase in the number of T bars per active zone without any gross changes in the overall ultrastructure (Figure 6D) (*DGluRIIA*$^{-/-}$, 1.26 ± 0.10, n = 7 boutons, $p < 0.001$). To complete our analysis of the relationship between quantal content and the number of T bars per active zone, we examined the effect of postsynaptic activation of CaMKII in *DGluRIIA*$^{-/-}$ mutants. Consistent with our hypothesis, expression of CaMKII$^{T287D}$ in *DGluRIIA*$^{-/-}$ mutants caused a significant decrease in the number of T bars per active zone (Figure 6E) (*DGluRIIA*$^{-/-}$/UAS-CaMKII$^{T287D}$ × MHC-Gal4, 0.76 ± 0.05, n = 8 boutons, $p < 0.001$). The correlation between quantal content and the number of T bars per active zone, based on our results, suggests that T bars may be involved in promoting vesicular release at active zones.

### Bone Morphogenic Protein Signaling Is Required for the Retrograde Signaling Pathway to Function

We have recently demonstrated that mutations in *wishful thinking* (*wit*), a presynaptic BMP type II receptor, lead to strong defects in synaptic structure, neurotransmitter release, and synaptic ultrastructure (Aberle et al., 2002). In light of these defects, we tested whether the retrograde signal that controls neurotransmitter release at the NMJ of *Drosophila* can function in *wit* mutants. First, we attempted to make double mutants of *wit* and *GluRIIA*$^{-/-}$. We could not produce any viable progenies of the double-mutant genotype; the lethality stage was earlier than third instar larval level. This suggested a possible role for *wit* in the retrograde signaling pathway.

We then tested whether postsynaptic expression of a dominant-negative GluRIIA (DGluRIIA$^{M/R}$) transgene would cause the same enhancement in quantal content in *wit* mutants as it does in wild-type. Postsynaptic expression of DGluRIIA$^{M/R}$ caused a decrease in the amplitude of mEPSP similar to when it is expressed in wild-type (0.41mV ± 0.03mV for *wit*$^{HA4}$;C142-Gal4 × *wit*$^{HA5}$;UAS-GluRIIA$^{M/R}$, n = 10, compared to 0.43mV ± 0.03mV for UAS-DGluRIIA$^{M/R}$ × C142-Gal4, n = 6; see also Figure 4). Postsynaptic expression of DGluRIIA$^{M/R}$ failed to cause any significant increase in quantal content in *wit* mutants (4.16 ± 0.44 for *wit*$^{HA4}$/*wit*$^{HA5}$ compared to 3.92 ± 0.49 for *wit*$^{HA4}$;C142-Gal4 × *wit*$^{HA5}$;UAS-GluRIIA$^{M/R}$) (Figures 7A and 7B), while it caused a 100% increase in quantal content when expressed in wild-type (31.02 ± 2.44 for wild-type, n = 8, compared to 60.62 ± 5.49 for UAS-DGluRIIA$^{M/R}$ × C142-Gal4, n = 6) (see also Figure 4). We further tested whether the enhancement of quantal content in response to inhibition of CaMKII could be reproduced in *wit* mutants. Inhibition of CaMKII in *wit* mutants did not cause an increase in the EPSP amplitude (2.77mV ± 0.29mV for *wit*$^{HA4}$/*wit*$^{HA5}$, n = 10, compared to 2.52mV ± 0.25mV for *wit*$^{HA4}$;G14 × *wit*$^{HA5}$;UAS-Ala, n = 10) or quantal content (4.16 ± 0.44 for *wit*$^{HA4}$/*wit*$^{HA5}$ compared to 3.79 ± 0.39 for *wit*$^{HA4}$;G14 × *wit*$^{HA5}$;UAS-Ala). This is in contrast to the effect of CaMKII inhibition in wild-type, which leads to a significant increase in EPSP size and quantal content (Figures 1 and 3). Importantly, the size and the kinetics of mEPSPs were not affected as a consequence of inhibiting CaMKII in muscles (0.68mV ± 0.04mV for

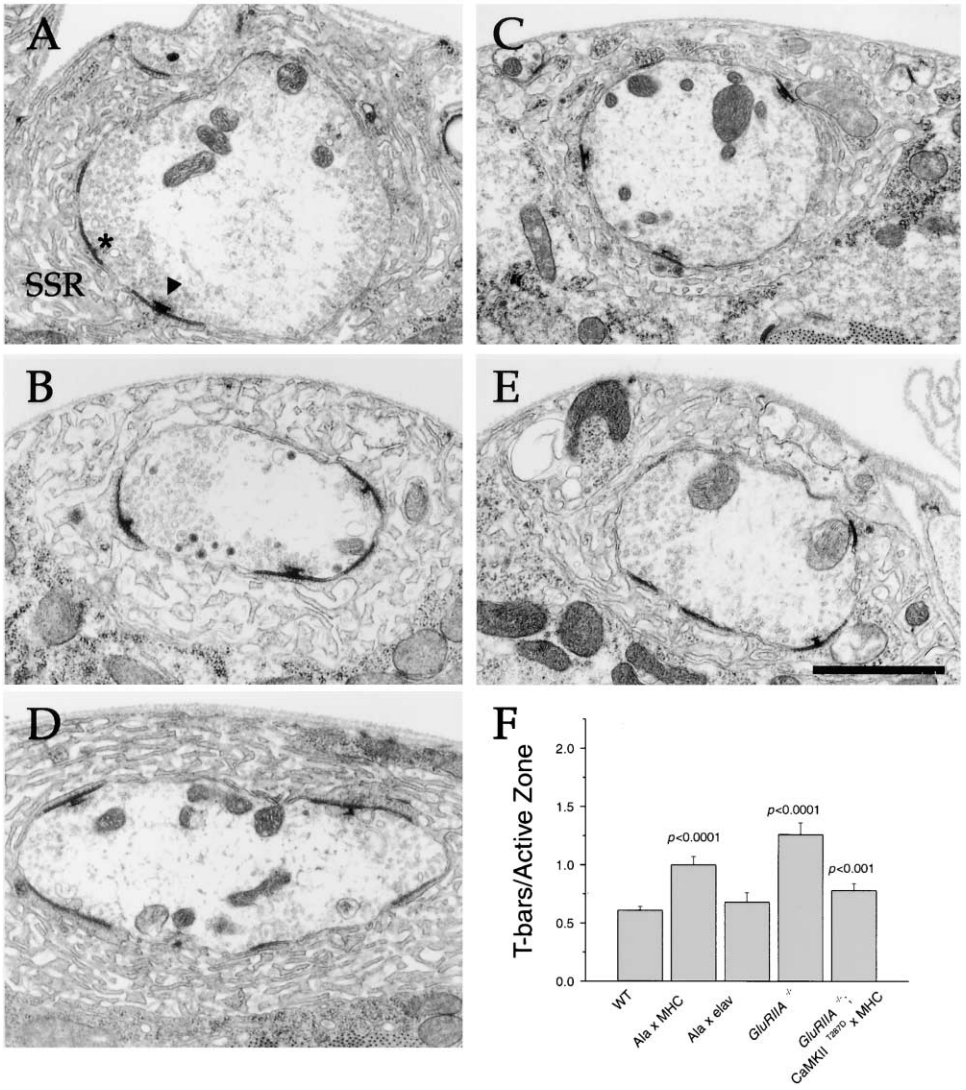


Figure 6. Postsynaptic Inhibition of CaMKII Does Not Alter the Overall Bouton Ultrastructure, but Increases the Number of T Bars per Active Zone

(A)–(E) correspond to electron micrographs of wild-type, UAS-Ala × MHC-Gal4, UAS-Ala × *elav*-Gal4, *DGluRIIA*$^{-/-}$, and *DGluRIIA*$^{-/-}$;CaMKII$^{T287D}$ × MHC-Gal4, respectively. Qualitatively, the overall bouton ultrastructure, including the number of vesicles and the extent of SSR, remain unchanged compared to wild-type; however, the number of T bars per active zone is increased in *DGluRIIA*$^{-/-}$ and UAS-Ala × MHC-Gal4. This increase is suppressed when CaMKII$^{T287D}$ is overexpressed in *DGluRIIA*$^{-/-}$ mutants postsynaptically. Scale bar is 1 μm for (A), (D), and (E); 1.1 μm for (B); and 1.2 μm for (C). (F) Graph shows the quantification of the number T bars per active zone are based on 239, 71, 42, 85, and 86 active zones from complete bouton series corresponding to the genetic combinations in (A), (B), (C), (D), and (E), respectively.

*wit*$^{HA4}$/*wit*$^{HA5}$ compared to 0.67mV ± 0.02mV for *wit*$^{HA4}$;G14 × *wit*$^{HA5}$;UAS-Ala). These results indicate an important role for *wit* in the retrograde signaling pathway.

## Discussion

We have previously demonstrated that reducing the function of postsynaptic glutamate receptors at the neuromuscular junction (NMJ) of *Drosophila* triggers a retrograde signal from the postsynaptic muscle to the presynaptic motor neuron, leading to an increase in the amount of neurotransmitter release (Davis et al., 1998; Petersen et al., 1997). Here, we show that this retrograde signal is regulated by the postsynaptic activity of CaMKII. Reducing postsynaptic CaMKII activity by expressing a CaMKII inhibitory peptide in somatic muscles increases quantal content, mimicking the effect of reducing postsynaptic glutamate receptor activity. Furthermore, in glutamate receptor *DGluRIIA*$^{-/-}$ mutants, constitutive activation of CaMKII in muscles inhibits the retrograde signal and decreases quantal content. These changes in retrograde signaling and neurotransmitter release are not accompanied by any significant changes in the number of synaptic boutons per muscle surface area or any gross structural or ultrastructural alterations. However,

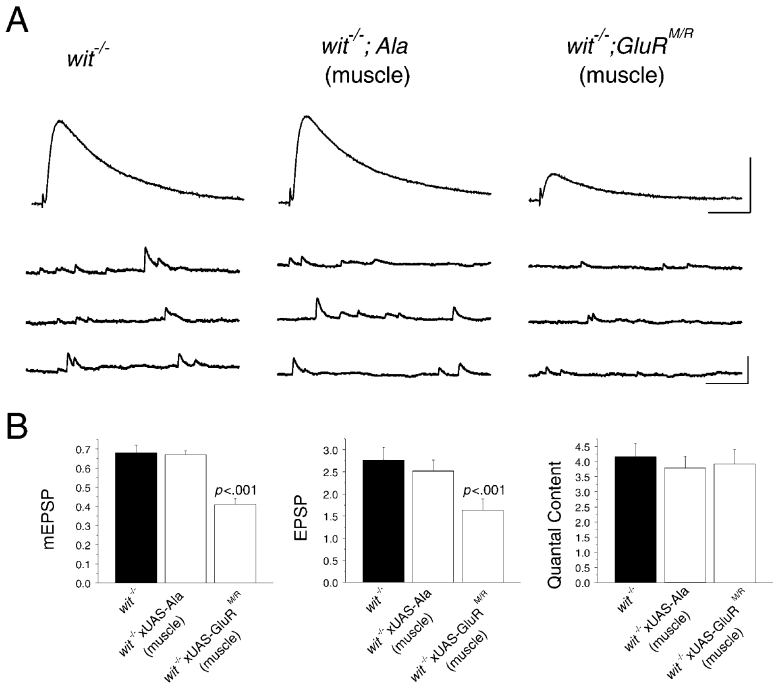


Figure 7. BMP Signaling through Type II Receptor *wit* Is Required for the Function of the Retrograde Signal

(A) Representative traces of evoked and spontaneous potentials from $wit^{HA4}/wit^{HA5}$, $wit^{HA4}$;G14-Gal4 × $wit^{HA5}$;UAS-Ala, and $wit^{HA4}$;C142-Gal4 × $wit^{HA5}$;UAS-GluRII$^{M/R}$. The upper trace in each panel shows an average of ten consecutive EPSPs (at 0.5 Hz) for each genotype. The lower traces in each panel show continuous recordings of mEPSPs in the absence of stimulation. Calibration: 2mV/40 ms in upper traces; 2mV/400 ms in lower traces.

(B) Bar graph representations of mean ± SEM for mEPSP amplitude, EPSP amplitude, and quantal content for the indicated genotypes. All recordings were performed at 0.6 mM $Ca^{2+}$. Postsynaptic inhibition of CaMKII or postsynaptic expression of dominant-negative DGluRIIA$^{M/R}$ do not cause an increase in quantal content, as seen when they are expressed in wild-type (see Figures 1 and 4).

we demonstrate that upon inhibition of CaMKII postsynaptically the number of T bars per active zone in presynaptic boutons is significantly increased. Similarly, we demonstrate that the number of T bars per active zone is doubled in *DGluRIIA*$^{-/-}$ mutant larvae. This increase was suppressed by constitutive activation of CaMKII in postsynaptic muscles in *DGluRIIA*$^{-/-}$ mutants. These results point to CaMKII as a key regulator of the retrograde signal controlling homeostasis of synaptic transmission at the NMJ of *Drosophila*.

### CaMKII Activity Controls Retrograde Signaling

Our findings demonstrate that postsynaptic inhibition of CaMKII activity is sufficient to increase presynaptic neurotransmitter release in a retrograde fashion. We show that this increase in quantal content can be potentiated by expressing additional doses of the inhibitory transgene and suppressed by expressing a constitutively active CaMKII transgene simultaneously. These results suggest a direct involvement of CaMKII in controlling the retrograde signal that maintains the homeostasis of neurotransmitter release at the *Drosophila* NMJ.

While increasing the postsynaptic activity of CaMKII in wild-type larvae has no effect on neurotransmitter release, once the retrograde signal is induced (i.e., in *DGluRIIA*$^{-/-}$ mutants), activation of CaMKII can inhibit the signal. This is consistent with the observation by Petersen et al. (1997) that while removal of DGluRIIA causes a decrease in quantal size and an increase in quantal content, overexpression of DGluRIIA, which leads to an increase in quantal size, does not change quantal content. These results suggest that quantal content may be increased only when CaMKII activity is reduced to a critical threshold. As long as CaMKII activity remains above this critical threshold, quantal content is unchanged. This could be a mechanism through which the synapse can compensate for any reduction in muscle activity and ultimately maintain homeostasis.

In a recent study, Kazama et al. (2003) have provided evidence for the involvement of postsynaptic CaMKII in the retrograde control of neurotransmission at the *Drosophila* NMJ. The authors show that changes in the activity of postsynaptic CaMKII affect both neurotransmitter release and synaptic structure in early first instar larvae. Kazama et al. also report an apparent change in localization of postsynaptic glutamate receptors in response to postsynaptic activation of CaMKII. This is in contrast to our observations; we did not find any changes in overall synaptic structure or localization of glutamate receptors in response to either inhibition or activation of postsynaptic CaMKII. The differences in our findings could be partially due to differences in the level and pattern of expression of transgenes (using different Gal4 lines) or due to the fact that we have examined the NMJ at very different developmental stages. We performed all our experiments in late third instar larvae, while Kazama et al. (2003) examined early first instar larvae. Interestingly, our results are in agreement in that we both observe no changes in the amplitude or kinetics of spontaneous potentials, indicating that CaMKII does not directly modulate glutamate receptors at the *Drosophila* NMJ, which is not the case with vertebrates.

### How Is the Retrograde Signal Triggered?

When the activity of postsynaptic glutamate receptors at the *Drosophila* NMJ (Petersen et al., 1997; Davis et al., 1998) is reduced, a retrograde signal from the muscle to the motor neuron is triggered that causes an increase in quantal content. It has been suggested that this retrograde signal could be triggered in response to changes in muscle depolarization or in response to $Ca^{2+}$ con-

ducted by glutamate receptors (Davis and Bezprozvanny, 2001; Petersen et al., 1997). Our data indicate that postsynaptic activity of CaMKII plays an important role in controlling the signal. Both muscle depolarization and $Ca^{2+}$ flux through glutamate receptors could be involved in changing the levels of intracellular $Ca^{2+}$ and thus that of CaMKII. We favor the idea that calcium influx through glutamate receptors is at least in part responsible for activating CaMKII and triggering the retrograde signal. There are several lines of evidence that support this hypothesis.

One line of evidence is based on the glutamate receptor ion channel properties and how they are changed in *DGluRIIA*$^{-/-}$ mutants. Compared to wild-type receptors, glutamate receptors in these mutants have a greatly reduced single-channel mean open time (DiAntonio et al., 1999); this also affects the kinetics of EPSPs (Petersen et al., 1997; DiAntonio et al., 1999; and in the present study). Therefore, evoked currents that give rise to similar EPSP peak amplitudes in wild-type and *DGluRIIA*$^{-/-}$ mutants will lead to less ion influx in the mutants (ion flux is a product of time and current). Considering the high $Ca^{2+}$ permeability of glutamate receptors ($P_{Ca}/P_{Na} = 9.55$) (Chang et al., 1994), due to this reduced ion influx, $Ca^{2+}$ influx will also be reduced in *DGluRIIA*$^{-/-}$ mutants both during spontaneous and evoked activities. Therefore, it is conceivable that this change in $Ca^{2+}$ influx, monitored by CaMKII, could act as a trigger for the retrograde signal.

This hypothesis is further supported by our previous data demonstrating that the retrograde increase in quantal content in *DGluRIIA*$^{-/-}$ mutants can be counteracted by overexpressing multiple copies of DGluRIIA, independent of the size of EPSPs (DiAntonio et al., 1999). The conclusion made from these results was that the retrograde control of presynaptic release for these genotypes was not directly related to muscle depolarization. In addition, we argue that if muscle depolarization were the sole trigger for the homeostatic retrograde signaling, then quantal content in *highwire* (*hiw*) mutants (Wan et al., 2000) should have been compensated for. *hiw* mutants have 60%–70% less quantal release, while we have shown previously that retrograde control of neurotransmission is still intact in these mutants (DiAntonio et al., 1999). Therefore, we propose that postsynaptic CaMKII regulates presynaptic release by responding to calcium influx through glutamate receptors during evoked and spontaneous neurotransmitter release.

The role of postsynaptic membrane depolarization in homeostatic control of presynaptic release at the *Drosophila* NMJ has been investigated by Paradis et al. (2001). This study demonstrates that the expression of an inward-rectifying potassium channel, Kir2.1, in postsynaptic muscles leads to an increase in quantal content. Kir2.1-expressing muscles show severe defects in muscle properties, including input resistance and membrane potential. More importantly, muscle excitability is affected to the point that mEPSP amplitude is reduced to less than half of wild-type levels (Paradis et al., 2001). The authors further show that mEPSCs are still wild-type under voltage-clamp conditions, suggesting no change in glutamate receptor function. However, considering the kinetics of membrane depolarization, it is conceivable that, under physiological conditions, GluRIIA function could be compromised during an evoked response that is severely reduced in duration. In other words, while glutamate receptor function is not affected directly, these results suggest that ion influx through glutamate receptors could be affected due to membrane defects. Therefore, the moderate increase in quantal content could be partially due to this apparent reduction in glutamate receptor activity.

### How Does the Motor Neuron Respond to the Retrograde Signal?

Based on our results, inhibition of postsynaptic CaMKII mimics the reduction in postsynaptic activity in glutamate receptor mutants and triggers the retrograde signal, leading to an increase in neurotransmitter release at the NMJ. This increase in neurotransmitter release does not appear to induce the NMJ to grow more synaptic boutons, as the numbers of synaptic boutons remained unchanged. Similarly, the overall ultrastructure of boutons remained indistinguishable from wild-type. In contrast, Koh et al. (1999) have reported an overdevelopment of the SSR in larvae expressing Ala.

In that study, Ala or CaMKII$^{T287D}$ were expressed in both muscles and the nervous system simultaneously, whereas we have manipulated CaMKII only in neurons or muscles exclusively. It is conceivable that the level and the pattern of expression of these transgenes could have led to our differences. Furthermore, Koh et al. analyzed boutons at the midline section only, while we looked at complete serial sections of boutons. These differences in the levels or pattern of Ala expression as well as differences in our analysis could underlie this discrepancy.

We found a 60% increase in the number of T bars per active zone in response to inhibition of CaMKII in muscles. Often present at active zones at the *Drosophila* NMJ, T bars are electron-dense structures associated with clusters of synaptic vesicles. Higher numbers of active zones and T bars appear to correlate with an increase in the strength of synaptic transmission (Atwood and Wojtowicz, 1999; Cooper et al., 1995; Jia et al., 1993; Prokop, 1999; Reiff et al., 2002). For example, hyperexcitable *eag shaker* mutants contain a higher number of T bars than wild-type at NMJ synapses (Jia et al., 1993). We have further demonstrated that induction of retrograde signaling in *DGluRIIA*$^{-/-}$ mutants leads to a doubling of the number of T bars per active zone, similar to the effect of postsynaptic inhibition of CaMKII. Reiff et al. (2002) have recently reported an increase in T bars in another allelic combination of glutamate receptor mutants. Finally, we were able to suppress the increase in T bars by postsynaptic activation of CaMKII. Since postsynaptic activation of CaMKII in glutamate receptor mutants also suppresses quantal content, these results further support a direct correlation between presynaptic T bars and neurotransmitter release at the *Drosophila* NMJ. Our findings suggest that postsynaptic reduction of CaMKII activity may boost presynaptic neurotransmitter release by upregulating T bars at active zones in presynaptic boutons, a potential mechanism for the control of synaptic transmission induced by the retrograde signal. The number of T bars per active zone could therefore be used as an index

for the presence of the homeostatic retrograde signal, independent from quantal content measurements.

We have recently demonstrated that a BMP type II receptor, *wishful thinking* (*wit*), is required for both growth and function of the NMJ in *Drosophila* (Aberle et al., 2002). To further explore the mechanism by which motor neurons respond to the retrograde signal, we examined whether the retrograde enhancement of quantal content can occur in *wit* mutants. Our results indicate that the retrograde signal cannot increase neurotransmitter release in the absence of Wit. Activation of the retrograde signal by either postsynaptic expression of $DGluRIIA^{M/R}$ or postsynaptic inhibition of CaMKII did not lead to any increase in quantal content. These results indicate a requirement for *wit* presynaptically for the functioning of the retrograde mechanism that controls the homeostasis of neurotransmitter release at the NMJ of *Drosophila* and that postsynaptic inhibition of CaMKII requires the function of presynaptic BMP signaling to enhance quantal release.

In an accompanying paper, we show that Glass bottom boat (Gbb), a BMP ortholog, can function as a retrograde ligand for Wit at the *Drosophila* NMJ (McCabe et al., 2003, this issue of *Neuron*). Mutations in *gbb* lead to NMJ defects similar to those observed in *wit* mutants, and postsynaptic transgenic expression of Gbb can rescue many of these defects. In light of these findings, it is possible that there is a link between postsynaptic activity of CaMKII and the level and function of Gbb at the NMJ of *Drosophila*.

Another candidate protein for interacting with CaMKII in controlling retrograde signaling is Discs large (DLG). DLG has been shown to be phosphorylated by CaMKII (Koh et al., 1999) and to be involved in synaptic transmission at the NMJ of *Drosophila* (Budnik et al., 1996). However, the defects in synaptic transmission in *dlg* mutants are rescued by presynaptic rather than postsynaptic expression of DLG (Budnik et al., 1996). This suggests that the role of DLG in neurotransmission is primarily presynaptic. Furthermore, we have previously shown that in *dpix* mutants quantal release is not greatly affected, while DLG levels are reduced by 80% (Parnas et al., 2001). Therefore, it seems unlikely that the effects we observe are due to changes in DLG phosphorylation levels. Additional experiments are needed to further elucidate the mechanism through which CaMKII activity controls the homeostasis of neurotransmitter release and to identify target proteins that CaMKII may interact with in the postsynaptic cell.

## Experimental Procedures

### Fly Stocks

UAS-Ala and UAS-$CaMKII^{T287D}$ transgenic fly stocks were provided by L. Griffith. Four muscle drivers (MHC-Gal4 [Schuster et al., 1996], Mef2-Gal4 [Ranganayakulu et al., 1996], C142-Gal4 [DiAntonio et al., 2001], and G14-Gal4 [Aberle et al., 2002]) and one panneuronal driver (*elav*-Gal4) (Luo et al., 1994) were used in our experiments, as indicated in the text. All crosses were set up using virgin females of Gal4 driver flies and were kept at 25°C. $DGluRIIA^{-/-}$ is null for DGluRIIA glutamate receptor subtype and corresponds to the following genotype in our experiments: $DGluRIIA^{SP16}/Df$(2L)clh4, as described by Petersen et al. (1997). Two null alleles of *wishful thinking* (*wit*), $wit^{HA4}$ and $wit^{HA5}$, were used (Aberle et al., 2002). The CaMKIINtide cDNA was constructed by amplifying the corresponding published sequence (Chang et al., 2001) from a partial rat cDNA (UI-R-BJI-ASX-F-07-0-UI, Research Genetics) representing CaM-KIIN$\alpha$ (Chang et al., 2001). The CaMKIINtide transgene contains a *Drosophila* Kozak sequence and 5′ EcoRI and 3′ XhoI sites that were added via PCR prior to cloning into pUAST. The translation product is a 28 amino acid-long peptide (MKRPPKLGQIGRSKRVVIED DRIDDVLK) that corresponds to the inhibitory region of CaMKIIN$\alpha$ (Chang et al., 2001).

### Immunocytochemistry

Analysis of bouton numbers was performed using anti-synaptotagmin (gift of K. Zinn or gift of T. Littleton) staining as previously described (Wan et al., 2000). Counts were performed blind from muscles 6 and 7 in segment A3 and were normalized by the surface area of muscle 6 and 7 in third instar larvae.

### Electrophysiology

Intracellular recordings were made from muscle 6, segment A3 of third instar wandering larvae, and dissected in Stewart saline HL3 (Stewart et al., 1994) containing varying concentrations of extracellular $Ca^{2+}$ (ranging from 0.25 mM for the failure analysis to 0.4 or 0.6 mM for EPSP recordings). The procedure has been described in Aberle et al. (2002) and Stewart et al. (1994). All crosses were kept at 25°C prior to recording. Cells with an input resistance of greater than 7 M$\Omega$ and a resting potential of less than −60mV were selected for further analysis. No significant differences were observed for input resistance or membrane potential among different genotypes. Nonlinear summation corrections were done where indicated (Martin, 1955).

Failure analysis was performed for several genetic combinations according to Petersen et al. (1997). In brief, 400 evoked responses were recorded in 0.25 mM $Ca^{2+}$, and failures were chosen when the amplitude of EPSP was smaller than 0.1mV. Quantal content was then measured by calculating the natural logarithm of the number of trials by the number of failures according to the following formula:

$$\text{quantal content} = ln\ (\text{trials/failures}).$$

Data were digitized and recorded to the hard drive of a PC computer using a Digidata 1200B analog-to-digital board and pClamp8 software (Axon Instruments). Both EPSP and mEPSP amplitudes were measured from the raw data using the off-line peak detection feature of the MiniAnalysis program (Synaptosoft, Inc.); all events were verified by eye as well. The decay time constant and half-width of mEPSPs and EPSPs were measured using the MiniAnalysis program. Half-width is a measure of the time between 50% of the maximum amplitude on the rise and fall of the mEPSP. Statistical analysis was performed using one-way ANOVA or unpaired Student's t test with Origin 6.0 software (Microcal software). All values are presented as mean ± SEM.

### Electron Microscopy

Conventional electron microscopy was performed on third instar larvae as described previously (Lin et al., 1994; Schuster et al., 1996). The number of samples corresponds to the number of boutons for every genetic combination.

### Acknowledgments

We would like to thank L. Griffith and T. Littleton and A. DiAntonio for fly stocks and reagents. We would like to thank J. Kaplan, S. Stowers, E. Isacoff, and G. Davis for critical reading of this manuscript and suggestions. We would also like to thank Beth Blankemeier for technical assistance; and members of the Goodman lab for their support. This work was supported by a Canadian Natural Sciences and Engineering Research Council fellowship to A.P.H. and by a Wellcome Trust Prize Traveling Fellowship (to B.D.M.). C.S.G. was an investigator with the Howard Hughes Medical Institute.

Received: August 6, 2002
Revised: March 7, 2003
Accepted: May 5, 2003
Published: July 16, 2003

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