Calcium-Dependent Isoforms of Protein Kinase C Mediate Posttetanic Potentiation at the Calyx of Held

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SUMMARY

High-frequency stimulation leads to a transient increase in the amplitude of evoked synaptic transmission that is known as posttetanic potentiation (PTP). Here we examine the roles of the calcium-dependent protein kinase C isoforms PKCα and PKCβ in PTP at the calyx of Held synapse. In PKCα/β double knockouts, 80% of PTP is eliminated, whereas basal synaptic properties are unaffected. PKCα and PKCβ produce PTP by increasing the size of the readily releasable pool of vesicles evoked by high-frequency stimulation and by increasing the fraction of this pool released by the first stimulus. PKCα and PKCβ do not facilitate presynaptic calcium currents. The small PTP remaining in double knockouts is mediated partly by an increase in miniature excitatory postsynaptic current amplitude and partly by a mechanism involving myosin light chain kinase. These experiments establish that PKCα and PKCβ are crucial for PTP and suggest that long-lasting presynaptic calcium increases produced by tetanic stimulation may activate these isoforms to produce PTP.

INTRODUCTION

At many synapses, a period of high-frequency (tetanic) stimulation can evoke a transient increase in synaptic strength known as posttetanic potentiation (PTP) (Feng, 1941; Griffith, 1990; Magleby, 1987; Magleby and Zengel, 1975; Zucker and Lara-Estrella, 1983; Zucker and Regehr, 2002). PTP is thought to provide an important means of synaptic regulation that can contribute to working memory and information processing (Abbott and Regehr, 2004; Silva et al., 1998). Many high-frequency stimuli are needed to induce PTP, and the frequency and duration of tetanic stimulation regulate the magnitude and duration of the enhancement (which lasts tens of seconds to minutes) (Habets and Borst, 2005, 2007; Korogod et al., 2005; Lev-Tov and Rahamimoff, 1980; Magleby, 1979; Zucker, 1989).

Tetanic stimulation also increases both the frequency and the magnitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) at many (Castillo and Katz, 1954; Delaney and Tank, 1994; Elliot et al., 1994; Habets and Borst, 2005; He et al., 2009; Korogod et al., 2005, 2007; Magleby, 1987), but not all (Brager et al., 2003) synapses. It is not known whether increases in the frequency and amplitude of spontaneous transmission and the increase in evoked release share a common presynaptic mechanism.

Numerous mechanisms could contribute to PTP. According to the leading hypothesis, known as the residual calcium hypothesis, tetanic stimulation leads to an accumulation of calcium in the presynaptic terminal, and an accompanying increase in the probability of release that persists for tens of seconds (Brager et al., 2003; Delaney and Tank, 1994; Delaney et al., 1989; Regehr et al., 1994; Zucker and Regehr, 2002). Other possibilities include an increase in the size of the readily releasable pool of vesicles (Habets and Borst, 2005; Lee et al., 2008), an increase in the size of mEPSCs as a result of vesicles fusing with each other before ultimately fusing with the plasma membrane (He et al., 2009), a change in action potential waveform (Eccles and Krnjevic, 1959; Habets and Borst, 2005) and an increase in calcium entry (Habets and Borst, 2005, 2006).

Pharmacological studies have implicated protein kinase C (PKC) in PTP. Phorbol esters, activators of PKC (Newton, 2001), increase the amplitude of evoked release and occlude PTP (Hori et al., 1999; Korogod et al., 2007; Lou et al., 2005, 2008; Malenka et al., 1986; Oleskevich and Walmsley, 2000; Rhee et al., 2002; Shapira et al., 1987; Virmani et al., 2005; Wierda et al., 2007). Phorbol esters also increase the frequency of mEPSCs (Hori et al., 1999; Lou et al., 2005, 2008; Oleskevich and Walmsley, 2000; Parfitt and Madison, 1993). In addition, PKC inhibitors reduce the magnitude of PTP at many synapses (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Korogod et al., 2007; Lee et al., 2007). Studies of the role of PKC in PTP have been limited by the lack of selectivity and ineffectiveness of pharmacological tools available to inhibit and activate PKC (e.g., Lee et al., 2008; for review see Brose and Rosenmund, 2002). Phorbol esters activate other synaptic proteins, including Munc13 (Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007). PKC inhibitors have highly variable effects on PTP: at the same synapse, different PKC inhibitors disrupt PTP to very different extents (Lee et al., 2008; D.F. and W.G.R., unpublished data);
at some synapses, PKC inhibitors do not affect PTP (Eliot et al., 1994; Lee et al., 2008; Reymann et al., 1988a, 1988b); and in some cases PKC inhibitors and their inactive analogs have similar effects on PTP (Lee et al., 2008). In addition, other proteins have been implicated in PTP, including Munc13 (Junge et al., 2004; Shin et al., 2010), calmodulin and CamKII (Chapman et al., 1995; Fiumara et al., 2007; Junge et al., 2004; Khoutorsky and Spira, 2009; Reymann et al., 1988a; Wang and Maler, 1998) and myosin light chain kinase (Lee et al., 2008). These findings have cast doubt on the involvement of PKC in PTP.

If PKC is involved in PTP, identifying which PKC isoform mediates PTP is of fundamental importance. Is it a classical, calcium-sensitive PKC isoform such as PKCα, PKCβ, or PKCγ, or one of the eight calcium-insensitive isoforms? The involvement of calcium-sensitive PKCs would be compatible with PKC being a sensor of calcium according to the residual calcium hypothesis, whereas if calcium-insensitive PKCs regulate PTP, tetanic stimulation would have to elevate presynaptic DAG or act through some unidentified pathway, and another presynaptic calcium sensor would need to respond to residual calcium. At the calyx of Held, calcium-insensitive PKCs have been implicated (Saitoh et al., 2001). These results suggest that if PKC plays a role in PTP, it does not serve as a calcium sensor.

Here we use knockout animals to examine the roles of PKCα and PKCβ in tetanus-induced enhancement of evoked and spontaneous transmission, and phorbol-ester-mediated enhancement at the calyx of Held synapse, where these forms PKCα and PKCβ produce PTP primarily by increasing the size of the readily releasable pool of vesicles that fuse in response to brief high-frequency stimulation; they do not facilitate presynaptic calcium currents. The small PTP remaining in double knockout animals is mediated in part by an increase in mEPSC amplitude and in part by a mechanism involving myosin light chain kinase (MLCK). In contrast to PTP, the increase in the mEPSC frequency following tetanic stimulation does not depend on PKCα/β, suggesting that tetanic enhancement of evoked and spontaneous release are mediated by different mechanisms. Finally, phorbol ester-dependent enhancement is greatly reduced in slices from double knockout animals. These findings establish the crucial role of calcium-sensitive PKCs in the enhancement of evoked synaptic responses induced by either tetanic stimulation or phorbol esters.

RESULTS

Calcium-Dependent PKC Isoforms in the Calyx of Held
We used immunohistochemistry to determine the localization of PKCα and PKCβ within the medial nucleus of the trapezoid body (MNTB) (Figure 1). Slices were colabeled with antibodies to either PKCα or PKCβ (green) and to the vesicular glutamate transporter vGlut1 to label glutamate-containing synaptic vesicles within presynaptic terminals (red). vGlut1 labeling was used to identify the calyces of Held, the large presynaptic terminals that provide a synaptic contact between globular bushy cells in the anteroventral cochlear nucleus and the principle neurons.
neurons in the MNTB. In slices from wild-type mice, antibody labeling for PKCα and PKCβ overlapped with vGlut1 labeling, consistent with presynaptic localization of these kinases at the calyx of Held synapse. No labeling was detected in MNTB primary neurons. Anti-PKCα antibody labeling appeared less restricted than anti-PKCβ antibody labeling, suggesting that PKCα might also be present in other structures, in addition to the presynaptic terminals.

In PKCα/−/− mice, labeling with antibodies to PKCβ and vGlut1 was similar to that observed in wild-type animals, but labeling with antibodies to PKCα was absent. Similarly, in PKCβ/−/− mice, labeling with antibodies to PKCα and vGlut1 was similar to that observed in wild-type animals, but labeling associated with antibodies to PKCβ was absent. In PKCα/−/−β/−/− mice no labeling was observed with antibodies to PKCα or PKCβ, but vGlut1 labeling was similar to that observed in wild-type littermate animals. These findings indicate that the targeted PKC isoforms are eliminated in knockout animals without noticeably affecting the synaptic distribution of vGlut1. They also suggest that the presence of one isoform is not required for the proper subcellular localization of the other.
and frequency of mEPSCs was the same in wild-types and PKC knockouts (see Figures S1A and S1B available online). We also measured the properties of use-dependent plasticity because changes in the initial probability of release alter the extent of use-dependent plasticity during high-frequency trains. These experiments were performed in the presence of kynurenate (1 mM) and cyclothiazide (0.1 mM) to reduce AMPA receptor saturation and desensitization, respectively, which can obscure changes in use-dependent plasticity. In Figure 3A, an example of excitatory postsynaptic currents (EPSCs) during 100 Hz train in a wild-type slice is shown. The average normalized EPSC amplitudes (Figure 3B) were similar in wild-type (black) (n = 22) and PKCα−/−β−/− (purple) (n = 18) animals. There was no significant difference in the use-dependent plasticity in wild-type and in PKCα−/−β−/− mice (p = 0.24 for the second stimulus, p = 0.13 for the third stimulus, p = 0.08 for the average of the 31st to 40th stimuli) (Figure 3B).

Synaptic currents evoked by stimulus trains can also be used to quantify the size of the vesicle pool that is readily released by a train (RRPtrain), as in Figure 3C. In this approach, the amplitudes of the EPSCs are measured and summed. In the plot of the cumulative EPSC, after approximately the first 10 EPSCs, RRPtrain is depleted, and the remaining steady-state EPSC is thought to reflect replenishment of RRPtrain. The cumulative EPSC (ΣEPSC0) can then be determined by extrapolating back to the first EPSC in the train, as in Figure 3C. ΣEPSC0 is proportional to RRPtrain [RRPtrain = ΣEPSC0/average mEPSC size]. The fraction of vesicles (f0) within RRPtrain that is liberated by the first action potential in a train can then be determined (f0 = EPSC0/ΣEPSC0). There was no significant difference in the size of RRPtrain for wild-type and double knockout animals, because there was no difference in either ΣEPSC0 (Figures 3D and 3E, p = 0.26) or in basal mEPSC size (Figure S1). There was also no significant difference in basal f0 (p = 0.66) (Figure 3F), or in the slope of the cumulative EPSC (p = 0.59) (Figure 3G). These findings indicate that the basal properties of synaptic transmission are similar in wild-type and double knockout animals.

Tetanically Induced Changes in RRPtrain and f0
Our studies indicate that calcium-dependent PKCs play a crucial role in PTP, but questions remain as to the mechanisms underlying this enhancement. One approach would be to determine the extent to which the size of the readily releasable pool (RRP), or the probability of releasing a vesicle (p) increases. Once RRP was determined, p would be calculated by dividing the number of vesicles that contribute to an evoked EPSC by

Figure 3. Deletion of PKCα/β Impairs PTP-Induced Increases in the Size of the Readily Releasable Pool and Release Probability without Affecting Basal Release Properties
(A) Representative example of EPSCs elicited by 100 Hz train of stimuli.
(B) Plot of normalized EPSC amplitude as a function of stimulus number during a 100 Hz, 0.4 s train delivered to slices from either wild-type (black) or double knockout (purple) animals.
(C) Cumulative EPSC plotted as a function of stimulus number. The linear fit (gray line) to the last 15 points, back-extrapolated to the y-intercept, is used to obtain ΣEPSC0, and provide a measure of the size of the readily releasable pool exocytosed by a train of stimuli [RRPtrain].
(D) Plot of normalized cumulative EPSCs for wild-type (black) and double knockout (purple) groups.
(E–G) Basal ΣEPSC0 (E), f0 (the fraction of the RRPtrain released by the first stimulus) (F), and slope (G) measurements for wild-type (black) and double knockouts (purple) groups.
(H and I) Cumulative EPSCs in response to the first 40 stimuli of a PTP-inducing train (first train; 100 Hz, 4 s; open symbols), and a second train (100 Hz, 0.4 s) delivered 10 s later (closed symbols), for wild-type (black) (H) and double knockout (purple) (I) groups.
(J) Plots of ratios for ΣEPSC0, f0, and slope for wild-type (black) and double knockout (purple) groups. Deletion of PKCα/β significantly impaired the increase in RRPtrain, as well as the slope, and to a lesser extent the increase in f0. *p < 0.01.
the number of vesicles in the RRP. However, different measures of the RRP do not agree: nonspecific PKC activators cause little or no increase in the size of the readily releasable pool (RRP) as determined by a strong and prolonged depolarization (Lou et al., 2005; Wu and Wu, 2001), but produce large increases in RRP_{train} (Lou et al., 2008). It is unlikely that differences between RRP and RRP_{train} can be accounted for by the stimulus frequency used to determine RRP_{train} (100 Hz trains in Lou et al. [2008] and in our study), because 300 Hz trains lead to only slightly larger estimates of RRP_{train} (Sakaba, 2006). One explanation for the differential effects of nonspecific PKC activators on RRP and RRP_{train} is that the RRP consists of different pools of vesicles, some that are located near calcium channels, and some that are located further from calcium channels (Neher and Sakaba, 2008). Whereas prolonged depolarization or large presynaptic calcium signals can release the entire RRP, presynaptic action potentials produce brief and local calcium transients that trigger fusion of vesicles near calcium channels, but are not effective at triggering the fusion of more distant vesicles. Increasing the size of the calcium transient, as when external calcium levels are elevated, can increase RRP_{train} by extending the spread of calcium entering through calcium channels to influence vesicle release. Alternatively, PKC could similarly extend the influence of calcium entering through calcium channels and increase RRP_{train} by increasing the calcium sensitivity of release (lowering the calcium cooperativity) (Lou et al., 2008). Thus, if activation of calcium-dependent PKCs produces PTP by increasing the calcium sensitivity of vesicles, it could lead to both an increase in RRP_{train} and an increase in the fraction of those vesicles that are liberated by the first action potential in a train (f_0).

We tested this possibility by measuring the effect of tetanic stimulation on ∑EPSC_0 and f_0. Experiments were performed in the presence of cyclothiazide (CTZ) and kynurenate to prevent receptor desensitization and saturation. Kynurenate and CTZ did not affect the magnitude of PTP in slices from either wild-type (1.79 ± 0.11, n = 9, p = 0.85) (Figure S2A) or double knockout mice (1.14 ± 0.08, n = 7, p = 0.78) (Figure S2B). To assess the effects of tetanization on ∑EPSC_0, synaptic currents were evoked by stimulating at 100 Hz for 4 s, followed by a brief train (100 Hz, 0.4 s) 10 s later. Plots of the cumulative EPSC were obtained for both trains, and used to calculate ∑EPSC_0 and f_0. As shown in representative experiments, tetanic stimulation increased ∑EPSC_0 in wild-type (Figure 3H), but not in double knockout mice (Figure 3I). Tetanic stimulation increased ∑EPSC_0 by 26% ± 0.7% and 2% ± 3% (Figure 3J, left; p < 0.01) and f_0 by 34% ± 5% and 23% ± 6% (Figure 3J, middle; p = 0.14), in wild-type and double knockout animals, respectively. Thus, the reduced PTP in double knockout mice arises primarily from decreases in the ∑EPSC_0 and perhaps f_0 (although the effect on f_0 is not statistically significant). This finding is consistent with calcium-dependent PKCs increasing the probability of release of vesicles located both near and far from calcium channels (see Discussion). Moreover, in wild-type animals the slope of the cumulative EPSC versus stimulus number was unaffected by tetanization (Figures 3H and 3J, right), but was reduced in double knockout animals (Figures 3I and 3J, right, p < 0.01). Impairment in the replenishment of the RRP_{train} or a decrease in steady-state release probability during the tetanus could contribute to decreased slope.

Previous studies suggest that myosin light chain kinase (MLCK) contributes to PTP through a mechanism that is distinct from calcium-dependent PKCs, raising the possibility that the PTP remaining in double knockout animals could be mediated by MLCK. This kinase is thought to be responsible for an activity-dependent increase in the RRP_{train}, that follows tetanic stimulation, but not the calcium-dependent increase in the probability of release (Lee et al., 2008, 2010). The time course of the action of MLCK has not been thoroughly characterized, although it is thought to be independent of the slow mitochondrial-dependent decay of presynaptic calcium following tetanic stimulation (Lee et al., 2008). According to a current model, calcium increases during tetanic stimulation activate calmodulin and MLCK, which contribute to PTP by increasing RRP_{train} without affecting the overall RRP (Lee et al., 2010).

We tested this model by examining the contribution of MLCK to PTP in both wild-type and double knockout mice. In wild-type mice, the MLCK inhibitor ML9 reduced PTP from 87% ± 2% (n = 17) to 26% ± 8% (n = 10, p < 0.0001) 5 s after the train, and from 81% ± 2% to 69% ± 2% (p = 0.21) 10 s after the train (Figure 4A). These findings confirm that MLCK contributes to PTP. They also indicate that the contribution of MLCK is short-lived compared to overall PTP, and that by 10 s after stimulation the contribution is much smaller than that of calcium-dependent PKCs. In double knockout animals, ML9 also influenced the magnitude of PTP, which at 10 s was reduced from 16% ± 2% (n = 16) to 9% ± 2% (n = 11, p < 0.05; Figure 4B). These findings indicate that MLCK-dependent mechanisms account for some, but not all, of the PTP remaining in double knockout mice.

In order to compare the mechanisms of action of MLCK and calcium-dependent PKCs in PTP, we examined the effect of MLCK inhibitors on f_0 and ∑EPSC_0 in both wild-type and double knockout mice. Inhibiting MLCK did not significantly change the basal properties of synaptic transmission in either wild-type or double knockout mice (Figure S3). In wild-type mice, inhibiting MLCK did not alter the tetanus-induced increases in ∑EPSC_0 (26% ± 7%, n = 13, compared to 21% ± 3% in the presence of ML9, n = 10, p = 0.51, Figures 4C and 4E), it reduced the increase in f_0 (35% ± 5% compared to 16% ± 5%, p < 0.05), and the slope was not statistically different (2% ± 4% compared to −8% ± 4%, p = 0.09). Similarly, in double knockout mice ML9 did not affect the tetanus-induced increase in ∑EPSC_0 (2.2% ± 4.4% n = 11 compared to 4.3% ± 4.9% n = 11 in ML9, p = 0.73; Figures 4D and 4F), but it reduced the increase in f_0 (23% ± 6% compared to 5% ± 4%, p < 0.05), and the slope was not statistically different (−13% ± 3% compared to −15% ± 7%, p = 0.82). Therefore, under our experimental conditions, MLCK did not contribute to PTP-induced increases in RRP_{train} but did contribute to increases in f_0. This finding indicates that calcium-dependent PKCs contribute to PTP through mechanisms that are distinct from MLCK.

**Tetanus-Induced Presynaptic Calcium Signals**

To further elucidate the manner in which calcium-dependent PKCs contribute to PTP, we examined the role of presynaptic calcium signaling in PTP. Previous studies suggest that...
First, PTP could involve the small but long-lasting presynaptic residual calcium (Ca\textsubscript{res}) signals that follow tetanic stimulation (Zucker and Regehr, 2002). At the calyx of Held, PTP and Ca\textsubscript{res} decay with similar time courses, and a roughly linear relationship between Ca\textsubscript{res} and PTP has been suggested (Habets and Borst, 2005; Korogod et al., 2005). This is consistent with the hypothesis that Ca\textsubscript{res} activates proteins that respond to modest Ca\textsubscript{res} levels to increase synaptic efficacy. Calcium-dependent PKCs could be the Ca\textsubscript{res} sensor that produces PTP. Another possibility is that tetanic stimulation increases presynaptic calcium entry by modulating calcium channels (Catterall and Few, 2008). At the calyx of Held, prolonged tetanic activation can increase presynaptic calcium entry under some circumstances (Habets and Borst, 2006). Indeed, tetanic stimulation for 4 s at 100 Hz can result in a 15% increase in presynaptic calcium entry (Habets and Borst, 2006). Calcium-dependent PKCs could mediate this calcium channel facilitation. Although there is controversy over the relative importance of Ca\textsubscript{res} and calcium channel facilitation, there is agreement that presynaptic calcium signaling plays an important role in PTP.

We therefore examined calcium signaling in calyces of Held from wild-type and double knockout animals. We introduced a low concentration of a calcium indicator (Calcium Green-1 dextran, K\textsubscript{D} = 326 nM) into the calyx of Held, as described previously for other synapses (Beierlein et al., 2004), and quantified calcium signals using established methodology (Brenowitz et al., 2006; Maravall et al., 2000). Brief loading times were used so that a small amount of indicator was introduced in order to minimize perturbations of presynaptic calcium signaling. A red dye (Alexa 594-dextran) was also used to allow visualization of calyces (Figure 5A), because basal Calcium Green-1 fluorescence is faint. As shown for an example experiment, single stimuli evoked fluorescence transients that decayed rapidly (Figure 5B). Single stimuli produced calcium increases of 20 ± 3 nM (n = 10) and 18 ± 2 nM (n = 10) in wild-type and double knockout animals, respectively (p = 0.53). Following tetanic stimulation of 100 Hz for 4 s, Ca\textsubscript{res} in wild-type was 132 ± 19 nM 5 s after the end of stimulation, and 164 ± 16 nM in double knockout animals (p = 0.21). Ca\textsubscript{res} decayed with a time constant of ~22 s in both wild-types (black) and double knockouts (purple). *p < 0.05.

We tested whether calcium channel facilitation contributes to PTP by measuring the effect of tetanic stimulation on increases in calcium transients evoked by single stimuli (Figure 5C, bottom). In wild-type animals tetanic stimulation elevated the calcium increases evoked by single stimuli by 60% ± 31% (n = 10; 5 s posttetanus), but the enhancement was short-lived (t < 10 s). This suggests that under our experimental conditions, tetanus-induced increases in calcium influx make only a short-lived contribution to PTP. In double knockout animals the calcium increases evoked by single stimuli (93% ± 46% of baseline at 5 s posttetanus; n = 10; p = 0.55 between wild-type and double knockout) show a similar short-lived increase following tetanic stimulation (t < 7 s). This suggests that the impairment of PTP in double knockout animals is not due to impaired facilitation of calcium currents in response to tetanic stimulation.
Activity-Dependent Increases in mEPSC Frequency

In addition to enhancing the amplitude of evoked synaptic transmission, tetanic stimulation also enhances the frequency of spontaneous release (Castillo and Katz, 1954; Eliot et al., 1994; Habets and Borst, 2005; Korogod et al., 2005, 2007; Magleby, 1987). We tested whether PKCα and PKCβ mediate this activity-dependent increase in mEPSC frequency. In these experiments the same tetanic stimulation was used as in our PTP experiments (4 s, 100 Hz), but without test stimuli. This allowed us to monitor mEPSCs before and after tetanic stimulation. Our tetanic stimulation protocol resulted in a large increase in mEPSC frequency, which is illustrated in a representative experiment in which the mEPSC frequency before and 2–6 s after stimulation increased from 1.0 to 3.5 Hz (Figure 6A, black). The extent of enhancement ranged from 1.7-fold to 6.7-fold (Figure 6B, black, the ratio of mEPSC frequency 2–6 s following tetanic stimulation to basal frequency). In wild-type animals the time course of mEPSC frequency enhancement decayed with a time constant of \( \tau = 12 \) s (Figure 6C). Tetanic stimulation also increased the frequency of spontaneous events in PKCα/−/−, PKCβ/−/−, and PKCαβ/−/− mice, as shown in representative experiments in which enhancement was 4.2-fold (increased from 0.67 to 2.8 Hz), 3.7-fold (0.74 to 2.77 Hz), and 3.9-fold (0.80 to 3.1 Hz), respectively (Figure 6A). There was no significant difference in the enhancement of mEPSC frequency among wild-type (3.5 ± 0.3, n = 15), PKCα/−/− (3.5 ± 0.5, n = 10), PKCβ/−/− (3.5 ± 0.3, n = 15), and PKCαβ/−/− (3.5 ± 0.5, n = 10), PKCαβ/−/− (3.5 ± 0.3, n = 15), and PKCαβ/−/− (3.5 ± 0.5, n = 10).
PKCβ−/− (4.4 ± 0.5, n = 16) and PKCα−/−β−/− groups (4.3 ± 0.6, n = 13) (p = 0.43) (Figures 6B and 6C). These results suggest that at the calyx of Held synapse, PTP and the enhancement of spontaneous release arise from different mechanisms. Calcium-dependent PKCs are crucial to PTP, but they do not mediate tetanus-evoked increases in mEPSC frequency.

**Activity-Dependent Increases in mEPSC Amplitude**

We tested whether PKCα and PKCβ mediate the increase in mEPSC amplitude that follows tetanic stimulation (He et al., 2009). In wild-type mice, tetanic stimulation altered the distribution of mEPSC sizes, and after tetanic stimulation the fraction of small mEPSCs was reduced and the fraction of large mEPSCs increased, as shown in a representative experiment (Figure 7A). In slices from PKC knockout animals, tetanic stimulation also increased mEPSC amplitude and produced similar effects on the mEPSC distributions, as illustrated in representative experiments from slices from PKCα−/− (Figure 7B), PKCβ−/− (Figure 7C), and PKCα−/−β−/− (Figure 7D) mice. As shown in the cumulative histograms (Figure 7E), tetanic stimulation significantly increased the mEPSC amplitude in slices from wild-type, PKCα−/−, PKCβ−/−, and PKCα−/−β−/− mice compared to their respective baseline (p < 0.05 for all paired comparisons). On average, enhancement was somewhat smaller in PKCβ−/− (10.1% ± 2.8%), and PKCα−/−β−/− (10.9% ± 4.7%) compared to wild-type (13.1% ± 3.5%) and PKCα−/− (18.7% ± 2.5%), but these differences were not statistically significant (p = 0.34). The time courses of the enhancement of mEPSC amplitude in the different genotypes (Figure 7F) can be approximated by single exponential decays with time-constants of 47 ± 9 s, 39 ± 4 s, 67 ± 17 s, and 35 ± 8 s for wild-type, PKCα−/−, PKCβ−/−, and PKCα−/−β−/− groups, respectively.

**Synaptic Enhancement Mediated by Phorbol Esters**

Phorbol esters activate PKC by binding to the diacylglycerol (DAG) binding site (Newton, 2001), leading to large synaptic enhancement that mimics and occludes PTP (Korogod et al., 2007; Malenka et al., 1986). Synaptic enhancement by phorbol esters has been studied extensively to provide insight into the mechanism mediating PTP. We examined the role of PKCα and PKCβ in phorbol ester-induced enhancement. As shown in representative experiments, the phorbol ester PDBu (1 μM) enhanced EPSC amplitude in slices from wild-type (Figure 8A; 2.5-fold), PKCα−/− (Figure 8B; 1.7-fold), PKCβ−/− (Figure 8C; 1.4-fold), and PKCα−/−β−/− (Figure 8D; 1.4-fold) mice, but the degree of enhancement was smaller in the knockout groups. Although there was variability in the extent of enhancement in the different genotypes (Figure 8F), the average extent of enhancement was clearly reduced in the PKC knockout groups (Figure 8F), and there was a significant difference in the

![Figure 7A](https://example.com/image1.png)

![Figure 7B](https://example.com/image2.png)

![Figure 7C](https://example.com/image3.png)

![Figure 7D](https://example.com/image4.png)

![Figure 7E](https://example.com/image5.png)

![Figure 7F](https://example.com/image6.png)
PDBu-dependent enhancement between wild-type (2.22 ± 0.14, n = 17) and PKCα/−/− (1.80 ± 0.12, n = 13, p < 0.05), PKCβ/−/− (1.46 ± 0.05, n = 13, p < 0.01), and PKCα/−/− β/−/− (1.44 ± 0.09, n = 7) groups. These experiments establish that calcium-dependent PKCs play an important role in phorbol ester-dependent enhancement at the calyx of Held. Compared to baseline, there is still significant enhancement remaining in slices from PKCα/−/− β/−/− mice (p < 0.01), which indicates that other target(s) of phorbol esters (Brose and Rose-nmund, 2002; Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007) are engaged at this synapse.

In addition to enhancing the amplitude of evoked EPSCs, phorbol esters increase mEPSC frequency. This is illustrated in a representative experiment by comparing spontaneous mEPSCs recorded under control conditions and in the presence of PDBu (Figure 8G, black). We tested whether PKCα and PKCβ also contribute to this enhancement of mEPSC frequency. As shown in the representative experiments, PDBu increased the mEPSC frequency in slices from PKCα/−/− (Figure 8G, green), PKCβ/−/− (Figure 8G, red), and PKCα/−/− β/−/− (Figure 8G, purple) mice. The range of mEPSC frequency enhancement was quite broad in all genotypes (Figure 8H). The average enhancement was 5.6 ± 0.7 in wild-type (Figure 8I, black, n = 13), 4.9 ± 0.4 in PKCα/−/− (Figure 8I, green, n = 14), 4.4 ± 0.5 in PKCβ/−/− (Figure 8I, red, n = 14), and 3.1 ± 0.6 in PKCα/−/− β/−/− (Figure 8I, purple, n = 7) groups. Although there was a trend suggesting that PKCα and PKCβ contributed to the phorbol ester-dependent enhancement in mEPSC frequency, the differences did not reach statistical significance (p = 0.054), despite the relatively large sample sizes. However, a pairwise comparison using a Kolmogorov-Smirnoff
2-sample test indicated that the mEPSC frequency distributions for wild-type and double knockout groups were significantly different \((p < 0.05)\).

**Summary of the Properties of Synaptic Transmission in Wild-Type and Knockout Animals**

Our findings indicate that PKC\(\alpha\) and PKC\(\beta\) play important roles in synaptic transmission at the calyx of Held synapse. Although there are no discernible effects on basal properties of synaptic transmission, there are profound differences in synaptic plasticity, with various synaptic properties affected differentially. This is illustrated in Figure 9, where the plasticity in different genotypes is expressed relative to the plasticity in the wild-type group. The largest effect was on PTP. In PKC\(\alpha\)/C0\(-/-\), PKC\(\beta\)/C0\(-/-\), and PKC\(\alpha\)/C0\(-/-\) groups PTP was 75% ± 10%, 26% ± 4%, and 20% ± 3%, respectively, of that observed in the wild-type group (Figure 9A, top). The primary effect of PKC\(\alpha\)/C0\(-/-\) was on \(\Delta EPSC_0\), which in double knockouts was reduced to 8% ± 13% of wild-type (Figure 9B, top). Deletion of PKC\(\alpha\)/C0\(-/-\) also modulated \(f_0\), which was reduced to 66% ± 19% of wild-type. The increases in mEPSC frequencies following tetanic stimulation in PKC\(\alpha\)/C0\(-/-\), PKC\(\beta\)/C0\(-/-\), and PKC\(\alpha\)/C0\(-/-\) were 87% ± 30%, 140% ± 41%, and 137% ± 43%, respectively, of that observed in the wild-type group (Figure 9C, top). Thus, the same genetic manipulation profoundly reduced PTP without reducing the frequency of spontaneous mEPSCs. Furthermore, the amplitude of mEPSC was also not significantly affected by the absence of calcium-sensitive PKCs (Figure 9D, top). The mEPSC amplitude changes in PKC\(\alpha\)/C0\(-/-\), PKC\(\beta\)/C0\(-/-\), and PKC\(\alpha\)/C0\(-/-\) after the tetanus were 143% ± 42%, 77% ± 29%, and 83% ± 42% respectively, of the wild-type group. Following application of PDBu, the increase in the amplitude of evoked synaptic responses in PKC\(\alpha\)/C0\(-/-\), PKC\(\beta\)/C0\(-/-\), and PKC\(\alpha\)/C0\(-/-\) was 66% ± 12%, 38% ± 6%, and 36% ± 9%, respectively, of that observed in the wild-type group (Figure 9E, top). In the PKC\(\alpha\)/C0\(-/-\) group a higher percentage of enhancement remains for PDBu-dependent enhancement (36%) than for PTP (20%).

**DISCUSSION**

Here we report that in the absence of both PKC\(\alpha\) and PKC\(\beta\), PTP is 20% of that observed in wild-type animals, indicating that calcium-dependent PKCs mediate most of PTP at the calyx of Held synapse. The remaining PTP appears to be mediated in part by an MLCK-dependent mechanism and in part by an increase in mEPSC size. Calcium-dependent PKCs enhance transmission primarily by increasing RRPtrain, and to a lesser extent by increasing the fraction of vesicles released in response to a stimulus; they also influence replenishment of RRPtrain.
following tetanic stimulation. Similar to PTP, phorbol ester-dependent enhancement was greatly reduced in slices from double knockout animals. The differential effects of PKCa and PKCb on evoked and spontaneous synaptic transmission are summarized in Figure 9 (top: group averages, bottom: individual examples).

**PTP Is Mediated Primarily by PKCa and PKCb**

Our finding that PTP is greatly reduced in the absence of PKCa and PKCb establishes an important role for these kinase isoforms in PTP at the calyx of Held. Our results resolve a long-standing controversy over whether PKC plays a role in PTP. Previous observations that phorbol esters occlude PTP (Korogod et al., 2007; Malenka et al., 1986) were thought to support a role for PKC in PTP until it was realized that in addition to activating PKC, phorbol esters activate other proteins such as Munc13 (Brose and Rosenmund, 2002; Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007). Similarly, the finding that PKC inhibitors reduce the magnitude of PTP (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Korogod et al., 2007; Lee et al., 2007) supported a role for PKC in PTP, but the observation that inactive analogs have similar effects on PTP (Lee et al., 2008) and that some PKC inhibitors do not affect PTP at all (Eliot et al., 1994; Lee et al., 2008; Reymann et al., 1988a, 1988b) have blurred the role of PKC in PTP. Using a molecular genetic approach allowed us to overcome the limitations associated with the lack of selectivity of PKC inhibitors and activators and establish that PKC plays a crucial role in PTP.

Our results establish that calcium-dependent PKC isoforms mediate most of the PTP at the calyx of Held, with PKCb playing a more prominent role than PKCa (Figure 9A, top). This challenges the previously-held view that a calcium-independent PKC isoform mediates PTP (Saitoh et al., 2001). Previous studies at the calyx of Held found that phorbol esters induce translocation of PKCa and suggested that this calcium-independent isoform mediates PKC-dependent plasticity at this synapse (Saitoh et al., 2001). Moreover, different PKC inhibitors were found to have very different effects on PTP. A broad-spectrum inhibitor (bisindolylmaleimide, BIS) and one that preferentially targets calcium-independent isoforms (Ro 31-8220) reduced PTP (expressed as fraction of PTP in control conditions) to ~40% and ~20%, respectively (Korogod et al., 2007). One interpretation of these results is that PTP involves calcium-independent PKCs, which might be activated by a tetanus-dependent elevation of DAG rather than by calcium. This interpretation is, however, complicated, because PKC inhibitors do not readily penetrate brain slices, and slices must be soaked in high concentrations of the inhibitors for long periods of time prior to the experiment. In some cases, broad-spectrum inhibitors (chelerythrine) do not reduce the magnitude of PTP (Lee et al., 2008). Limitations associated with the use of PKC inhibitors in slice preparation raise the possibility that the differential efficacy of PKC inhibitors may reflect their ability to penetrate the slice, rather than their isoform selectivity (Brose and Rosenmund, 2002). This seems to be a plausible explanation for the differential effects of PKC inhibitors, in light of our observation that the calcium-dependent isoforms PKCa and PKCb account for most of the PTP.

**Mechanisms of PTP Mediated by PKCa and PKCb**

Our experiments provide insight into the mechanisms underlying PTP. Calcium measurements suggest that although calcium channels are briefly facilitated, this facilitation makes a short-lived contribution to PTP (Figure 5C). Facilitated calcium entry is still present in double knockout animals, indicating that it is not mediated by calcium-dependent PKCs. PTP at cultured superior cervical ganglion neurons is also mediated primarily by mechanisms that are independent of calcium channel facilitation (Mochida et al., 2008).

The effect of tetanic stimulation on responses evoked by brief 100 Hz stimulus trains was revealing with respect to how calcium-dependent PKCs contribute to PTP. The use-dependent increase in the size of RRPtrain is absent in double knockout animals (Figures 3I and 3J), suggesting that PKCa and PKCb mediate the increase in the size of the pool of vesicles following tetanic activation. This appears to be the primary mechanism by which calcium-dependent PKCs produce PTP, although they also appear to be partially responsible for the increase in the fraction of vesicles exocytosed by an action potential. The substantial increase in RRPtrain is compatible with the observation that phorbol esters have only minor effects on the overall RRP size, provided the properties of different vesicle pools at the calyx of Held are considered (Lou et al., 2008). When an action potential invades a presynaptic bouton, vesicles that are located near calcium channels are exposed to a larger calcium signal than more distant vesicles (Neher and Sakaba, 2008). Any increase in the sensitivity of a vesicle to calcium could increase both the size of the vesicle pool that can be exocytosed by a train of action potentials, and the fraction of the vesicles that are released by the first action potential (Lou et al., 2008). The relative contributions of these two mechanisms depend on the detailed ultrastructure of the synapse, the spatiotemporal calcium signal, and the calcium sensitivity of the vesicles (Branco and Staras, 2009; Neher and Sakaba, 2008). In the case of PTP, our findings suggest that PKCa/β act primarily to increase the size of the readily releasable pool.

The involvement of calcium-dependent PKC isoforms in PTP raises the question: are PKCa and PKCb the calcium sensors that, according to the residual calcium hypothesis of PTP, detect presynaptic calcium signals evoked by tetanic stimulation to phosphorylate downstream targets to increase the probability of release? We find that Cares decays (~ 22 s) more quickly than PTP (~ 45 s), suggesting that for our experimental conditions PTP is longer-lived than Cares at the calyx of Held, as is the case at hippocampal and cerebellar synapses (Beierlein et al., 2007; Brager et al., 2003). Furthermore, we find that PTP is produced by tetanic stimulation that increases Cares by several hundred nanomolar. Can calcium-dependent PKCs respond to such small calcium increases? In the absence of lipid membranes, the Ca2+-binding affinities for PKCa and PKCb are ~ 40 μM (Kohout et al., 2002), which is much higher than the observed residual calcium signals. However, in the presence of phosphatidyserine and/or PIP2-containing membranes or in model systems, cooperative Ca2+-binding is observed for both isoforms, and calcium affinities range from 0.1 to 5 μM (Corbalan-Garcia et al., 1999; Corbin et al., 2007; Guerrero-Valero et al., 2007; Kohout et al., 2002). It is also possible that factors in the
intracellular milieu raise the binding affinity of PKCs for calcium, as is the case for calmodulin (Xia and Storm, 2005). Thus, it is plausible that PKCα and PKCβ could be sufficiently sensitive to detect residual calcium. Alternatively, PKCs could be initially activated by the calcium signals during the train and then, because of positive cooperative binding, become sensitive to residual calcium. Once activated, PKC could phosphorylate proteins such as Munc18 to increase the probability of release (Wierda et al., 2007). Further studies are needed to determine if PKCα and PKCβ are indeed the calcium sensors in PTP, and if they influence release by phosphorylating Munc18.

**Increases in mEPSC Frequency and Amplitude Evoked by Tetanic Stimulation**

Tetanic stimulation increases the frequency of mEPSCs several-fold at the calyx of Held synapse and at other synapses (Figure 6) (Castillo and Katz, 1954; Eliot et al., 1994; Groffen et al., 2010; Habets and Borst, 2005; Korogod et al., 2005, 2007; Magleby, 1987). The increase in the frequency of spontaneous release and PTP are both dependent on presynaptic calcium increases (Bao et al., 1997; Korogod et al., 2005; Nussinovitch and Rahamimoff, 1988; Zucker and Lara-Estrella, 1983), suggesting that they share a common mechanism. However, the elevation in mEPSC frequency does not last as long as the enhancement of evoked EPSCs (τ ~ 12 s and 45 s, respectively) (see also Korogod et al., 2007). In addition, pharmacological inhibitors of PKC that reduce the increase in evoked EPSC amplitude do not prevent the increase in mEPSC frequency at calyx of Held synapses (Korogod et al., 2007). Here, using a genetic approach, we also find that the frequency of MEPS and the amplitude of evoked EPSCs are regulated independently. Indeed, potentiation of evoked EPSCs is reduced by 80% in slices from PKCα−/−β−/− mice compared to controls (Figure 9A) whereas the increase in mEPSC frequency is largely unaffected (Figure 9C). Therefore, the activity-dependent regulation of mEPSC frequency is not mediated by PKCs, and is likely regulated by other calcium-sensitive proteins in the presynaptic terminal, such as Doc2a and Doc2b (Groffen et al., 2010; but see Pang et al., 2011).

Tetanic stimulation also results in increased mEPSC amplitude in slices from wild-type animals (Figure 7). Although modest, this increase has a time course (τ ~ 47 s) that is similar to that of PTP (τ ~ 45 s, compare Figure 2F and Figure 7F), and it is thought to contribute to PTP (He et al., 2009). The increase in mEPSC amplitude appears to reflect the fusion of vesicles with each other prior to ultimate fusion with the plasma membrane (He et al., 2009). We find that the increase in mEPSC amplitude persists in the absence of PKCα, PKCβ or both isoforms (Figure 7). This suggests that calcium-dependent isoforms of PKC do not regulate vesicle-to-vesicle fusion within the calyx of Held. The 10% increase in mEPSC amplitude that remains in PKCα/β double knockout animals could account for some of the remaining PTP observed in this group (Figure 9A).

**Synaptic Enhancement by Phorbol Esters**

Previous studies have supported a role for PKCs in the phorbol ester-induced enhancement of evoked EPSCs at the calyx of Held (Hori et al., 1999; Lou et al., 2008), but the isofrom responsible for this enhancement was not known. A broad-spectrum PKC inhibitor, though with questionable selectivity (Lee et al., 2008), reduced enhancement by PDBu to ~40% of control, and inclusion of a more selective PKC blocking peptide in the presynaptic terminal reduced the enhancement to less than 20% of that observed in control conditions (Hori et al., 1999). Previous studies suggested that the calcium-insensitive isoform PKCβ mediates this enhancement because it is present at the calyx of Held and is activated by phorbol esters (Saitoh et al., 2001). However, our observation that phorbol ester-induced potentiation of evoked EPSCs is reduced by ~70% in PKCβ double knockouts compared to controls indicate that these two isoforms account for the bulk of the contribution of PKCs to EPSC enhancement by phorbol esters. Moreover, our results are consistent with the observation that ~50% of phorbol ester-induced potentiation in the hippocampus is impaired in PKCβ knockout mice (Weeber et al., 2000). The component of phorbol ester-induced enhancement that is not mediated by PKCs is likely mediated by the synaptic protein Munc13, either as a result of phorbol esters directly activating Munc13, or as a result of phorbol ester binding to the N-terminal domain of Doc2α, thereby allowing it to interact with Munc13 (Hori et al., 1999; Lou et al., 2008).

Phorbol esters enhance mEPSC frequency ~6-fold in wild-type animals (Figure 8). In the absence of PKCα and PKCβ, this enhancement is reduced by ~50% (compare black and purple traces in Figure 8I). This result agrees with previous observations using pharmacology (Lou et al., 2008; Oleskevich and Walsmsley, 2000) and suggests that PKC plays a less important role in potentiating spontaneous release compared to evoked release. In double knockout animals, the impairment of the phorbol ester-induced increase in mEPSC frequency (Figure 8I), although moderate, contrasts with the lack of effect on tetanus-induced increase in mEPSC frequency (Figure 9C). Further studies are needed to understand this potential difference in the regulation of spontaneous activity.

**EXPERIMENTAL PROCEDURES**

**Animals**

PKCα (in 129S2 genetic background) and PKCβ (in C57BL/6J genetic background) single knockout animals, generated by M. Leitges (Leitges et al., 1996, 2002), were bred together to obtain offspring heterozygous for both genes (het-het animals). Crosses of het-het animals generated z+/+ β+/+ (WT), z−/− β+/+ (αKO), z+/+ β−/− (βKO), and z−/− β−/− (double knockout) animals with a frequency of 1:1:6 each. All animal handling and procedures abided by the guidelines of the Harvard Medical Area Standing Committee on Animals.

**Preparation of Brain Slices**

Transverse 180- to 200-μm-thick brainstem slices containing the region of the medial nucleus of the trapezoid body (MNTB) were made with a vibratome slicer (VT1000S, Leica) from postnatal day P11–14 mice deeply anesthetized with isoflurane. Brains were dissected and sliced at 4°C in cutting solution consisting of the following (in mM): 125 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 0.1 CaCl2, 3 MgCl2, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O2/5% CO2 (pH 7.4). Slices were incubated at 32°C for at least 30 min in a bicarbonate-buffered solution composed of the following (in mM): 125 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 2 CaCl2, 1 MgCl2, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O2/5% CO2 (pH 7.4).
Electrophysiology
Slices were transferred to a recording chamber at room temperature (21–24 °C) in an upright microscope (Olympus, Center Valley, PA) equipped with a 60×, 0.9 N.A. objective. During recordings, the standard perfusion solution consisted of the bicarbonate-buffered solution (see above) with 1 μM strychnine and 25 μM bicuculline to block inhibitory synaptic transmission. Slices were superfused at 1–3 ml/min with this external solution. Whole-cell postsynaptic patch-clamp recordings were made from visually identified cells in the MNTB region using glass pipettes of 2–3 MΩ resistance, filled with an internal recording solution of the following (in mM): 20 CsCl, 140 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 EGTA, 5 Na₂-phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, pH: 7.3, 315–320 mOsm. Series resistance (Rs) was compensated by up to 70% and the membrane potential was held at −70 mV.

Excitatory postsynaptic potentials (EPSCs) were evoked by stimulating presynaptic axons with a bipolar stimulating electrode (custom-made or from FHC, Bowdoin, ME) placed midway between the medial border of the MNTB and the midline of the brainstem. Multiclamp 700A and 700B (Axon Instruments/Molecular Devices, Union City, CA) amplifiers were used. Recordings were digitized at 20 kHz with an ITC-18 A/D converter (Instrutech, Port Washington, NY) using custom macros (written by M.A. Xu-Friedman) in Igor Pro (WaveMetrics, Lake Oswego, OR) and filtered at 8 kHz.

The protocol for inducing PTP was as follows: an estimate of baseline synaptic strength was obtained through low-frequency stimulation at 0.2 Hz for 25 s. PTP was induced with a 4 s stimulus train at 100 Hz, followed by low-frequency stimulation to test for PTP. Changes in miniature EPSCs (mEPSCs) were measured by delivering the same PTP-inducing train, but without the low-frequency stimulation. For phorbol ester experiments, basal synaptic strength was evaluated by paired (50 ms interval) stimuli, repeated every 20 s. During the interval intervals, 10 s stretches of postsynaptic current were recorded to assess the frequency and amplitude of mEPSCs. For all recordings, the access resistance and leak current were monitored, and experiments were rejected if either of these parameters changed significantly.

Presynaptic Calcium Measurements
Alexa 594 dextran and Calcium Green-1 dextran (10 kDa, Invitrogen, Carlsbad, CA) were loaded into presynaptic boutons as described previously (Beierlein et al., 2004). Loading times were 3–5 min and the loading solution contained 0.025%–0.1% Alexa 594 dextran and 0.5% Calcium Green-1 dextran. Fluorescence transients from calyces were monitored with a 2-photon microscope as described previously (Brenowitz et al., 2006). Fluorescence signals were converted to calcium by determining the F_max/F_min ratio (F_max/F_min = 5.5) in a cuvette, determining F_max using high frequency stimulation according to the approach presented previously (Maravall et al., 2000). In general, calyces that had bright green fluorescence at rest were found to be unsuitable for further study, either because they had elevated resting calcium levels, or they were overloaded with calcium indicator and the calcium transients were slowed.

Data Analysis
Data analysis was performed using routines written in IgorPro (WaveMetrics). PTP magnitude was calculated as the ratio of EPSC amplitude 10 s after the 100 Hz train over the average baseline. mEPSCs were detected using a threshold (average peak-to-peak noise in the baseline) of the first derivative of the raw current trace, and confirmed visually. mEPSC frequency measurements were made during the baseline (25 s before PTP induction) and starting 8 s after PTP induction. The observed increases in mEPSC size cannot be attributed to the near synchronous fusion of 2 vesicles because, assuming a Poisson distribution and a peak mEPSC frequency (ν) of 12 events/s (as observed following tetanic stimulation), we estimate that only (1 − exp(−νΔt))/ν = 2.4% of mEPSCs occur within 2 ms of each other following tetanic stimulation (a conservative upper bound for the timing of two closely spaced mEPSCs that can be both detected). Statistical analyses were done using one-way ANOVA tests for multiple group comparisons followed by Tukey post-hoc analysis. Pairwise comparisons were performed with Student’s paired t tests or Wilcoxon signed rank tests. Level of significance was set at p < 0.05.

Immunohistochemistry
Transverse brainstem slices (150 μm thick) were prepared from P12 animals as described above and fixed with 4% paraformaldehyde for 2 h at 4 °C. At the end of fixation, slices were transferred to phosphate buffer (Sigma-Aldrich, St. Louis, MO) and stored at 4 °C until further processing. Slices were then incubated in blocking solution (phosphate buffered solution + 0.25% Triton X-100 [PBST] + 10% normal goat serum) for 1 h at room temperature. Slices were incubated with primary antibodies in PBST overnight at 4 °C, followed by incubation with secondary antibodies in PBST for 2 h at room temperature. Slices were mounted to Superfrost glass slides (VWR, West Chester PA) and air-dried for 30 min. Following application of DAPI-containing Prolong anti-fade medium (Invitrogen), slices were covered with a top glass coverslip (VWR) and allowed to dry for 24 hr prior to imaging. The following antibodies were used: anti-vGlut1 guinea pig polyclonal (Synaptic Systems, Göettingen, Germany), anti-PKCa rabbit monoclonal (Abcam, Cambridge, MA), anti-PKCβ rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz CA), goat anti-guinea pig rhodamine-conjugated and goat anti-rabbit FITC-conjugated secondary antibodies (Santa Cruz Biotechnology). All antibodies were used at 1:500 dilution.

Images were acquired with a Zeiss 510 Meta confocal microscope using a Plan-achromat 63 × 1.4 N.A. oil lens. Excitation was set at 543 nm for rhodamine (vGlut1) and 488 nm for FITC (PKCs). Emission filters were LP560 for vGlut1 and BP505-530 for PKCs. An optical zoom of 2 was used. Single optical sections at 1024 × 1024 (Kalman average of four scans) were obtained sequentially for the different channels. Experiments with slices from different animals of all genotypes were repeated three times.

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.neuron.2011.04.019.

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