

# Control of Inhibitory Synaptic Outputs by Low Excitability of Axon Terminals Revealed by Direct Recording

## Highlights

- Cultured Purkinje cell (PC) axon terminals contain a large RRP of synaptic vesicles
- Release probability of RRP vesicles is extremely low in cultured PC axon terminals
- Action potentials that conduct reliably along the axon are attenuated close to terminals
- Attenuated action potentials control short-term synaptic depression

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## In Brief

By directly recording from the axon and terminal of a cultured cerebellar Purkinje cell, Kawaguchi and Sakaba demonstrate that action potential amplitudes are attenuated around axon terminals upon high-frequency activation, leading to short-term depression.

# Control of Inhibitory Synaptic Outputs by Low Excitability of Axon Terminals Revealed by Direct Recording

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## SUMMARY

An axon is thought to faithfully conduct action potentials to its terminals. However, many features of the axon and axon terminals, especially at inhibitory synapses, remain unknown. By directly recording from the axon and terminal of a cultured cerebellar Purkinje cell (PC), we demonstrate that low membrane excitability of axon terminals shapes synaptic output. Simultaneous measurements of presynaptic capacitance and evoked IPSCs revealed PC axon terminals contained large readily releasable synaptic vesicles that exhibited a low release probability. Nevertheless, IPSCs evoked by stimulating a PC soma underwent frequency-dependent depression. Direct axonal recordings showed that high-frequency action potentials were faithfully conducted over axonal bifurcations but were attenuated around terminals. Sparse  $\text{Na}^+$  channels relative to enriched voltage-gated  $\text{K}^+$  channels in terminals caused short-term depression of IPSCs by reducing  $\text{Ca}^{2+}$  influx. Together with confirmation in slice recordings, our findings reveal a presynaptic mechanism that shapes short-term synaptic depression without depleting releasable vesicles.

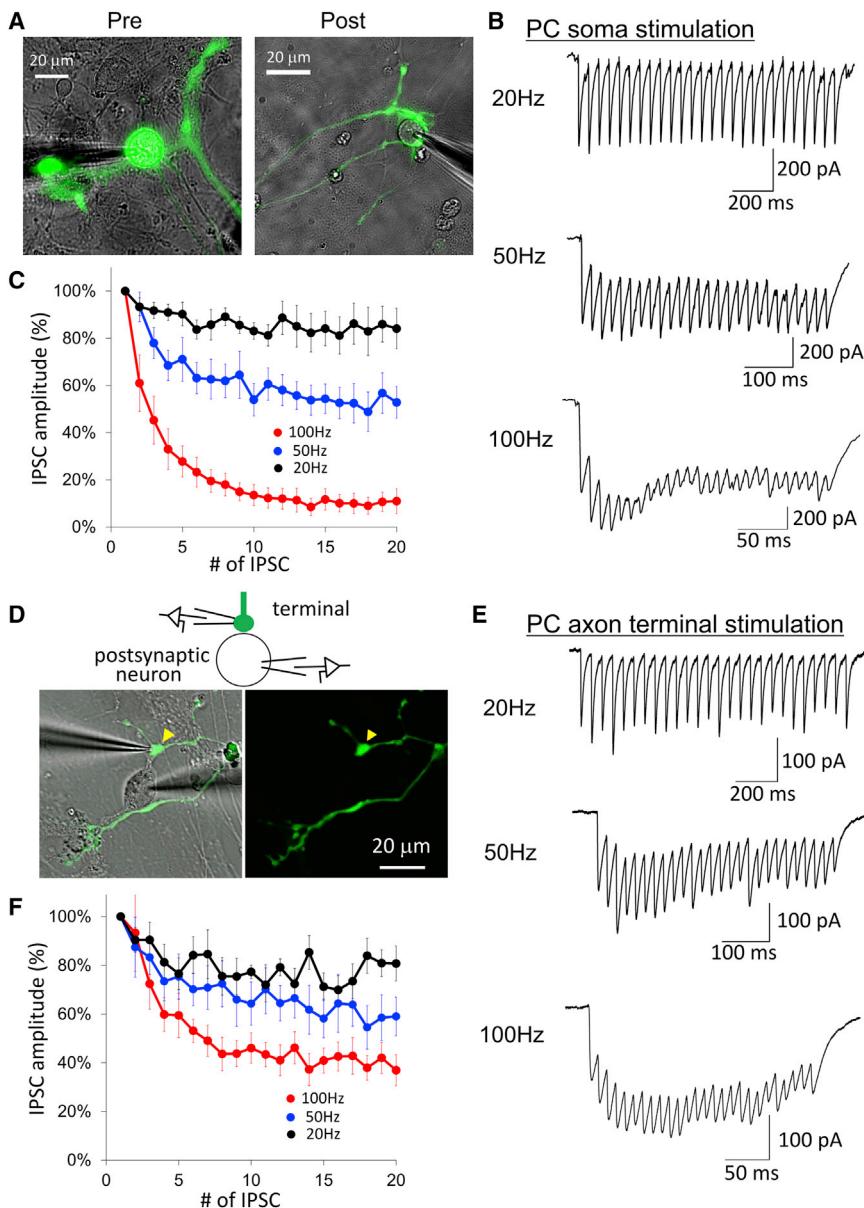
## INTRODUCTION

Efficacy and activity-dependent plasticity of synaptic transmission impact computations of the nervous system. Short-term synaptic plasticity lasts over periods of milliseconds or minutes and is crucial for information processing (Abbott and Regehr, 2004). At many central synapses, frequent and repetitive activity leads to a decrease in synaptic strength, termed short-term synaptic depression. At glutamatergic synapses, depletion of the readily releasable pool (RRP) of synaptic vesicles is considered to be one of the major mechanisms underlying such synaptic depression (Zucker and Regehr, 2002; Fioravante and Regehr, 2011). Other mechanisms at presynaptic varicosities include inactivation of release sites (Neher and Sakaba, 2008; Pan and Zucker, 2009),  $\text{Ca}^{2+}$  current inactivation, and action potential (AP) conduction failure at axonal branches (Xu and Wu, 2005;

Catterall and Few, 2008; Brody and Yue, 2000; Prakriya and Mennerick, 2000). In addition, postsynaptic mechanisms such as receptor desensitization shape synaptic depression (Zucker and Regehr, 2002). Many types of inhibitory synapses also exhibit short-term depression (Vincent and Marty, 1996; Galarreta and Hestrin, 1998; Kraushaar and Jonas, 2000; Sakaba, 2008). However, compared to excitatory synapses, the mechanisms of short-term plasticity remain largely unknown at inhibitory synapses, despite their functional importance (Lawrence and McBain, 2003; Jonas et al., 2004). One possible mechanism is the activity-dependent depletion of the RRP (Moulder and Mennerick, 2005), as reported for excitatory synapses. In addition, unidentified mechanisms upstream of exocytosis (release-independent component of depression) or the properties associated with neurotransmitter release may be responsible (Kraushaar and Jonas, 2000; Hefft et al., 2002; Kirischuk et al., 2002; but see Volynski et al., 2006).

To clarify the mechanism of frequency-dependent modulation of inhibitory synaptic output, it is necessary to directly record from presynaptic boutons. However, the small size of presynaptic terminals has hindered their direct functional analysis, with the exception of a few large presynaptic structures (Borst et al., 1995; Geiger and Jonas, 2000; Southan and Robertson, 1998) and synaptosomes (Smith et al., 2004, but see a recent study by Novak et al., 2013). In addition, technical challenges have hampered the study of information transmission from the axon to its terminals. Specific questions remain concerning whether the transmission is faithful or plastic and how it might affect synaptic transmission and/or plasticity (Debanne, 2004). To address such questions, we performed direct patch-clamp recordings from the axon and/or axon terminals of cultured Purkinje cells (PCs). These neurons form synapses similar to that observed in intact preparations (Hirano and Kasuno, 1993), allowing for simultaneous recording of the postsynaptic response. From such recordings, we were able to examine the electrical properties of axons and terminals as well as the relatively unknown properties of transmitter release at inhibitory synapses.

PCs are the sole output neurons of the cerebellar cortex, and they potently inhibit neurons in the deep cerebellar nuclei (DCN). PC axons are relatively thick and can faithfully transmit sodium APs up to over 200 Hz (Khaliq and Raman, 2005; Monsivais et al., 2005). Synaptic transmission at PC-DCN neuron synapses exhibit short-term depression upon repetitive stimulation in a manner dependent on stimulation frequency (Telgkamp and



**Figure 1. Frequency-Dependent Depression of PC Outputs**

(A) Images of a synaptically connected EGFP-labeled PC (left) and postsynaptic neuron (right). Whole-cell recordings were performed from both.

(B) Representative traces of IPSCs elicited by train stimulation at 20, 50, and 100 Hz. Each trace was the average of 20 traces.

(C) Peak IPSC amplitudes normalized to the first IPSC amplitude;  $n = 7$  pairs for each.

(D) Images of a paired patch-clamp recording from an EGFP-expressing PC axon terminal (yellow arrowhead) and postsynaptic cell.

(E) Representative average traces of IPSCs evoked by current injection directly into a presynaptic terminal at 20, 50, and 100 Hz.

(F) Peak amplitudes of IPSCs caused by direct activation of the presynaptic terminal were normalized to the first IPSC amplitude;  $n = 7$  pairs for each. Data are presented as mean  $\pm$  SEM. See also Figure S1.

## RESULTS

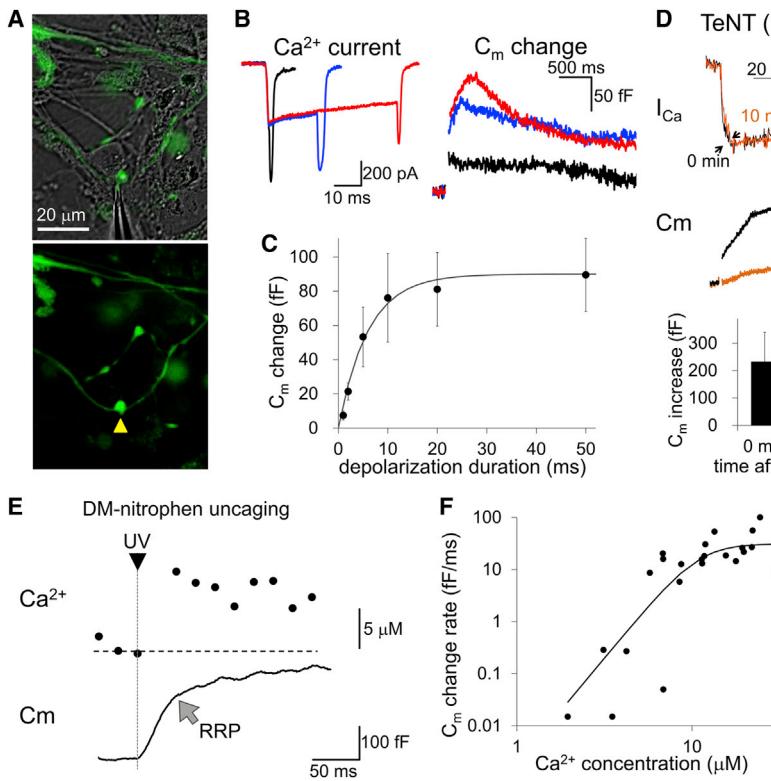
### Frequency-Dependent Depression of PC Outputs

We first performed paired recordings from a PC and a target neuron in culture. To visualize the axons of PCs specifically, EGFP was expressed using an adeno-associated virus (AAV) vector that preferentially infected PCs in the cerebellum (Kaneko et al., 2011) (Figure 1A). Each PC had an extended axon with many bifurcations, which tended to form synapses onto a particular cell type in the DCN (Figures 1A and S1; see Supplemental Experimental Procedures). PCs were voltage clamped at  $-70$  mV and the paired target neuron clamped at voltages between  $-50$  and  $-100$  mV. APs were elicited at 20, 50, or 100 Hz in the PC soma, and the resulting evoked IPSCs in the target cell exhibited a frequency-

dependent depression (20 Hz,  $84\% \pm 6\%$ , average of 16<sup>th</sup>–20<sup>th</sup> IPSCs, 50 Hz,  $53\% \pm 7\%$ , 100 Hz,  $10\% \pm 4\%$ ,  $n = 7$  pairs) (Figures 1B and 1C). This was in agreement with previous studies conducted at PC-DCN synapses in slice (Telgkamp and Raman, 2002; Pedroarena and Schwarz, 2003).

To examine mechanisms of the frequency-dependent depression of PC outputs, we used the EGFP fluorescence as a guide to directly patch-clamp a PC axon terminal, which usually ranged in size from 1 to 3  $\mu$ m. Although technically demanding, the success of this method was due largely to the visibility and high accessibility of boutons in the culture preparation (Figure 1D). IPSCs were elicited by direct activation of the presynaptic terminal following either a depolarizing pulse (0 mV for 1 to 2 ms) or current injection (400–1,000 pA for 1 to 2 ms) (Figure 1E). The resulting IPSC was similar in amplitude and time course to the

Raman, 2002; Pedroarena and Schwarz, 2003; Telgkamp et al., 2004), although the underlying mechanisms are unknown. We have taken advantage of a cultured PC expressing enhanced green fluorescent protein (EGFP) to directly visualize its axon and presynaptic varicosities and have performed direct recordings from the axon and its terminal. Paired recordings of presynaptic membrane capacitance ( $C_m$ ) and postsynaptic currents demonstrated that PC axon terminals, in spite of undergoing frequency-dependent depression, contain a large number of RRP vesicles and a very low release probability. Therefore, vesicle pool depletion cannot account for short-term depression at this synapse. Instead, AP conduction to the axon terminals is negatively regulated by the low membrane excitability of boutons in an activity-dependent manner, leading to short-term synaptic depression.



**Figure 2.  $C_m$  Increase by Direct Depolarization or  $\text{Ca}^{2+}$  Uncaging in a PC Axon Terminal**

(A) Images of a presynaptic terminal recorded in the whole-cell patch-clamp configuration.

(B) Representative traces of presynaptic  $\text{Ca}^{2+}$  currents and the resultant change in  $C_m$  caused by 2 (black), 20 (blue), and 50 ms (red) depolarizing pulses.

(C) Amplitudes of  $C_m$  changes (mean  $\pm$  SEM, 200 ms after the end of the  $\text{Ca}^{2+}$  current) were plotted against depolarization pulse duration;  $n = 9$ .

(D) Top: Presynaptic  $\text{Ca}^{2+}$  currents and  $C_m$  change upon a 50 ms depolarization pulse immediately or 10 min after the start of whole-cell recording with 100 nM TeNT included in the patch pipette. Bottom: Averaged  $C_m$  changes immediately or 10 min after the TeNT application;  $n = 4$  cells.

(E) Representative time courses of  $[\text{Ca}^{2+}]_i$  and  $C_m$  increase caused by UV-mediated  $\text{Ca}^{2+}$  uncaging. Sharp  $C_m$  increase corresponds to the RRP, and the subsequent gradual increase presumably corresponds to the release of re-plenished vesicles.

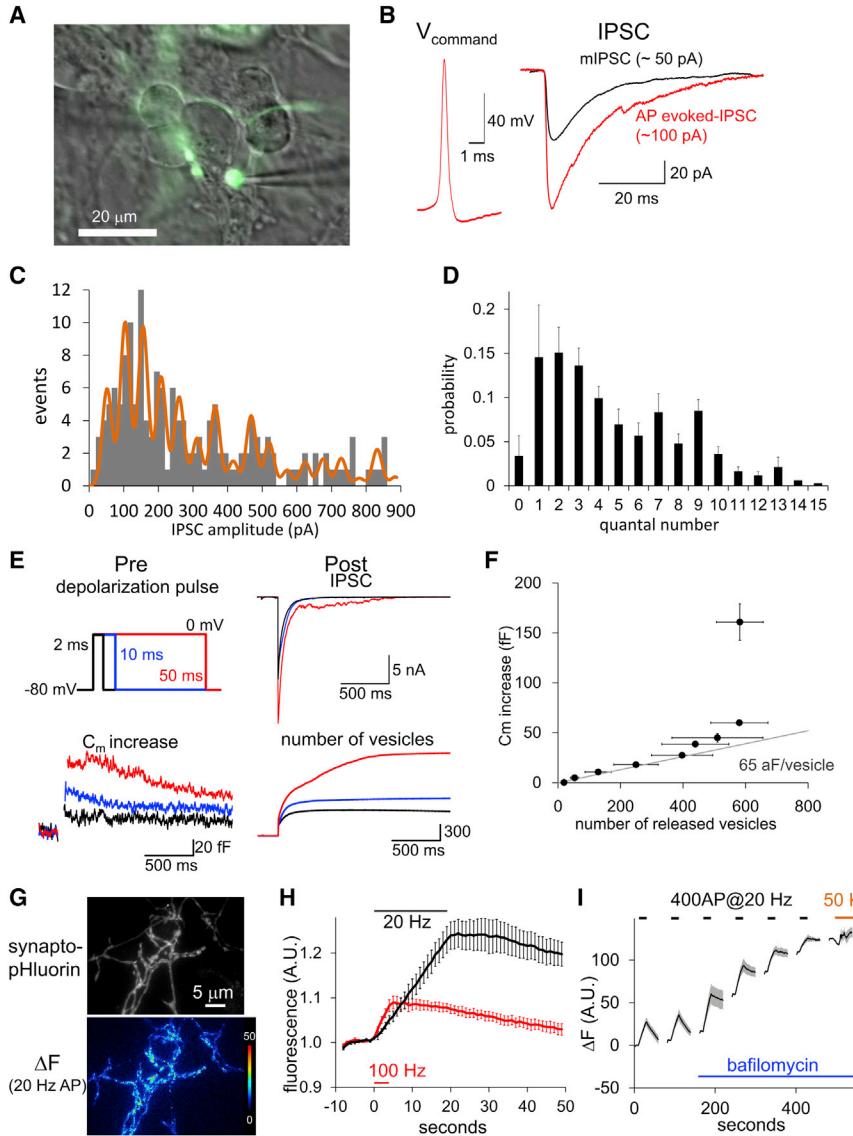
(F) The peak rate of  $C_m$  increase was plotted against  $[\text{Ca}^{2+}]_i$ . Hill equation curve ( $K_{\text{Ca}^{2+}}$  of 11  $\mu\text{M}$  and Hill coefficient of 4.04) obtained by fitting the data with the least square method is shown by the black line.

IPSC triggered by a spontaneous AP (see Figure S1). Surprisingly, when IPSC trains were triggered by repetitive direct stimulation of a presynaptic varicosity, the depression of IPSC amplitude by 100 Hz stimulation was markedly reduced compared to somatic stimulation (20 Hz,  $81\% \pm 7\%$ , average of 16<sup>th</sup>–20<sup>th</sup> IPSCs,  $p = 0.54$  by Student's t test, compared with soma stimulation; 50 Hz,  $60\% \pm 9\%$ ,  $p = 0.53$ ; 100 Hz,  $40\% \pm 6\%$ ,  $p = 0.001$ ,  $n = 7$  pairs) (Figure 1F). Thus, a large part of the high-frequency-dependent depression at PC-DCN synapses is presynaptic in origin. However, the depression might be due not to the release mechanisms such as depletion of the releasable vesicles pool, but to the activity-dependent changes at the axon or terminals, such as altered presynaptic  $\text{Ca}^{2+}$  influx, conduction failure, or attenuation of APs.

#### Characterization of the RRP, Release Probability, and Intracellular $\text{Ca}^{2+}$ Sensitivity of Transmitter Release at a PC Terminal

To examine the kinetics of transmitter release from a PC terminal, we measured  $C_m$  of a presynaptic varicosity under the whole-cell configuration (Neher and Marty, 1982) before and after (~200 ms, average period of 100 ms) the depolarization pulse (Figure 2A). TTX (1  $\mu\text{M}$ ) and TEA (2 mM) were applied to the external solution to block  $\text{Na}^+$  and  $\text{K}^+$  channels, respectively. In addition, Cs-based intracellular solution was used to isolate presynaptic  $\text{Ca}^{2+}$  currents. To limit the  $C_m$  change to single presynaptic terminal, we recorded from a bouton with few neighboring varicosities (Figure 2A). Considering experimental conditions (space-clamp of the terminal and neighboring axonal regions),

$C_m$  changes likely reflect exocytosis and endocytosis of vesicles at the patch-clamped terminal (see [Supplemental Experimental Procedures](#)). The duration of the depolarization pulse was altered to change the duration of  $\text{Ca}^{2+}$  influx (Figure 2B). We found that the  $C_m$  of PC terminals increased depending on the duration of the depolarization pulse (2 ms,  $21 \pm 5$  fF; 10 ms,  $76 \pm 26$  fF; 50 ms,  $90 \pm 21$  fF) (Figures 2B and 2C). For longer pulses, a continuous increase in  $C_m$  was observed lasting for approximately 1 s after the depolarization ceased, probably reflecting asynchronous release. When SNARE complex function for vesicle exocytosis was inhibited by VAMP2 cleavage by applying tetanus toxin (TeNT, 100 nM) into the terminal through a patch pipette, the  $C_m$  increase was suppressed by about 80%–90% within 10 min after whole-cell rupture ( $15\% \pm 4\%$ ,  $p = 0.0002$ , paired t test) (Figure 2D). Therefore, the increase in  $C_m$  mostly reflected exocytosis of synaptic vesicles. In Figure 2C, the time course of  $C_m$  increase could be fitted by a single exponential with a time constant of 6 ms and a maximal amplitude of 90 fF, though the latter was variable (ranging from 30 fF to 250 fF). The maximal amplitude corresponds to the RRP of synaptic vesicles. Assuming the  $C_m$  of a single synaptic vesicle is 65 aF (estimated from the value of Figure 3F), on average, about 1,000–1,500 synaptic vesicles were released in response to a single 10 ms depolarization pulse. Thus, the RRP size is very large in a cultured PC terminal. Increasing the intracellular EGTA concentration from 0.5 to 5 mM altered neither the time course nor maximal amplitude of  $C_m$  ( $n = 4$  cells, time constant = 6 ms and a maximal amplitude of 110 fF; data not shown), in agreement with other inhibitory synapses where transmitter



**Figure 3. Paired Recordings of Presynaptic Exocytosis and IPSCs**

(A) Image of a dual recording from a presynaptic terminal and a connected postsynaptic neuron.

(B) Presynaptic AP-like voltage command and the corresponding evoked IPSC (average of 42 traces, red). For comparison, the mIPSC trace (average of 217 traces) recorded from the same cell is overlaid.

(C) IPSC amplitude distribution histogram with approximately ten apparent discrete steps recorded from a paired recording as shown in (A). The orange distribution shows fitting of the IPSC distribution with a combination of single Gaussian distributions exhibiting a mean and SD of 60 and 15 pA, respectively.

(D) Distribution of quantal number averaged from six pairs.

(E) Time course of the presynaptic  $C_m$  increase, resulting IPSC, and cumulative number of released vesicles estimated by deconvolution, in response to different durations of terminal depolarization.

(F) The  $C_m$  increase was plotted against total released vesicles estimated by deconvolution. Data were obtained from ten synapses. The  $C_m$  increase showed a linear relation to the number of released vesicles for <400 released vesicles (gray line).

(G) Fluorescent images of synaptophysin expressed in a PC. The lower panel shows the increase in fluorescence during a 20 Hz AP train stimulation.

(H) Normalized time courses of synaptophysin fluorescence intensity over time following 400 APs at 100 (red) or 20 Hz (black);  $n = 40$  terminals from eight cells.

(I) Time course of fluorescence increase during rounds of AP train stimulation (400 APs at 20 Hz) before and after bafilomycin application (indicated by blue bar). To confirm that the majority of releasable vesicles were depleted, 50 mM K<sup>+</sup>-containing solution was applied at the end of the recording;  $n = 25$  terminals from five cells. Data are presented as mean  $\pm$  SEM. See also Figure S2.

release was found to be EGTA insensitive (Bucurenciu et al., 2008). On the other hand, loading 5 mM of the relatively fast Ca<sup>2+</sup> chelator BAPTA (with a similar  $K_d$  to EGTA) into the PC axon terminal reduced the  $C_m$  increase by about 80% ( $C_m$  maximally increased by  $19 \pm 11$  fF with a time constant of 9 ms,  $n = 5$  cells). It should be noted that the amplitude of  $C_m$  increase was substantially smaller in PC terminals of slice preparation at P9–P16 (see Figure S6A), suggesting that the RRP size of small axon terminals in intact preparation at young developmental age is much smaller.

In order to examine the release mechanism quantitatively, we next applied the Ca<sup>2+</sup> uncaging method to the PC terminal (Delaney and Zucker, 1990; Heidelberger et al., 1994). The caged Ca<sup>2+</sup> compound DM-Nitrophen (2–5 mM) and the Ca<sup>2+</sup> indicator Fura2-FF (200  $\mu$ M) were applied to the terminal via the patch pipette. Flash photolysis of DM-Nitrophen uniformly elevated the intra-terminal Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ) (Fig-

ure 2E), which caused a linear increase in  $C_m$  when  $[Ca^{2+}]_i$  was low. When the  $[Ca^{2+}]_i$  was high, the  $C_m$  showed a sharp increase (Figure 2E), which peaked at an average of  $80 \pm 20$  fF ( $n = 10$  terminals). The maximal  $C_m$  increase reflects the RRP size, and the value attained was similar to that elicited by a depolarizing pulse (see above, Figure 2C), suggesting a homogenous pool of vesicles. As illustrated in Figure 2E, the  $C_m$  showed a slow increase following the initial phase, presumably reflecting vesicle replenishment. As a result, the entire time course could be fitted by the sum of an exponential and a linear function. By changing the  $[Ca^{2+}]_i$  in the terminal, we have also measured the intracellular Ca<sup>2+</sup> sensitivity for transmitter release. When plotted as a function of  $[Ca^{2+}]_i$ , the release rate showed an approximate 4<sup>th</sup> power dependency and the  $[Ca^{2+}]_i$  required for half-maximal activation of release was approximately 11  $\mu$ M (Figure 2F). The relationship was similar to that observed for the calyx of Held and cerebellar basket

cell (BC) terminals (Bollmann et al., 2000; Schneggenburger and Neher, 2000; Sakaba, 2008).

To further examine the size of the RRP and release probability ( $Pr$ ), we performed simultaneous recordings of presynaptic Cm and IPSCs (Figure 3A). Presynaptic terminals were voltage clamped with a Cs-based internal solution in the presence of TTX and TEA, stimulated with an AP voltage waveform previously recorded from another terminal. As shown in Figure 3B, the amplitude of evoked IPSCs recorded from the postsynaptic neuron was only about twice that of miniature IPSCs (mIPSCs), which presumably corresponded to the synaptic response caused by single vesicle release. Thus, several vesicles ( $4.8 \pm 1.7$ ,  $n = 8$  pairs) are released at a single synapse in response to a single AP. Representative IPSCs recorded from a pair showed approximately ten discrete steps in response to the AP-like voltage command (Figure 3C; also see Figure S2). The distribution of IPSC amplitudes could be fitted by combinations of quantal IPSC amplitude distributions with multiple integer amplitudes. The quantal distribution obtained from six pairs suggested that, on average,  $4.8 \pm 0.5$  quanta are released from a single AP (Figure 3D).

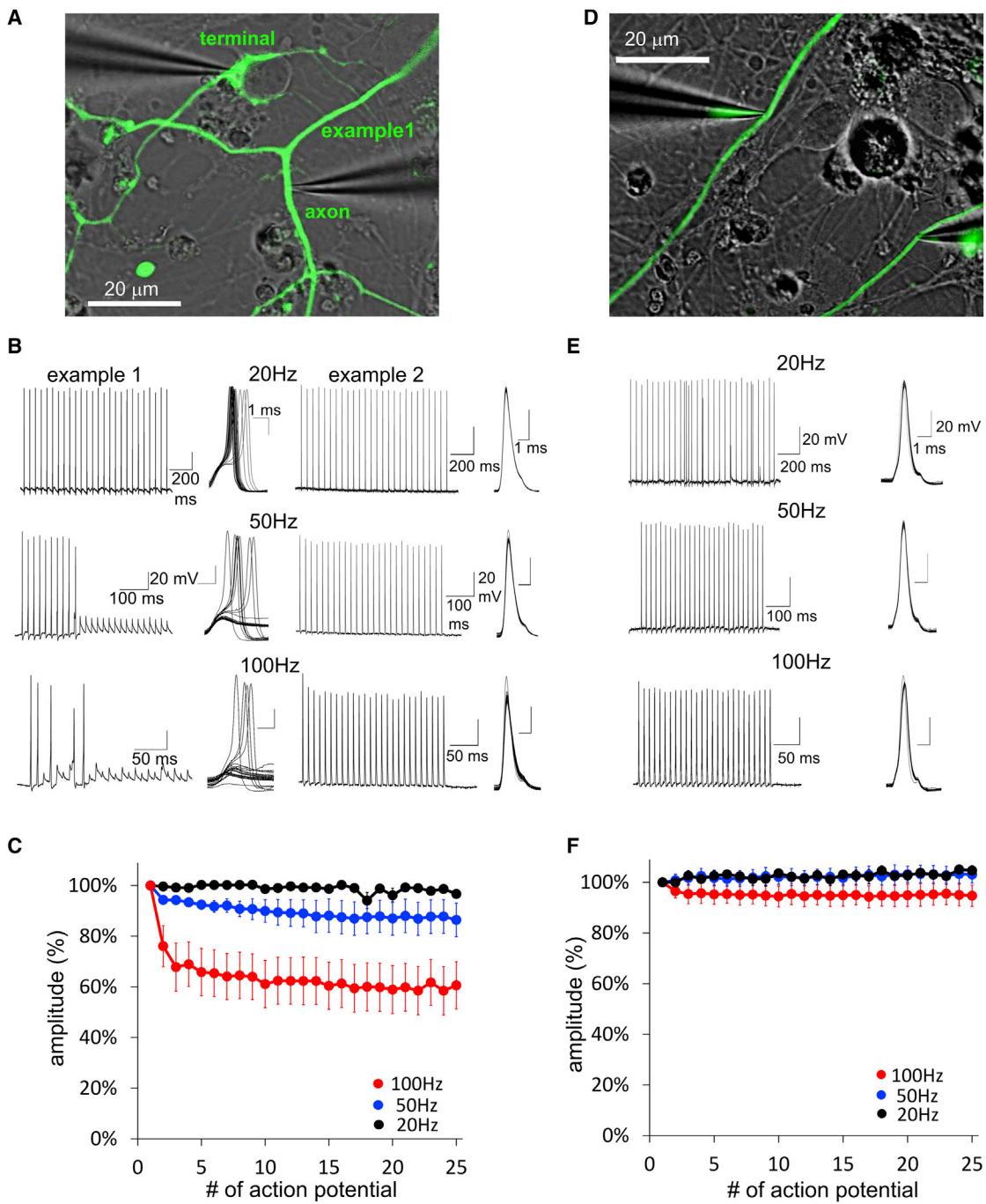
Our findings that cultured PC terminals have a low  $Pr$  and a large RRP were supported by the following experiments. Prolonged square depolarization pulses (to 0 mV) produced a >100-fold increase in both vesicle fusion events and IPSC amplitude (Figure 3E). The total number of released synaptic vesicles, estimated by deconvolution of each IPSC trace with the mIPSC trace (Sakaba, 2008), showed a linear relationship to the measured Cm jump, with an initial slope of 65 aF/vesicle (Figure 3F). This value is toward the higher end of the range (30–85 aF) estimated from a synaptic vesicle with a size of 35–50 nm in PC terminals (Takei et al., 1992), as well as values calculated from on-cell capacitance measurements (He et al., 2006). It is possible that the number of released vesicles even following limited exocytosis might be underestimated in deconvolution analysis due to high receptor occupancy at GABAergic synapses (Auger and Marty, 2000). We also note that the relationship between Cm and the estimated number of vesicles has some variability. For large amounts of exocytosis (>400 vesicles), the slope steeply increased, indicating that postsynaptic responses did not follow linearly to vesicle release. This is most likely because of strong postsynaptic receptor saturation and/or desensitization (Telgkamp et al., 2004; Pugh and Raman, 2005). Nevertheless, the postsynaptic recordings confirmed a large RRP (at least >500 vesicles). Taken together, the simultaneous recordings of presynaptic Cm and IPSCs at a single synapse revealed thousands of RRP vesicles that have an extremely low (~0.003) release probability in response to a single AP.

From the relationship between  $[Ca^{2+}]_i$  and release rates (Figure 2F), we estimated the  $[Ca^{2+}]_i$  during an AP at the transmitter release site using the five-step release model (Bollmann et al., 2000; Schneggenburger and Neher, 2000). The peak  $[Ca^{2+}]_i$  was estimated to be 7  $\mu M$  (data not shown), which is low compared to other depressing synapses (10–50  $\mu M$ ) (Schneggenburger and Neher, 2000; Millar et al., 2005). Together with the observation that release is EGTA insensitive, transmitter release from PC terminals is likely regulated by the openings of a low density of tightly coupled  $Ca^{2+}$  channels.

We further attempted to validate our estimates of large RRP size and low  $Pr$  at PC terminals by using an imaging technique, which had the advantage of being less invasive compared with patch-clamp recordings. For this end, synaptopHluorin (Sankaranarayanan and Ryan, 2001; Fernández-Alfonso and Ryan, 2004) was expressed in a PC, which allowed us to monitor the increase in fluorescence upon vesicle release that results from pHluorin being exposed to the neutral pH of the extracellular space relative to the acidic vesicle lumen. As shown in Figure 3G, 20- or 100-Hz AP trains elicited by somatic depolarization increased the fluorescence in axonal varicosities. Consistent with the frequency-dependent depression of IPSCs, the increase in synaptopHluorin-based fluorescence following 400 APs at 20 Hz was approximately three times larger than that at 100 Hz (Figure 3H,  $p < 0.005$ ). Thus, high-frequency stimulation resulted in depressed synaptic vesicle release, but the availability of vesicles does not seem to be the key determining factor. When acidification of the vesicle lumen following endocytosis was inhibited by baflomycin, a specific inhibitor of vesicular ATPase (Sankaranarayanan and Ryan, 2001; Fernández-Alfonso and Ryan, 2004), the increase in fluorescence following 400 APs at 20 Hz was augmented by 60%, suggesting that about 40% of released vesicles are endocytosed and re-acidified during the 20 s stimulation period (Figure 3I). In addition, we observed that though vesicle re-cycling was blocked by baflomycin, the number of remaining vesicles available for release still exceeded the number of vesicles released during the 1<sup>st</sup> round of 400 APs at both 20 Hz (see Figure 3I) and 100 Hz (not shown), suggesting the number of vesicles is not a key constraint. Taken together, our data suggest that cultured PC terminals have a large releasable synaptic vesicle pool, which is not readily depleted by repetitive AP arrival, and thus is unlikely to limit synaptic transmission.

### AP Attenuation around Axon Terminals Weakens Synaptic Transmission

The large RRP and low  $Pr$  characteristic of PC axon terminals suggest that the frequency-dependent depression of PC outputs to the target neuron is not mediated by RRP depletion but by some activity-dependent mechanism that limits vesicle release. One obvious candidate is the attenuation of the AP and/or its failure in conduction along the axon or at axon terminals. To test this possibility, we recorded simultaneously from both the axon and its terminal separated by at least 300  $\mu M$  (Figures 4A and S3A). APs were evoked at 20, 50, or 100 Hz by either a 2 ms depolarizing pulse (to 0 mV) or a 1 to 2 ms current injection of 200–700 pA (Figure S3C). Supporting our hypothesis, the propagation of APs to the terminal showed either conduction failure (a representative single sweep shown as example 1) or attenuation in the peak amplitude (example 2) in a frequency-dependent manner (20 Hz,  $97\% \pm 2\%$  at 20<sup>th</sup> AP relative to the 1<sup>st</sup>; 50 Hz,  $86\% \pm 7\%$ ; 100 Hz,  $61\% \pm 9\%$ ,  $n = 14$  cells) (Figures 4B and 4C; also see Figure S3D). In contrast, dual recordings of APs from two distinct sites along a single axon (separated by ~300  $\mu M$  and several branching points, Figure S3B) showed reliable AP propagation (20 Hz,  $103\% \pm 2\%$  at 20<sup>th</sup> AP,  $p = 0.1$  by Mann-Whitney U test compared with terminal; 50 Hz,  $103\% \pm 3\%$ ,  $p = 0.013$ ; 100 Hz,  $95\% \pm 4\%$ ,  $p = 0.008$ ,  $n = 6$  cells) (Figures 4D–4F). This result is consistent with previous studies of faithful AP

**Figure 4. Attenuation of APs at Axon Terminals**

(A) An image of a paired recording from an axon and its terminal.

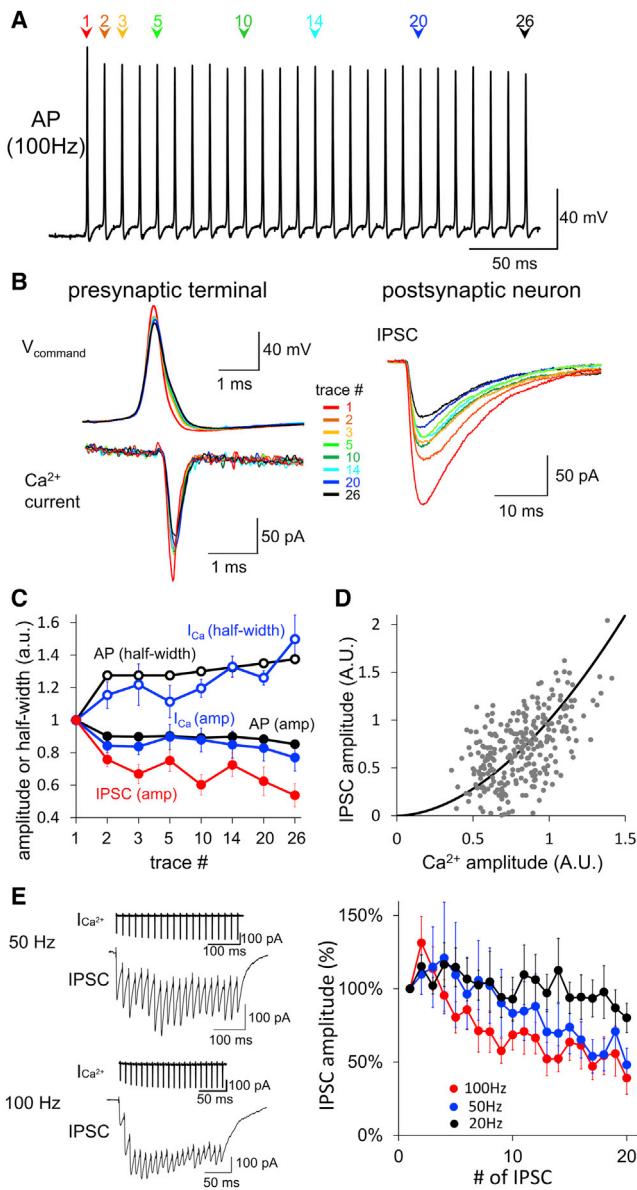
(B) Representative traces of AP trains conducted from the axon to the terminal in two example cells. The recording shown in example 1 corresponds to the image shown in (A). APs were triggered from the patch-clamped axon at frequencies of 20, 50, or 100 Hz.

(C) Peak amplitudes of APs were normalized by that of the first AP (mean  $\pm$  SEM);  $n = 14$  cells.

(D) An image of paired recordings from two different sites along a single axon.

(E) Representative traces showing AP conduction when evoked by trains of up to 100 Hz at axonal sites.

(F) Plot of AP amplitude normalized to the first;  $n = 6$  cells. See also [Figure S3](#).



**Figure 5. Impact of AP Attenuation on Synaptic Transmission**

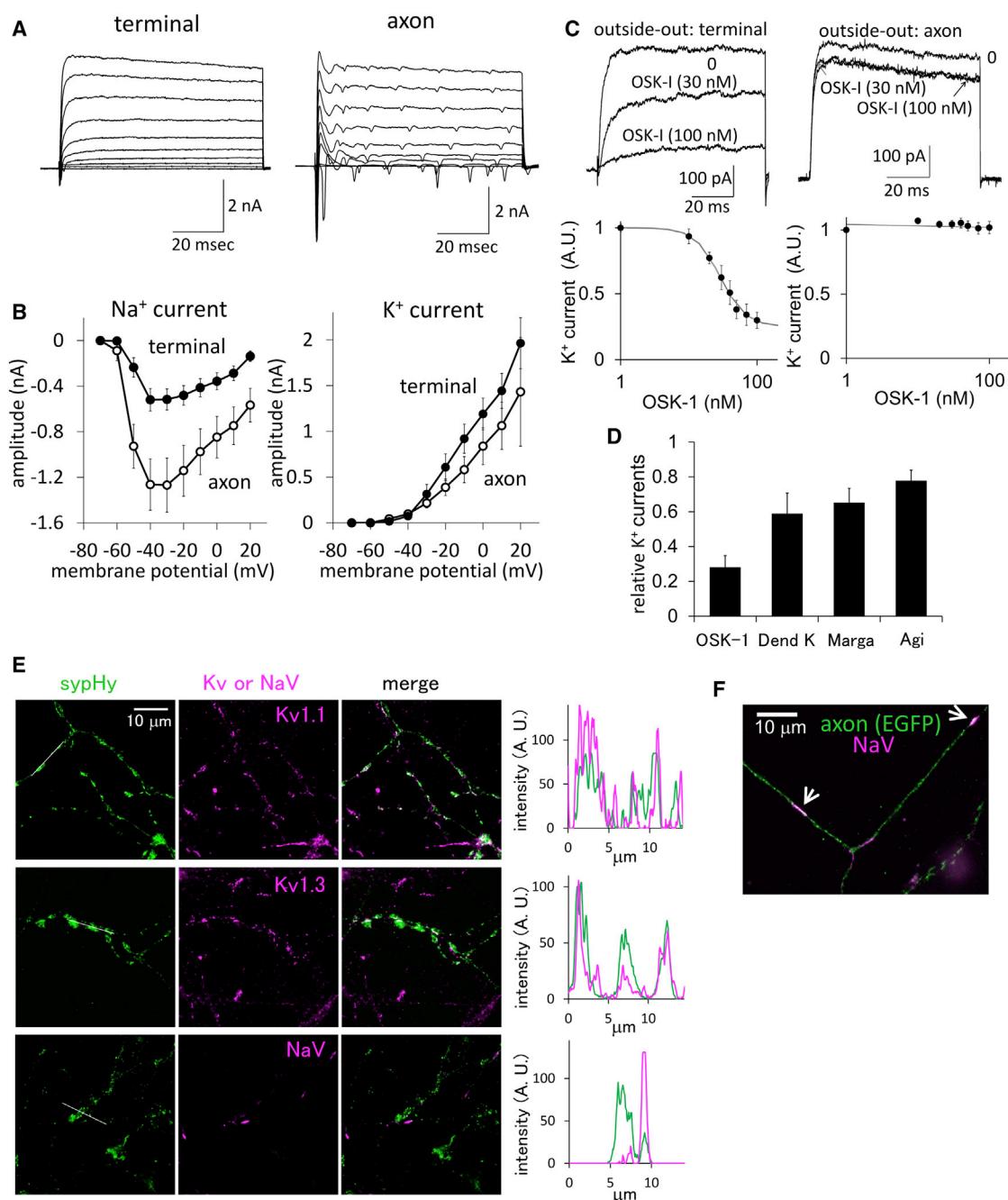
- (A) AP trains recorded from a terminal stimulated at a distant axonal site at 100 Hz. From the AP trains, eight different AP waveforms with different amplitudes (indicated by colored arrowheads) were selected for voltage commands used in (B).
- (B) Representative presynaptic  $\text{Ca}^{2+}$  currents and IPSCs in response to the eight different AP-like voltage commands.
- (C) Average plot of AP amplitude, AP half-height width, presynaptic  $\text{Ca}^{2+}$  current amplitude, presynaptic  $\text{Ca}^{2+}$  current half-width, and IPSC amplitude in response to the eight different AP-like voltage commands;  $n = 6$  cells.
- (D) Relationship between IPSC amplitude and  $\text{Ca}^{2+}$  current. Each gray dot represents individual presynaptic  $\text{Ca}^{2+}$  current and the resultant IPSC. A black curve represents the relationship between  $\text{Ca}^{2+}$  and IPSC amplitudes, characterized by a 1.8 power dependency. Peak  $\text{Ca}^{2+}$  current and IPSC amplitude in each cell were normalized to the average elicited by AP waveform #1.
- (E) Left: Representative traces of presynaptic  $\text{Ca}^{2+}$  currents and IPSC trains elicited by 20 AP-like voltage commands with an identical waveform. Right: IPSC amplitude resulting from trains of identical AP waveforms evoked at 20, 50, or 100 Hz. Data are presented as mean  $\pm$  SEM.

conduction along PC axons when recorded extracellularly (Khaliq and Raman, 2005; Monsivais et al., 2005). Similarly, when we performed extracellular axonal stimulation and recorded from the terminal, we also observed a frequency-dependent attenuation and/or conduction failure of action sodium currents (Figures S3E–S3G). These results indicate that APs safely propagate along PC axons over many branches but are attenuated around the terminals.

We next examined how the observed AP attenuation affected both the  $\text{Ca}^{2+}$  influx into the presynaptic terminal and the resulting synaptic transmission (Figure 5). AP waveforms were recorded from PC terminals in response to 100 Hz axonal stimulation (Figure 5A). In this particular case, AP attenuation was modest ( $\sim 10\%$ ). Eight AP waveforms (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup>, 20<sup>th</sup>, and 26<sup>th</sup>) are superimposed in Figure 5B (shown as  $V_{\text{command}}$ ). Compared with the first AP, the following APs showed smaller peak amplitudes and greater half-height width. We then applied these AP waveforms as voltage commands (stimulated sequentially at 0.5 Hz) to the voltage-clamped presynaptic terminal and observed the subsequent  $\text{Ca}^{2+}$  currents (Figure 5B). As the amplitude of evoked AP waveform became smaller, so did the  $\text{Ca}^{2+}$  current amplitude, in a linear fashion (Figures 5B and 5C). In addition, the half-width of  $\text{Ca}^{2+}$  currents became wider as the AP waveform became wider (Figures 5B and 5C). As was expected, a reduction in the peak  $\text{Ca}^{2+}$  current was associated with a reduction in the amplitude of evoked IPSCs (Figure 5C). This is consistent with previous studies showing that the  $\text{Ca}^{2+}$  current amplitude more strongly affects the amount of transmitter release compared to its half-width (Bollmann and Sakmann, 2005). Within the range we examined, transmission at six synapses showed, on average, a  $\sim 1.8^{\text{th}}$  power dependency on the  $\text{Ca}^{2+}$  current amplitude (Figure 5D), similar to previous estimates at other inhibitory presynaptic terminals (Kraushaar and Jonas, 2000; Bucurenciu et al., 2008). To further assess the role of AP attenuation, a train of identical AP waveform commands was applied to the terminal, and the resulting IPSCs were recorded from the postsynaptic neuron. As shown in Figure 5E, direct stimulation of a terminal with such AP trains weakened the depression of IPSCs (20 Hz,  $91\% \pm 13\%$ , average of 16<sup>th</sup>–20<sup>th</sup> IPSCs,  $p > 0.6$  compared with control, 50 Hz,  $59\% \pm 12\%$ ,  $p > 0.6$ , 100 Hz,  $52\% \pm 12\%$ ,  $p < 0.05$ ,  $n = 5$  cells) (please compare with Figure 1C). Presynaptic  $\text{Ca}^{2+}$  currents showed slight facilitation during the train (Figure 5E). On the other hand, when the peak amplitude of AP trains gradually reduced to 60% (similarly to that shown in Figure 4C), the  $\text{Ca}^{2+}$  current amplitude was reduced to  $30\% \pm 11\%$  ( $n = 3$ ) of its initial value (data not shown). Collectively, attenuation of APs during high-frequency stimulation reduces  $\text{Ca}^{2+}$  influx to the presynaptic terminal, resulting in the depression of IPSCs.

### Low Excitability of PC Axon Terminals

Why do APs attenuate in close proximity to the terminals in spite of their successful conduction along the axon across many branch points? To address this question, we compared membrane excitability of an axon and a terminal by direct whole-cell recording of voltage-gated currents (see *Supplemental Experimental Procedures* for the issue of space-clamp). In response to depolarizing pulses, an axon showed large transient



**Figure 6. Low Excitability of Presynaptic Terminal Membrane Relative to an Axon**

(A) Representative voltage-gated currents recorded from an axon (right) and a presynaptic varicosity (left). Membrane potentials were depolarized with a 10 mV step from  $-70$  mV to  $+20$  mV for 80 ms.

(B) Current-voltage relations for voltage-gated Na<sup>+</sup> (left) and K<sup>+</sup> currents (right) recorded from an axon (open circles) and a terminal (filled circles); Data are presented as mean  $\pm$  SEM. n = 30 for terminals and 12 for axons.

(C) Representative traces of voltage-gated K<sup>+</sup> currents recorded by outside-out membrane patches excised from a terminal (left) and an axon (right) with (30 or 100 nM) or without OSK-1, a peptide inhibitor of Kv-1 family K<sup>+</sup> channels. Dose-response curves for inhibitory effects of OSK-1 on K<sup>+</sup> currents are shown at bottom; n = 3 for terminal and 4 for axon.

(D) Inhibition of K<sup>+</sup> currents recorded from excised terminal membrane patches by OSK-1 (100 nM, n = 3), Dendrotoxin K (50 nM, n = 5), Margatoxin (500 nM, n = 4), or Agitoxin (750 nM, n = 4).

(E) Immunofluorescent images of synaptophysin-pHluorin (sypHy, green) expressed in PCs and either Kv1.1, Kv1.3, or NaV (magenta). Line plots of signal intensities along white lines indicated in sypHy images are also shown. To estimate co-localization of each channel with synaptophysin signal, the similarity of signal

(legend continued on next page)

inward currents mediated by voltage-gated  $\text{Na}^+$  channels ( $1.3 \pm 0.2$  nA at  $-40$  mV), followed by sustained outward currents through voltage-gated  $\text{K}^+$  channels ( $1.1 \pm 0.3$  nA at  $10$  mV) (Figures 6A and 6B). On the other hand,  $\text{Na}^+$  currents recorded at PC presynaptic varicosities were relatively small ( $0.5 \pm 0.1$  nA at  $-40$  mV,  $p = 0.008$ , Student's t test), whereas  $\text{K}^+$  currents tended to be large ( $1.4 \pm 0.2$  nA at  $10$  mV) (Figures 6A and 6B). It should be noted that the  $\text{Na}^+$  current amplitude might be slightly underestimated at positive membrane potentials due to the large  $\text{K}^+$  conductance (see Figure S4A). Thus, the terminal appears able to conduct little  $\text{Na}^+$  current relative to its large  $\text{K}^+$  permeability. Given that the  $\text{Na}^+/\text{K}^+$  conductance ratio determines the membrane excitability, we predict the terminal to be relatively less excitable. In accordance with the data, immunocytochemistry demonstrated that  $\text{Na}^+$  channels were accumulated at specific parts of the axon, which were likely the nodes of Ranvier (Figure 6F), but exhibited sparse labeling at the terminals (Figure 6E). Consistently, compared with axons, axon terminals did not exhibit AP firing in response to current injection (18 out of 30 cells), presumably because of their weak  $\text{Na}^+$  conductance relative to the large shunting effect by  $\text{K}^+$  channels (Figure S4B). Direct recording from a PC axon terminal in slice showed similar results and is consistent with low excitability of the terminal (see Figure 8 and S6). To summarize, axon terminals are less excitable than the axon, which leads to the attenuation of APs.

### Low Terminal Excitability Responsible for Frequency-Dependent Synaptic Depression

To test whether the low excitability of an axon terminal underlies the depressive property of PC terminal synapses, we searched for a pharmacological agent to increase the excitability of the terminal but not the axon. For this, we performed outside-out recordings of voltage-gated currents from membrane patches excised from either the terminal or axon. As shown in Figure 6C, the voltage-gated  $\text{K}^+$  current recorded from a terminal patch was attenuated by application of OSK-1, an inhibitor of the Kv1 family (Kv1.1, Kv1.2, and Kv1.3) of voltage-gated  $\text{K}^+$  channels ( $29\% \pm 6\%$  of basal at  $100$  nM). In contrast, OSK-1 did not affect the  $\text{K}^+$  current recorded from the axonal membrane ( $100\% \pm 18\%$  of basal,  $p = 0.027$ , Student's t test compared with terminal) (Figure 6C). Thus, the composition of voltage-gated  $\text{K}^+$  channels differs between the axon and the terminal. In addition, the voltage-gated  $\text{K}^+$  current recorded from the terminal was inhibited by  $50$  nM of the Kv1.1 and 1.2 blocker Dendrotoxin K, ( $59\% \pm 12\%$ ,  $n = 5$ ),  $500$  nM of the Kv1.3 blocker Margatoxin ( $65\% \pm 8\%$ ,  $n = 4$ ), and  $750$  nM of the Kv1.3 blocker Agitoxin ( $78\% \pm 6\%$ ,  $n = 4$ ) (Figure 6D). Because the  $\text{K}^+$  conductance in the terminal was highly sensitive to  $2$  mM TEA (not shown), Kv1.2 seemed not to be involved. Taken these results together, Kv1.1 and Kv1.3 are likely to be the predominant subtypes that mediate the voltage-gated  $\text{K}^+$  current at PC terminals. Accordingly, immunocytochemistry showed localization of both Kv1.1 and Kv1.3 at PC axonal varicosities (visualized by sypHy, synap-

tophysin tagged with a GFP variant pHluorin), but not at axonal locations (Figure 6E). The area positive for sypHy was also positive for VGAT (not shown). Intensity profiles in the line scan showed colocalization of sypHy with Kv, but not  $\text{Na}_v$  (Figure 6E). Rather,  $\text{Na}_v$  was mainly localized in the axon (Figure 6F).

In the following sets of experiments, we confirmed that OSK-1 suppresses AP attenuation by selectively altering membrane excitability. First, recordings from the terminal showed that OSK-1 weakened the extent of frequency-dependent AP attenuation ( $n = 4$ , data not shown) without affecting the resting membrane potential of the terminal (before,  $-69 \pm 2$  mV; after OSK-1 application,  $-68 \pm 2$  mV,  $p = 0.82$ ,  $n = 4$ ). Second,  $\text{Ca}^{2+}$  imaging using Oregon green BAPTA-1 ( $500$   $\mu\text{M}$  applied to a PC soma through a patch pipette) demonstrated that the  $\text{Ca}^{2+}$  increase around the terminals following  $100$  APs at  $100$  Hz was augmented by OSK-1 application (Figure S5). Importantly, the augmenting effect of OSK-1 became evident not at the beginning of the AP train, but at later points during the stimulation. Thus, the  $\text{Ca}^{2+}$  imaging data further suggests that OSK-1 weakened the high-frequency-dependent negative regulation of AP and the resulting reduction in presynaptic  $\text{Ca}^{2+}$  influx.

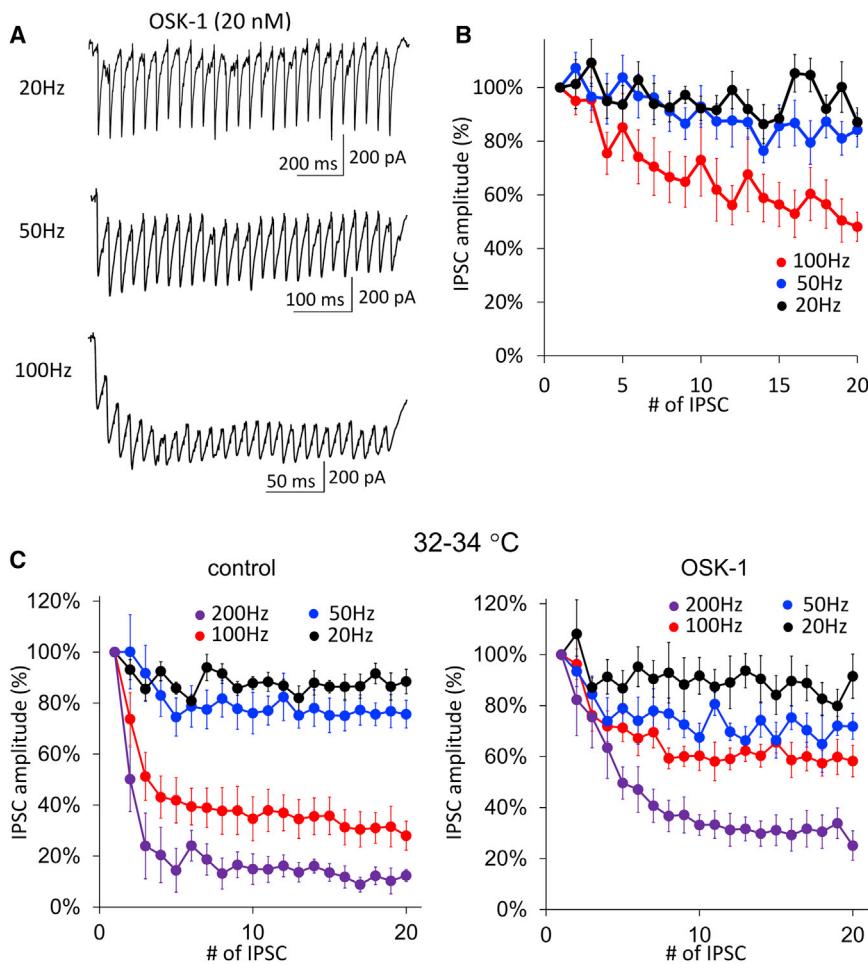
We next examined how the increase in presynaptic excitability by  $20$  nM OSK-1 affected the frequency-dependent depression of synaptic transmission at PC synapses. As shown in Figures 7A and 7B, depression of synaptic transmission upon  $50$  Hz stimulation of the PC soma was almost abolished by OSK-1 treatment ( $84\% \pm 6\%$ , average of  $16^{\text{th}}\text{--}20^{\text{th}}$  IPSCs,  $p = 0.007$ , Student's t test compared with control). Similarly, in response to  $100$  Hz trains, the synaptic depression was also markedly reduced by OSK-1 ( $54\% \pm 8\%$ ,  $p = 0.0003$ ). Thus, increased membrane excitability by inhibition of  $\text{K}^+$  channels in the presynaptic terminal weakened frequency-dependent depression of IPSCs. These results were in accord with the idea that the AP attenuation around the presynaptic terminals contributed to depression of synaptic transmission. Temperatures may affect electrical and synaptic properties (Kushmerick et al., 2006; Fernández-Alfonso and Ryan, 2004), but the frequency-dependent depression of IPSC was also evident at physiological temperature ( $32^\circ\text{C}$ – $34^\circ\text{C}$ ) ( $20$  Hz,  $87\% \pm 3\%$ , average of  $16^{\text{th}}\text{--}20^{\text{th}}$  IPSCs;  $50$  Hz,  $81\% \pm 5\%$ ;  $100$  Hz,  $30\% \pm 7\%$ ;  $200$  Hz,  $11\% \pm 3\%$ ) and was suppressed by inhibition of Kv1 channels with OSK-1 ( $20$  Hz,  $87\% \pm 7\%$ ;  $50$  Hz,  $71\% \pm 5\%$ ;  $100$  Hz,  $59\% \pm 5\%$ ,  $p < 0.001$  compared with control;  $200$  Hz,  $30\% \pm 6\%$ ,  $p < 0.05$ ) (Figure 7C). Under physiological temperature, we also found attenuation of APs at the presynaptic terminal when an axon was stimulated at  $100$  Hz ( $n = 3$ ).

### Low Presynaptic Excitability Determines Synaptic Depression in Acute Slices at Multiple Types of Synapse

We next attempted to confirm our results at PC-DCN synapses in acute slices prepared from postnatal day 9–16 rats. To examine the excitability of the terminal, we fluorescently labeled PC axons in the DCN with DiO ( $0.5\%$  in external solution) applied locally to

distribution pattern was assessed by calculating the Pearson product-moment correlation coefficient, which ranges between  $-1$  and  $1$  (higher value represents higher co-localization; Kv1.1,  $0.55 \pm 0.15$ ; Kv1.3,  $0.64 \pm 0.09$ ;  $\text{Na}_v$ ,  $0.23 \pm 0.11$ ;  $p < 0.05$ ).

(F) Immunocytochemistry for  $\text{Na}_v$  (magenta) and EGFP (green, a PC axon). High signals for  $\text{Na}_v$  are observed in an axon as white or magenta signals (highlighted by white arrows). See also Figure S4.



**Figure 7. Frequency-Dependent Depression of IPSCs Was Attenuated by Blocking Terminal-Specific Voltage-Gated K<sup>+</sup> Channels**

(A) Representative traces of IPSC trains (averaged from 20 traces) stimulated at 20, 50, or 100 Hz in the presence of 20 nM OSK-1. IPSCs were triggered from PC soma in the whole-cell configuration.

(B) IPSC amplitudes were normalized to that of the first;  $n = 8$ . The effect of OSK-1 can be assessed by comparing the data to that performed in the absence of OSK-1 (shown in Figure 1C).

(C) Plot of average IPSC amplitudes evoked by train stimulation at 20, 50, 100, and 200 Hz with (right) or without (left) 20 nM OSK-1 at 32°C–34°C;  $n = 8$  in control conditions, except for 200 Hz ( $n = 5$ );  $n = 5$  for OSK-1-treated recordings. Data are presented as mean  $\pm$  SEM. See also Figure S5.

the white matter of sagittal cerebellar slices through a glass pipette (for 30 min). Once the PC axons could be visualized, we performed direct patch-clamp recordings from the terminals (Figure 8A). It was likely that we recorded from presynaptic terminals, because Cm jumps were observed in response to depolarizing pulses though, notably, the peak Cm was smaller than in the culture preparation (Figure S6A). APs were elicited at a frequency of 100 Hz by extracellular electrical stimulation (100  $\mu$ sec) through a glass electrode placed in the white matter. Although APs were reliably recorded at the beginning of trains, they tended to fail over the course of stimulation, in line with our observations in culture. On average ( $n = 5$ ), the success probability of presynaptic spikes dropped by 40%–50% during 100 Hz train stimulation (Figure 8B).

Next, we examined whether weak excitability of PC axon terminals mediated frequency-dependent depression of synaptic transmission *in situ*. Whole-cell recordings were made from a neuron with a large cell body (>15  $\mu$ m diameter), and evoked IPSCs were recorded following electrical stimulation in the white matter. Consistent with previous studies, IPSCs from PC axon terminals were identified by their characteristic all-or-none response to increasing stimulation intensity and their relatively large amplitude (Telgkamp and Raman, 2002; Pedroarena and

Schwarz, 2003). IPSC amplitudes were somewhat larger in slice recordings, which may be due to multiple varicosities along an axon innervating a single postsynaptic neuron. However, we do not exclude the possibility that Pr was somewhat higher in slices. Because cultured PC axon terminals fired at ~5 Hz at rest (see Figure S6), test stimulations of 20, 50, or 100 Hz were applied to the white matter following 10 pre-stimulations at 5 Hz. In accordance with previous studies (Telgkamp and Raman, 2002; Pedroarena and Schwarz, 2003), recordings of IPSCs at PC-DCN synapses in acute sli-

cles exhibited similar frequency-dependent depression to that observed at cultured PC outputs (20 Hz, 66%  $\pm$  11%, average of 16<sup>th</sup>–20<sup>th</sup> IPSCs; 50 Hz, 28%  $\pm$  11%; 100 Hz, 16%  $\pm$  5%) (Figure 8). After application of 50 nM OSK-1, IPSC amplitude was increased by 1.72  $\pm$  0.31 fold, and the subsequent IPSCs were all larger than the original level. If depletion of the RRP was responsible for the extent of synaptic depression, the increase in the 1<sup>st</sup> IPSC amplitude would lead to decrease of the amplitude of subsequent IPSCs, and hence enhance the short-term depression. In contrast, we observed that the synaptic depression induced by repetitive stimulation was weakened by treatment of OSK-1 (20 Hz, 77%  $\pm$  6% at 20<sup>th</sup> IPSC,  $p = 0.11$  by paired t test compared with basal; 50 Hz, 47%  $\pm$  15%,  $p = 0.012$ ; 100 Hz, 27%  $\pm$  9%,  $p = 0.019$ ) (Figure 8). Such data support the idea that low membrane excitability of PC axon terminals contributed to frequency-dependent depression. Without pre-stimulation, the depression of IPSC trains relative to the first IPSC was stronger (10 Hz, 40%  $\pm$  4%, average of 16<sup>th</sup>–20<sup>th</sup> IPSCs; 20 Hz, 30%  $\pm$  2%; 50 Hz, 17%  $\pm$  2%; 100 Hz, 11%  $\pm$  1%, Figure S6), in accordance with previous studies (Telgkamp et al., 2004). In this condition, 50 nM OSK-1 weakened IPSC depression when the stimulation frequency was set to either 10 Hz (55%  $\pm$  5%,  $p = 0.004$  by paired t test), 20 Hz

(42%  $\pm$  4%,  $p = 0.004$ ), or 50 Hz (22%  $\pm$  2%,  $p = 0.038$ ), but not during 100 Hz stimulation (11%  $\pm$  1%,  $p = 0.5$ , Figure S6). The absence of OSK-1-mediated effect at 100 Hz might be possibly due to a K<sup>+</sup>-channel-independent mechanism that favorably operates even at relatively low stimulation frequencies.

It is possible that K<sup>+</sup> channel blockade increased Ca<sup>2+</sup> influx, thereby augmenting Ca<sup>2+</sup>-dependent synaptic vesicle replenishment (Dittman and Regehr, 1998; Stevens and Wesseling, 1998). However, while increasing external Ca<sup>2+</sup> concentration from 2 to 4 mM increased the IPSC amplitude (1.99  $\pm$  0.19 times), similar to OSK-1, the depression of IPSC amplitude during train stimulation did not markedly change (Figure S6H). To summarize, low excitability of PC axon terminals enriched with OSK-1-sensitive Kv1.1 and Kv1.3 channels underlies the depression of PC outputs. In contrast, depletion of the RRP appears to play no major role at this synapse.

Finally, we tested whether weak membrane excitability also causes synaptic depression at other inhibitory synapses. We performed paired recordings from cerebellar fast-spiking BCs and PCs at physiological temperature. A 100 Hz train of presynaptic stimulation (0 mV for 2 ms) caused synaptic depression at this inhibitory connection (Vincent and Marty, 1996; Sakaba, 2008). Application of TEA (10 mM), a K<sup>+</sup> channel blocker, augmented the first IPSC amplitudes to 234%  $\pm$  57% ( $n = 6$ ) and, importantly, converted synaptic depression into facilitation (Figure 8F,  $p < 0.01$ ). If RRP depletion primarily mediates the depression, an increase in the Pr should have augmented the depression. The results are similar to the PC-DCN synapse and consistent with the idea that excitability controls synaptic depression. Accordingly, extracellular recordings of AP trains at 50 and 100 Hz showed that the AP amplitude tended to be attenuated (Figure S6I). AP propagation along the axon is reported to be reliable in fast-spiking neurons (Hu and Jonas, 2014), and hence, AP attenuation is likely to occur around the terminals.

We also recorded IPSCs from cultured hippocampal neurons where release-independent depression has been previously suggested (Kraushaar and Jonas, 2000). As shown in Figure 8G, IPSCs were strongly depressed upon repetitive stimulation. Interestingly, inhibition of K<sup>+</sup> channels by 10 mM TEA weakened the high-frequency-dependent depression (20, 50, and 100 Hz,  $p < 0.05$ ) but did not affect synaptic depression when stimulation frequency was lowered to 10 Hz (data not shown). It should be noted that unlike PC output synapses, the normalized IPSC amplitudes after the 8<sup>th</sup> stimulation became similar to those without TEA treatment, suggesting that the RRP would be depleted after high-frequency stimulation.

Taken these results together, low presynaptic membrane excitability contributes to depression of inhibitory synaptic transmission not only in cerebellar PCs but also in cerebellar BCs and hippocampal neurons.

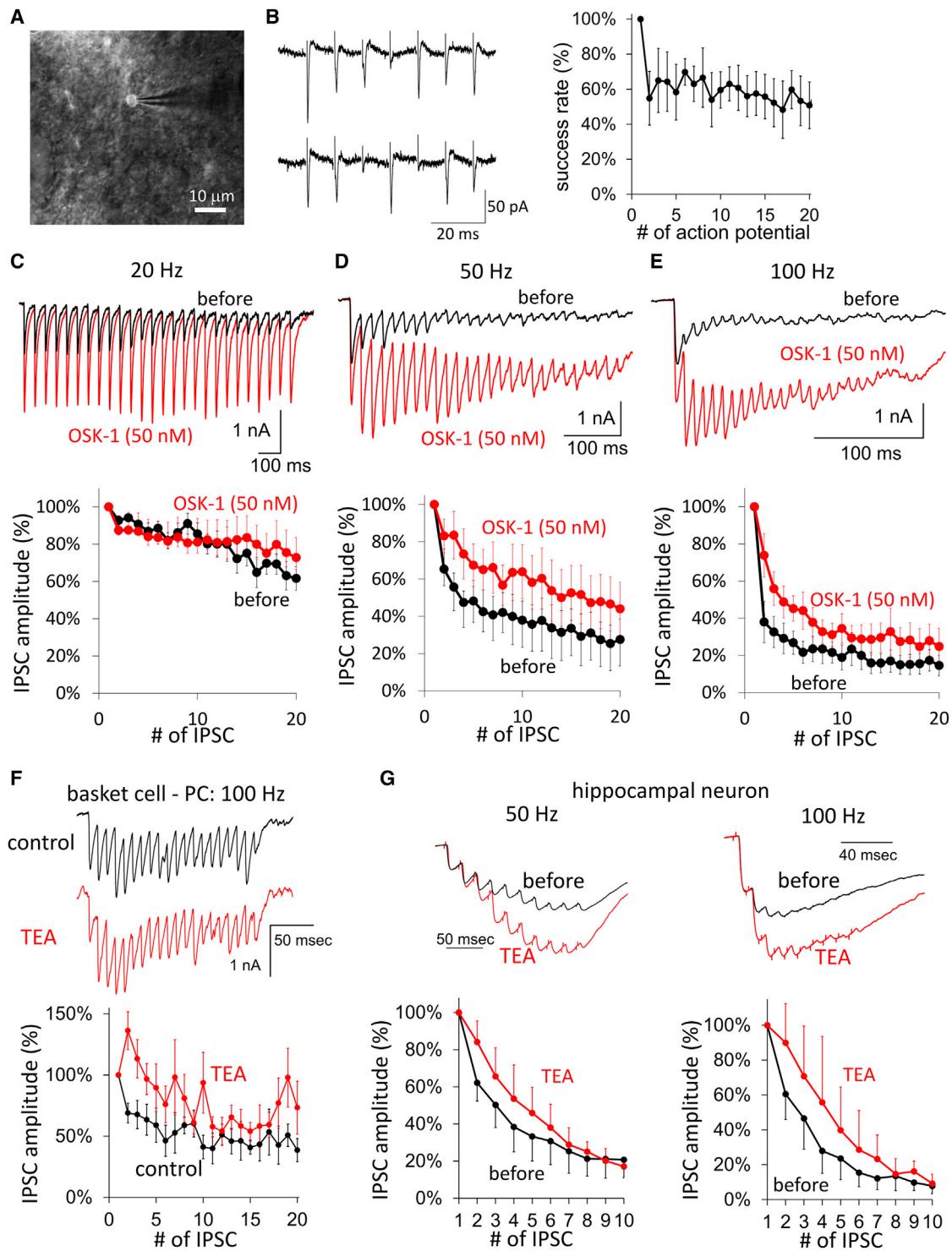
## DISCUSSION

By developing a technique to directly record from inhibitory pre-synaptic terminals of cultured cerebellar PCs, we have demonstrated an information processing mechanism that occurs in close proximity to axon terminals. By taking advantage of a cere-

bellar culture preparation and aided by visualization of axons and small terminals expressing EGFP, we succeeded in overcoming the difficult task of direct recordings from axons and small varicosities (1–3  $\mu\text{m}$ ) (but see Novak et al., 2013). This technique allowed us to reveal a series of findings. (1) We demonstrated the basic mechanism of transmitter release at an inhibitory terminal. Measurement of presynaptic Cm, deconvolution of evoked IPSCs, and Ca<sup>2+</sup> uncaging revealed the presence of a large pool of releasable vesicles, of which <1% were exocytosed upon a single AP. (2) We identified low membrane excitability to be a key determinant of short-term depression. Paired patch-clamp recordings from an axon and a terminal demonstrated that APs were conducted reliably along the axon and over bifurcations, but tended to be attenuated around terminals. This resulted in a reduction of Ca<sup>2+</sup> influx within the terminal, thus causing weaker synaptic transmission. Our data further suggest that the low membrane excitability of axon terminals resulted from sparse expression of Na<sup>+</sup> channels relative to the abundant localization of the Kv1-familiy of voltage-gated K<sup>+</sup> channels. Inhibition of terminal K<sup>+</sup> channels suppressed the frequency-dependent depression of PC outputs in both culture and slice. Moreover, overall results were confirmed by a less invasive, imaging approach. Thus, our data show that information conducted along an axon is subject to frequency-dependent modulation at sites close to terminals and is then transmitted to postsynaptic neurons with altered strength.

## The RRP at PC Terminals

The RRP of synaptic vesicles and the kinetics of transmitter release have been measured at excitatory synapses where Cm measurements and simultaneous recordings from presynaptic and postsynaptic compartments are possible (von Gersdorff and Matthews, 1994; Sun and Wu, 2001; Hallermann et al., 2003). In spite of the important roles of inhibitory synapses in information processing in the circuit (Lawrence and McBain, 2003; Jonas et al., 2004), a study of inhibitory terminals has been lacking. Here we have simultaneously measured the presynaptic Cm and IPSCs from PC output synapses that exhibit frequency-dependent depression. Postsynaptic GABA receptors tend to be saturated or desensitized at inhibitory synapses (Pugh and Raman, 2005); therefore, we used presynaptic Cm measurements that reflect exocytosis of synaptic vesicles more reliably. Surprisingly, we observed a very large RRP (>1,000 vesicles) with an extremely low Pr (~0.005). At most excitatory and inhibitory synapses that undergo depression, Pr has been considered to be relatively high (>0.1) (Kraushaar and Jonas, 2000), and depletion of the RRP considered to determine the time course of synaptic depression during a train of APs (Saviane and Silver, 2006). In contrast, the RRP size is large and Pr low at facilitating synapses (Millar et al., 2005; Hallermann et al., 2003). The PC synapse is surprising in this respect, because this depressing synapse has a low Pr and a large RRP, typical features of facilitating synapses. Nevertheless, the observation is consistent with morphological data at the PC output synapse and other inhibitory synapses, where the release sites are less well defined but accumulation of synaptic vesicles are observed (Telgkamp et al., 2004; Palay and Chan-Palay, 1974).



**Figure 8. IPSC Depression at PC-DCN Synapses in Cerebellar Slices Was Suppressed by K<sup>+</sup> Channel Blockade**

(A) An image of a patch-clamped PC varicosity visualized by DiO applied to the white matter of a cerebellar slice.

(B) Left: Example traces of extracellular spike recordings from a PC terminal upon the white matter stimulation at 100 Hz. Right: Average success rate of pre-synaptic spikes during a 100 Hz train was plotted against stimulus number.

(C–E) Representative traces (average of 10 traces) and amplitudes of IPSC trains, normalized to the first, stimulated at 20 (C), 50 (D), and 100 Hz (E) before (black) and after (red) OSK-1 (50 nM) application. Prior to train stimulation, PC axons were stimulated ten times at 5 Hz to mimic spontaneous firing in a cultured PC (4–6 Hz); n = 7.

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The time constant of the RRP depletion at PC synapses was slower than the 0.5–3 ms previously observed for glutamatergic synapses (Sun and Wu, 2001; Hallermann et al., 2003; Sakaba and Neher, 2001; Mennerick and Matthews, 1996). Slow release from the PC synapse is consistent with a low Pr under basal conditions. Because the  $\text{Ca}^{2+}$  sensitivity for release is similar to that at excitatory synapses, the low Pr of PC synapses could be explained by a relatively small number of  $\text{Ca}^{2+}$  channels at release sites during an AP, or by weaker coupling between  $\text{Ca}^{2+}$  channels and release machinery. The EGTA insensitivity of release suggests that  $\text{Ca}^{2+}$  channels are coupled tightly to synaptic vesicles, but that the peak  $[\text{Ca}^{2+}]_i$  is much lower than that postulated at other inhibitory synapses (Bucurenciu et al., 2008; Sakaba, 2008). While this study shows that release probability per synaptic vesicle is low, it is consistent with the finding that release probability per synapse is high (Telgkamp et al., 2004). The low Pr of a PC terminal contrasts to the relatively high Pr at inhibitory synapses formed by fast-spiking interneurons, as was demonstrated by both statistical analysis of IPSCs (Kraushaar and Jonas, 2000) and analysis using  $\text{Ca}^{2+}$  uncaging (Sakaba, 2008). However, no direct measurements of exocytosis have been performed at these synapses so far, and thus it remains to be investigated whether the low Pr and large RRP are general features of inhibitory synapses or unique features at PC synapses.

### The Mechanisms of Synaptic Depression at the PC Output Synapse

Short-term synaptic depression is seen at many central synapses. At glutamatergic synapses, the RRP depletion is considered to be one of the major mechanisms of depression (Zucker and Regehr, 2002), though other mechanisms such as  $\text{Ca}^{2+}$  current inactivation and AP conduction failure at axonal branches also contribute (Xu and Wu, 2005; Brody and Yue, 2000; Prakriya and Mennerick, 2000). Characteristics of inhibitory synapses are clearly different from those of excitatory synapses. For example, depression time course is relatively insensitive to changes in the initial Pr, which can be caused by alterations in the external  $[\text{Ca}^{2+}]$  (Kraushaar and Jonas, 2000). In contrast, glutamatergic synapses show deeper depression at higher Pr (Dittman and Regehr, 1998). Thus, it was hypothesized that inhibitory synapses either have a release-independent component of synaptic depression or are engendered with special release mechanisms (Kraushaar and Jonas, 2000, Kirischuk et al., 2002; but see Moulder and Mennerick, 2005). By direct recordings from axons and terminals, we have identified the release-independent mechanism of synaptic depression, that low membrane excitability specifically at the terminal, but not the axon, attenuates the presynaptic AP waveform. Axonal branch failure (Brody and Yue, 2000; Prakriya and Mennerick, 2000) is unlikely to contribute to the depression because of the high reliability of AP conduction in PC axons (Khaliq and Raman, 2005; Monsivais et al., 2005), a finding that we confirmed using dual recordings

from two different sites along the axon. Due to the non-linear dependence of transmitter release on  $\text{Ca}^{2+}$ , attenuation of APs is a powerful mechanism of synaptic depression, though we emphasize the importance of other depression mechanisms previously identified at this synapse (Telgkamp et al., 2004).

We propose that the low membrane excitability causing the synaptic depression at PC axon terminals was due to a relatively low  $\text{Na}^+$  channel number and an abundance of  $\text{K}^+$  channels. This contrasts with the view that synaptic depression is generally mediated by vesicle pool depletion, release site inactivation, or  $\text{Ca}^{2+}$  channel inactivation, which all assume that APs are conducted faithfully to the terminal (Fioravante and Regehr, 2011). Because a release-independent component of synaptic depression is observed in many inhibitory synapses, one may speculate that low excitability may be a general feature of inhibitory terminals. Consistently, we have shown that  $\text{K}^+$  channel blockers weakened synaptic depression both at the BC-PC synapses in acute cerebellar slices and at cultured hippocampal inhibitory synapses. We should note that AP attenuation is less drastic at BC terminals compared with PC terminals (Figure S6), and the impact of excitability over other factors might differ between synapse types.

Sparse  $\text{Na}^+$  channel clustering in axon terminals has also been reported in other preparations such as the calyx of Held (Leão et al., 2005) and at the neuro-muscular junction (Lindgren and Moore, 1989). The axon heminode near the terminal has abundant  $\text{Na}^+$  channels, yet the terminal does not, which might also contribute to low membrane excitability around the terminal. Nevertheless, large  $\text{Na}^+$  currents originating from the heminode can be measured at the calyx terminal, thus ensuring reliable AP firing. Absence of  $\text{Na}^+$  channels in the terminals seems to limit the duration of APs. Reduction of  $\text{Na}^+$  currents by TTX greatly diminishes the EPSCs (Wu et al., 2004), suggesting that the ratio of  $\text{Na}^+$  currents over  $\text{K}^+$  currents crucially determines the fidelity of AP firing and synaptic transmission at the calyx synapse.  $\text{K}^+$  channel expression at the calyx synapse is strong but is considered to shorten the AP waveform rather than causing the AP attenuation. Taken together, large  $\text{Na}^+$  and  $\text{K}^+$  currents around the calyx of Held terminal contribute to reliable and fast AP firing up to kHz frequencies (Taschenberger and von Gersdorff, 2000). En-passant-type mossy fiber terminals in hippocampus, another model system of glutamatergic terminals, express abundant  $\text{Na}^+$  and  $\text{K}^+$  channels (Engel and Jonas, 2005; Geiger and Jonas, 2000), which amplify the presynaptic AP amplitude and limit the AP duration. Cerebellar mossy fiber terminals also have abundant  $\text{Na}^+$  and  $\text{K}^+$  channels and follow APs up to hundreds of Hz (Rancz et al., 2007). Therefore, the localization pattern of  $\text{Na}^+$  and  $\text{K}^+$  channels around terminals impacts the reliability of presynaptic AP firing and synaptic transmission.

### Physiological Implications

PCs provide the sole output from the cerebellar cortex and are considered to form strong inhibitory synapses onto DCN

(F and G) Top panels show representative traces of IPSCs at BC-PC synapses (F) and at cultured hippocampal inhibitory synapses (G) (average of 10–30 traces) with (red) and without (black) 10 mM TEA. The presynaptic neuron was stimulated at 50 or 100 Hz by depolarizing the soma to 0 mV for 2 ms. Bottom panels show the amplitude of IPSC trains (normalized to the 1<sup>st</sup> amplitude), with (red) and without (black) TEA. Differences between the two plots were statistically significant ( $p < 0.01$  in [F] and  $p < 0.05$  in [G]). To isolate the high-frequency component of depression in hippocampal neurons, pre-stimulation (five pulses at 10 Hz) was applied before the train stimulation (50 or 100 Hz). Error bars represent SEM. See also Figure S6.

neurons. Axonal AP conduction was shown to be reliable up to hundreds of Hz (Khaliq and Raman, 2005; Monsivais et al., 2005). However, the properties of axon terminals have not been examined in depth. Synaptic strengths under low frequency stimulation seem to be reliable at this synapse. Reliability is aided by multivesicular release, spillover of transmitter, postsynaptic saturation (Telgkamp et al., 2004), as well as a large RRP size (this study). As a consequence of the large RRP size and low Pr, the vesicle pool is kept far from depletion (Kraushaar and Jonas, 2000; Taschenberger and von Gersdorff, 2000). More importantly, synaptic strength becomes very unreliable at high-frequency stimulation, as shown in this study.

What is the advantage of such a synaptic design with low excitability, low Pr, and a large RRP? We consider three possibilities. First, regulation of excitability might be an easy method to establish target-dependent differences in synaptic strengths and plasticity. In addition to the DCN, PC axons make synapses within other regions such as the vestibular nucleus (Shin et al., 2011) as well as other PCs (Orduz and Llano, 2007), where short-term facilitation takes place. Facilitation at such synapses might be due to a greater expression of  $\text{Na}^+$  channels. Inhibition of  $\text{K}^+$  channels by OSK-1 makes the frequency-dependent depression at the PC-DCN synapse somewhat frequency-independent between 20 and 100 Hz (see Figure 7), similar to synapses in the vestibular nucleus where PCs also make an inhibitory contact (Bagnall et al., 2008). As a second possibility, low Pr may support transmission under high rates of spontaneous firing. Low Pr and large RRP may limit the extent of synaptic vesicle depletion and support transmission during long-lasting stimulation (Kraushaar and Jonas, 2000; Taschenberger and von Gersdorff, 2000). Because basal firing frequency is high *in vivo* and dynamically changes during motor control (Loewenstein et al., 2005), the filtering characteristics are functionally important. The frequency-dependent depression due to the low excitability of presynaptic boutons might function to fine-tune the working range of postsynaptic neuronal activity, without depleting the presynaptic RRP. As a third possibility, a large RRP may allow room for short- and long-term synaptic plasticity, and vesicle pool depletion may become rate limiting only after induction of plasticity.

Potent inhibition of the postsynaptic DCN neurons caused by burst firing of PCs is followed by a rebound depolarization, accompanied by an increase in  $[\text{Ca}^{2+}]_i$ . This inhibition-triggered rebound depolarization leads both to synaptic plasticity at the homosynaptic PC synapses (Aizenman et al., 1998) and modulation of plasticity at excitatory mossy fiber synapses (Pugh and Raman, 2006). Thus, frequency-dependent signal modulation around axon terminals might play a role in adaptive changes of neuronal processing in the DCN. It remains to be investigated how the characteristic information processing at PC axon terminals affects the overall neuronal computation in the cerebellar networks *in vivo*.

## EXPERIMENTAL PROCEDURES

Animal experiments were performed in accordance with the guidelines regarding care and use of animals for experimental procedures of the NIH (U.S.A.) and Doshisha University and were approved by the local committee for handling experimental animals in Doshisha University.

## Culture and Slice Preparation

The method for preparing primary cultures of cerebellar neurons and cerebellar sagittal brain slices were similar to those in previous studies (Kawaguchi and Hirano, 2007; Sakaba, 2008). Hippocampal neurons were cultured in Neurobasal medium (GIBCO) with B27 supplement (GIBCO), and used for experiments 10–16 days after plating.

## DNA Construction and Transfection

CAG promoter-based AAV vector (Kaneko et al., 2011) for EGFP transfection was a generous gift from Dr. Kengaku (Kyoto University). cDNAs of syph and synaptophysin were obtained from Addgene (plasmid #24478) and from Dr. T. Hirano (Kyoto University). Transfection was performed by direct micro-injection of cDNA into the nucleus of a PC through a sharp glass pipette or by AAV infection.

## Electrophysiology

Electrophysiological experiments were performed similarly to previous studies (Kawaguchi and Hirano, 2007; Sakaba, 2008) at room temperature (20°C–24°C) or at physiological temperature (32°C–36°C). Cm measurements were carried out using sine + DC technique (Neher and Marty, 1982). The deconvolution analysis and  $\text{Ca}^{2+}$  uncaging were performed as previously described (Sakaba, 2008). For details, see Supplemental Information.

## Statistics

Data are presented as mean  $\pm$  SEM. Statistical significance was assessed by paired t test, unpaired Student's t test, Mann-Whitney U test or ANOVA.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2015.02.013>.

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