The potassium channel subunit Kvβ1 serves as a major control point for synaptic facilitation

In Ha Cho \textsuperscript{a,b,*}, Lauren C. Panzera \textsuperscript{a,b,*}, Morven Chin \textsuperscript{a,†}, Scott A. Alpizar \textsuperscript{a,b,*}, Genaro E. Olveda \textsuperscript{a,b,*}, Robert A. Hill \textsuperscript{a,b,*}, and Michael B. Hoppa \textsuperscript{a,b,2,*}

\textsuperscript{a}Department of Biology, Dartmouth College, Hanover, NH 03755; and \textsuperscript{b}Molecular and Cellular Biology Graduate Program, Dartmouth College, Hanover, NH 03755

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Analysis of the presynaptic action potential’s (AP\textsubscript{syn}) role in synaptic facilitation in hippocampal pyramidal neurons has been difficult due to size limitations of axons. We overcame these size barriers by combining high-resolution optical recordings of membrane potential, exocytosis, and Ca\textsuperscript{2+} in cultured hippocampal neurons. These recordings revealed a critical and selective role for Kv1 channel inactivation in synaptic facilitation of excitatory hippocampal neurons. Presynaptic Kv1 channel inactivation was mediated by the Kvβ1 subunit and had a surprisingly rapid onset that was readily apparent even in brief physiological stimulation paradigms including paired-pulse stimulation. Genetic depletion of Kvβ1 blocked all broadening of the AP\textsubscript{syn} during high-frequency stimulation and eliminated synaptic facilitation without altering the initial probability of vesicle release. Thus, using all quantitative optical measurements of presynaptic physiology, we reveal a critical role for presynaptic K\textsubscript{+} channels in synaptic facilitation at presynaptic terminals of the hippocampal upstream of the exocytic machinery.

Significance

Nerve terminals generally engage in two opposite and essential forms of synaptic plasticity (facilitation or depression) that play critical roles in learning and memory. While the molecular components of both types of terminals are similar with regards to vesicle fusion, much less is known about their molecular control of electrical signaling. Measurements of the electrical impulses (action potentials) underlying these two forms of plasticity have been difficult in small nerve terminals due to their size. In this study we deployed optical physiology measurements to overcome this size barrier. Here, we identify a unique mechanism (Kvβ1 subunit) that enables broadening of the presynaptic action potentials that selectively supports synaptic facilitation, but does not alter any other aspects of nerve terminal function.

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The authors declare no competing interest.

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1Present address: Program in Neuroscience, Harvard University, Cambridge, MA 02138.
2To whom correspondence may be addressed. Email: michael.b.hoppa@dartmouth.edu.

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he action potential (AP) firing pattern or “spike code” as typically measured from the soma is a gold standard for neural excitability within circuits. However, at presynaptic terminals the quantitative relationship between the input AP spike code and the magnitude of exocytosis, or vesicle fusion events per AP, can change dynamically as a result of stimulation frequency or firing pattern. Increased firing frequency can significantly increase the number of vesicles that fuse from an identical number of APs. This phenomenon is known as short-term synaptic facilitation, which can significantly enhance information transfer at synapses influencing several aspects of learning and memory (1). Thus, it is important to completely understand the underlying molecular and cellular mechanisms of synaptic facilitation.

A critical initial step in exocytosis is the arrival of AP\textsubscript{syn} at boutons, whose waveform can exhibit plasticity based on firing frequency. Repetitive firing may cause inactivation of axonal voltage-gated sodium (Na\textsubscript{v}) channels and voltage-gated potassium (K\textsubscript{+}) channels that control the depolarization and hyperpolarization of the waveform, respectively. K\textsubscript{+} inactivation primarily leads to an increase in AP width or broadening (2–9). The width of the AP\textsubscript{syn} controls the fraction of time that Ca\textsuperscript{2+} channels open and the driving force of Ca\textsuperscript{2+} entry (10). These changes in voltage kinetics during the AP\textsubscript{syn} will also impact the shape or profile of the Ca\textsuperscript{2+} microdomain envelope that builds locally around open Ca\textsuperscript{2+} channels in the terminal (11, 12). The highly nonlinear influence of Ca\textsuperscript{2+} on exocytosis (13, 14) thus dictates that modest AP\textsubscript{syn} broadening has the potential to critically impact synaptic facilitation (15–17). Indeed, AP\textsubscript{syn} broadening during repetitive firing has been demonstrated to cause the facilitation of exocytosis in the pituitary nerve (3), dorsal root ganglion (18), and mossy fiber bouton (2), all due to K\textsubscript{+} channel inactivation. However, the AP\textsubscript{syn} waveform in Purkinje cells has also been shown to undergo frequency-dependent decreases in amplitude that substantially contribute to synaptic depression (19). Thus, it is best to consider the AP\textsubscript{syn} as a plastic signal that can powerfully modulate exocytosis bidirectionally, rather than as a digital spike acting solely as an initiation signal. We therefore reason that the somatic AP has proven to be a poor predictor of exocytosis magnitude as a result of a failure to resolve the AP\textsubscript{syn} waveform and its molecular regulators in the majority of brain regions.

As opposed to the majority of large synapses, en passant terminals are most commonly involved in brain regions associated with synaptic plasticity. Investigating the molecular regulation of AP\textsubscript{syn} in the common en passant nerve terminals of the cortex and hippocampus remains elusive due to the small size of these structures (<1 μm), which makes them inaccessible for whole-cell patch clamp recording. An innovative initial strategy to overcome these size restrictions was the use of voltage dyes, which failed to detect use-dependent changes in the AP\textsubscript{syn} in hippocampal slices (20). However, these dyes were limited by very low voltage sensitivity (<0.5% change in fluorescence for an AP) requiring population averaging, and these dyes were unable to report a stable resting voltage during stimulation (20). Moreover, it was found only later that this class of voltage dyes were phototoxic and altered membrane physiology, limiting their usefulness in small axons (21). As a result of these complications, the question of AP\textsubscript{syn} plasticity as a modulator of synaptic plasticity

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facilitation remains unanswered for hippocampal neurons. Our group has overcome the size barrier of hippocampal axons, while also avoiding cell population averaging and dye toxicity by pioneering the use of genetically encoded rhodopsin-based voltage indicators. Here, we measure the APsyn of individual en passant terminals from both inhibitory and excitatory hippocampal neurons. These measurements demonstrate a striking contrast between facilitating excitatory and depressing inhibitory nerve terminals in the hippocampus. We discovered that excitatory axons and terminals are uniquely enriched with a combination of Kv1.1/1.2 heteromers and Kvβ1 subunits. This combination of Kv subunits causes rapid APsyn broadening during brief periods of high-frequency firing. This broadening was essential for enabling synaptic facilitation without altering initial exocytosis. We also found that simply overexpressing this Kvβ1 subunit made inhibitory neurons switch from depressing during high-frequency stimulation to facilitation. Taken together, these results suggest that the molecular control of presynaptic Kv channel inactivation is an important modulator of synaptic facilitation upstream of vesicle release machinery.

**Results**

Previously, our measurements of the APsyn in cultured hippocampal neurons found a very high ratio of Kv to Na channels with Kv1.1/1.2 channels dominating repolarization (22), similar to in vivo measurements from CA3 neurons (23). We measured the sensitivity of exocytosis to changes in Kv1.1/1.2 conductance using an optical probe of exocytosis (vGLUT1-pHluorin; vG-pH) (14, 24). Blockade of Kv1.1/1.2 channels by application of dendrotoxin (DTX) greatly enhanced exocytosis in excitatory hippocampal nerve terminals by 61 ± 18% when stimulated with 1 AP (Fig. 1A and B). The sensitivity of exocytosis to DTX application was mirrored in the optical recordings of the APsyn from neurons expressing the indicator QuasAr (25) with a characteristic broadening as measured by the full width at half max (FWHM) of the waveform (30 ± 4%) (Fig. 1C and D) in agreement with previous findings (22). We measured this phenomenon in both excitatory and inhibitory neurons of the hippocampus without prior knowledge of their identity. Interestingly, the inhibitory neurons did not display any sensitivity to DTX treatment as assayed by exocytosis (Fig. 1E and F). Furthermore, the APsyn waveform displayed no changes in amplitude or width from DTX treatment (Fig. 1G and H). We note

**Fig. 1.** Kv1 channels are expressed exclusively in excitatory presynaptic terminals. (A–D) Measurements of exocytosis using vG-pH (A and B) for both control (black) and DTX-treated (magenta) excitatory neurons; arrows indicate when stimulation was applied. Example of recording of voltage using QuasAr (C) and corresponding FWHM (D). (Scale bar, 10 μm.) (vG-pH, n = 16 cells, *P < 0.001, paired t test; QuasAr, n = 20 cells, *P < 0.01, paired t test). (E–H) Measurements of exocytosis using vG-pH (E and F) in control (blue) and DTX-treated (orange) inhibitory neurons. Representative recording of voltage using Quasar (G) and the corresponding averaged FWHM (H). Arrow in G indicates when stimulation was applied (vG-pH, n = 10 cells; QuasAr, n = 5 cells). (I and J) Representative images of vGAT antibody live staining in excitatory terminals (I) and inhibitory terminals (J). Colocalization of vGAT-staining signal with active synapses marked by vG-pH response is indicated by the white arrows. (Scale bar, 10 μm.) (K) Representative ΔF image of GluSnFR upon single stimulation. Average trace of GluSnFR for both control (black) and DTX-treated (magenta) excitatory neurons; arrow indicates when stimulation was applied; black dotted line indicates the peak of control, and magenta dotted line indicates the peak of DTX-treated neurons, showing the latency (n = 7 cells). (Scale bar, 10 μm.) (L and M) The corresponding averaged time to peak after stimulation (L) and normalized peak to control (M) are shown. (*P < 0.05, paired t test). Extracellular Ca2+ concentration is 2 mM in all experiments.
that QuasAr’s reporting of voltage is linear and the most rapid voltage indicator to date; nonetheless, QuasAr has a sampling delay of ~300 μs for changes in voltage as previously demonstrated (25, 26). As a result of this inherent filtering of the reporter, the peak is reduced compared to electrophysiological recordings, and measurements of FWHM will systematically appear broader. However, as QuasAr’s delay is uniform in combination with its linear sensitivity to voltage across a large physiological range (±100 mV), it has been shown to comparatively display relative voltage changes in vitro and in vivo with high fidelity in neurons (25–27). We confirmed the identity of neurons after measuring exocytosis or APsyn in vitro and in vivo with high fidelity in neurons (25).

We created bicistronic expression vectors to measure voltage paired with Ca²⁺ (Fig. 3 A and B) in single nerve terminals. These measurements had limited signal to noise under such restrictions, but even in the presence of GCaMP, voltage measurements demonstrated robust APsyn broadening during paired-pulse (50 Hz) stimulation (Fig. 3 C–G). We found that the width of the first AP was correlated with the magnitude of Ca²⁺ entry at the single AP level (Fig. 3H). However, under paired-pulse stimulation during AP broadening by blocking Kv channels introduced a latency in synaptic transmission (29). We used a fast variant of the glutamate sensor GluSnFR to study the kinetics of glutamate release during AP broadening by DTX treatment and found that the peak of GluSnFR was delayed by 40 ± 6% relative to field stimulation, while also exhibiting a 57 ± 16% increase in neurotransmitter release in good agreement with previous measurements (Fig. 1 K–M). Taken together, these experiments demonstrate a very selective enrichment of presynaptic Kᵥ1.1 channels in excitatory nerve terminals of hippocampal neurons.

We hypothesized that terminals enriched with Kᵥ1.1/1.2 channels might exhibit APsyn broadening during high frequency (>10 Hz) stimulation due to Kᵥ1 channel inactivation. We observed a robust (39 ± 4%) broadening during a train of stimulation with 100 APs stimulated at 50 Hz (Fig. 2A), with example binned recordings of the APsyn shown in Fig. 2B and the corresponding quantifications of the FWHM in Fig. 2C. Next, we examined if APsyn broadening also took place in short paired-pulse stimulation protocols associated with synaptic facilitation. We measured the FWHM of the APsyn waveform during paired-pulse stimulation to compare how stable the shape of the waveform is across basal firing rates (4 to 10 Hz) and those typically associated with facilitation (50 Hz) (30, 31) (Fig. 2D and E). The FWHM paired-pulse ratio (PPR) is plotted as a function of the interspike interval in Fig. 2F, demonstrating that APsyn broadening is reliably triggered by stimulation frequencies of 50 Hz. This broadening behavior for the APsyn during paired-pulse stimulation was exclusive to excitatory terminals and was not seen in inhibitory terminals that exhibited a strong hyperpolarization (12 of 17 neurons; SI Appendix, Fig. S1). When we measured the FWHM of each spike individually during paired-pulse stimulation for both inhibitory and excitatory nerve terminals, we noticed that the broadening at 50 Hz was prominent only in excitatory nerve terminals and absent in inhibitory terminals (SI Appendix, Fig. S1 A, B, E, and F). However, the measurement of individual spikes from their independent baselines failed to account for important changes in voltage for the second AP from the optical voltage recordings, particularly with respect to hyperpolarization. As such, we used the amplitude of the FWHM from the first AP to measure the FWHM for all subsequent recordings to account for absolute changes in voltage that are more relevant to considering the opening time for voltage-gated Ca²⁺ channel behavior. Measurements based on the half-max of the first AP that account for this hyperpolarization demonstrate a significant narrowing of the APsyn in inhibitory neurons during paired-pulse stimulation at 50 Hz, while also accounting for AP broadening in excitatory neurons (SI Appendix, Fig. S1 C–H). We will refer to this measurement from here on as a normalized FWHM (nFWHM). Taken together, these results suggest that presynaptic terminals enriched with Kᵥ1.1/1.2 channels undergo frequency-dependent broadening of the APsyn even in minimal conditions of paired-pulse stimulation, which could influence vesicle fusion.

Fig. 2. Activity-dependent broadening of APsyn in excitatory nerve terminals of hippocampal neurons. (A) Example APsyn traces evoked by 50-Hz stimulation. (B) Representative average AP traces from four separate bins of 25 APs during a 100-AP stimulation train delivered at 50 Hz. (C) Plot of normalized nFWHM for four separate bins from excitatory cells. Asterisk indicates significance relative to first AP bin in excitatory cells (*P < 0.05, ANOVA with Tukey’s post hoc comparisons). (D) Representative measurement of QuasAr with 2 AP at 4, 10-, or 50-Hz stimulation in excitatory neurons. Peaks are normalized to the first peak of each measurement. Blue arrow indicates the first stimulation, and red arrow indicates the second stimulation. Red dashed line represents the half maximum of the peak where the width was measured. (E) Average nFWHM for the first (blue) and second (red) AP waveform in paired-pulse stimulation of excitatory neurons (n = 10 cells for 4-Hz condition; n = 8 cells for 10-Hz condition; n = 13 cells for 50-Hz condition; *P < 0.05, paired t test). Error bars indicate mean ± SEM. (F) Average nFWHM ratio (second/first AP) is plotted as a function of a stimulation interval (*P < 0.05, ANOVA with Tukey’s post hoc comparisons). Extracellular Ca²⁺ concentration is 2 mM in all experiments.
stimulation, the PPR for GCaMP (Fig. 3I) was not correlated with the PPR of the APsyn nFWHM. We suspect that these subtle changes were too small for detection by GCaMP6F whose relatively high affinity is best at detecting bulk changes in slow Ca\(^{2+}\) transients and not in the local Ca\(^{2+}\) where microdomains can collapse quickly (32, 33). Indeed, no indicator or measurement can tell the difference if the number of channels that open or close with different fluxes during changing voltage commands that total calcium could be the same, but the localized microdomains that influence vesicle fusion could be altered dramatically. We suspected that the most significant changes to Ca\(^{2+}\) influx during APsyn broadening with respect to influencing vesicle fusion occurs at the level of the Ca\(^{2+}\) microdomain profile. As no genetically encoded Ca\(^{2+}\) indicators can be localized to detect these changes, we probed the influence of Ca\(^{2+}\) microdomains using Ca\(^{2+}\) buffers and vG-pH, our sensitive indicator of vesicle fusion. The high-affinity intracellular ethylene glycol tetraacetic acid (EGTA) molecules strongly restrict Ca\(^{2+}\) diffusion to within the microdomain and prevent global diffusion (12, 34). Thus, DTX sensitivity would be diminished after EGTA treatment only if slow changes in Ca\(^{2+}\) diffusion outside of a microdomain were responsible for enhanced vesicle fusion instead of changes to the microdomain profile. We found that, while EGTA loading inhibited vesicle fusion by half as compared to control conditions (35), this buffering was quite ineffective in blocking a 50% increase in vesicle fusion after DTX treatment as shown in Fig. 3J and K. The finding that DTX enhancement to vesicle fusion persisted in the presence of EGTA suggests that Ca\(^{2+}\) entry and vesicle fusion are likely to be largely regulated at the level of the Ca\(^{2+}\) microdomain by APsyn broadening, as long as overall changes in cytosolic Ca\(^{2+}\) are small, as when brief stimulation such as paired-pulse is applied.

While many changes in ionic conductances could underlie the rapid broadening of the APsyn during stimulation, the most significant changes were too small for detection by GCaMP6F whose relatively high affinity is best at detecting bulk changes in slow Ca\(^{2+}\) transients and not in the local Ca\(^{2+}\) where microdomains can collapse quickly (32, 33). Indeed, no indicator or measurement can tell the difference if the number of channels that open or close with different fluxes during changing voltage commands that total calcium could be the same, but the localized microdomains that influence vesicle fusion could be altered dramatically. We suspected that the most significant changes to Ca\(^{2+}\) influx during APsyn broadening with respect to influencing vesicle fusion occurs at the level of the Ca\(^{2+}\) microdomain profile. As no genetically encoded Ca\(^{2+}\) indicators can be localized to detect these changes, we probed the influence of Ca\(^{2+}\) microdomains using Ca\(^{2+}\) buffers and vG-pH, our sensitive indicator of vesicle fusion. The high-affinity intracellular ethylene glycol tetraacetic acid (EGTA) molecules strongly restrict Ca\(^{2+}\) diffusion to within the microdomain and prevent global diffusion (12, 34). Thus, DTX sensitivity would be diminished after EGTA treatment only if slow changes in Ca\(^{2+}\) diffusion outside of a microdomain were responsible for enhanced vesicle fusion instead of changes to the microdomain profile. We found that, while EGTA loading inhibited vesicle fusion by half as compared to control conditions (35), this buffering was quite ineffective in blocking a 50% increase in vesicle fusion after DTX treatment as shown in Fig. 3J and K. The finding that DTX enhancement to vesicle fusion persisted in the presence of EGTA suggests that Ca\(^{2+}\) entry and vesicle fusion are likely to be largely regulated at the level of the Ca\(^{2+}\) microdomain by APsyn broadening, as long as overall changes in cytosolic Ca\(^{2+}\) are small, as when brief stimulation such as paired-pulse is applied.

While many changes in ionic conductances could underlie the rapid broadening of the APsyn during stimulation, the most
suggestive possibility from the previous experiments was that frequency-dependent Kv1.1/1.2 channel inactivation was responsible for broadening. The dominant mechanism of Kv,1 family channel inactivation is the “ball-and-chain” mechanism, in which the N-terminal structures of either the K+ channel’s α- or β-subunits occlude the channel pore from the cytosol (36-38) (Fig. 4A). Kv1.1/1.2 channels are known to most prominently undergo inactivation when associated with cytosolic β-subunits (39). As such, we investigated the role of the Kβ1 subunit for AP broadening using short hairpin RNA (shRNA) (Fig. 4B). We verified previous reports (7, 23, 40) of Kv1.1 expression and histochemical staining in brain slices. Kv1.1 channels are function in the axons of the hippocampus in vivo using immunohistochemical staining in brain slices. Kv1.1 channels are strongly expressed throughout the hippocampus including the axonal Schaffer collaterals, supporting their role in neurotransmission (Fig. 4D and E). Next, to explore the physiological role of these channels, we combined shRNA targeting the Kβ1 subunits with QuasAr to determine the involvement of Kβ1.1/1.2 inactivation through the β-subunit in the broadening of the AP_syn during paired-pulse stimulation. The average waveforms are shown for excitatory neurons expressing scrambled shRNA (wild type [WT]; Fig. 4F) or shRNA directed against Kβ1 (β1KD; Fig. 4G). We also combined Kβ1 KD with expression of a human variant of Kβ1 to rescue KD expression levels and check for off-target effects of KD (+hKβ1; Fig. 4H). Genetic depletion of Kβ1 not only stopped AP broadening, but also caused a small amount of narrowing (−7.0 ± 2.6% in the nFWHM) compared to control and rescue terminals (+10.4 ± 4.1% and +8.9 ± 2.6%, respectively, in the nFWHM) (Fig. 4F). This decrease in nFWHM was accompanied by a more prominent hyperpolarization in KD neurons (Fig. 4F–H) and an overall relative decrease (~11%) in presynaptic AP amplitude (Fig. 4J), suggesting an enhancement of presynaptic Kv1 currents when Kβ1 was not expressed. Furthermore, blocking the Kβ1/1.2

**Fig. 4.** Kβ1-induced Kv1 inactivation is critical to AP_syn broadening with paired-pulse stimulation. (A) Cartoon of Kβ1-induced inactivation of Kβ1.1/1.2 complexes. (B) Immunofluorescence staining for GCaMP (using anti-GFP antibody) and endogenous Kβ1 in primary cultured hippocampal neurons. Solid circles indicate the soma of a neuron cotransfected with GCaMP and Kβ1 shRNA, and dashed circles indicate those of untransfected neurons. (Scale bar, 20 μm.) (C) Quantification of relative expression of Kβ1 in soma of Kβ1 shRNA transfected neurons compared with WT neurons (n = 50 for control; n = 19 cells for Kβ1 shRNA transfected neurons; *P < 0.001, Student’s t test). (D and E) Immunohistochemical staining of adult mouse brain slices with antibodies against Kβ1.1 channels (green) and the nuclear marker DAPI (blue). (Inset in D) The transition into the CA1 region where Schaffer collateral (SC) axons are prominent with magnification of Inset shown in E. DG: dentate gyrus; CA1 and CA3: regions of the hippocampus. (F and H) Average traces of AP waveforms in response to 2 AP at 50-Hz stimulation from control (F), Kβ1 KD (G), and hKβ1 rescue (H) neurons. Insets provide a representative QuasAr 4F image from each condition. (Scale bar, 20 μm.) (I and J) Average nFWHM (I) and amplitude (J) for the first (blue) and second (red) AP waveform as shown in F–H (WT, n = 13 cells; Kβ1 KD, n = 17 cells; hKβ1 rescue, n = 16 cells; *P < 0.05, Student’s t test for amplitude comparison between different conditions, paired t test for nFWHM). (K) Average traces of exocytosis for 1 AP (black) or 2 AP delivered at 50 Hz (cyan) from control and Kβ1 KD neurons as measured with vG-pH. Black and cyan arrow(s) in K indicates when stimulation was applied for each corresponding trace respectively. (L) Average vG-pH ratio from control and Kβ1 KD neurons (control, n = 8 cells; Kβ1 KD, n = 9 cells; *P < 0.01, Student’s t test). Error bars indicate mean ± SEM. Extracellular Ca²⁺ concentration is 2 mM in all experiments.
isoforms of K\textsubscript{β}1 using DTX inhibits AP broadening in both WT and K\textsubscript{β}1 KD neurons during paired-pulse stimulation at 50 Hz (SI Appendix, Fig. S2), supporting the argument that the inactivation of the K\textsubscript{β}1 channel, and not the addition of other K\textsubscript{C} channels, is responsible for the phenotype that we measured. We used vG-pH to investigate the consequences of these changes in AP\textsubscript{syn} broadening on the facilitation or depression of exocytosis during 50-Hz paired-pulse stimulation (Fig. 4K). We found that control (WT) terminals displayed a 42 \pm 10\% increase in exocytosis (paired-pulse ratio of vG-pH is 1.42; facilitation >1) when comparing stimulation from paired pulses at 50 Hz to a single AP, but this enhancement or facilitation in vesicle fusion was completely abolished in K\textsubscript{β}1 KD neurons (\(\sim 9 \pm 8\%\); Fig. 4L). We found that inhibitory nerve terminals mimicked the excitatory K\textsubscript{β}1 KD terminals and exhibited paired-pulse narrowing of the AP\textsubscript{syn} (SI Appendix, Figs. S3 A and B). We were curious about whether the inhibitory nerve terminals that exhibited narrowing displayed facilitation or depression. We found that this AP\textsubscript{syn} narrowing was accompanied by depression of neurotransmission (vG-pH ratio <1), akin to K\textsubscript{β}1 KD excitatory neurons (SI Appendix, Figs. S3 C and D). Taken together, these results indicate that K\textsubscript{β}1 subunits play a critical role in AP broadening in excitatory nerve terminals during paired-pulse stimulation and suggest an important modulatory role for presynaptic K\textsubscript{β}1.1/1.2 currents in facilitating glutamate release.

The impaired response during 50-Hz paired-pulse stimulation for cells lacking K\textsubscript{β}1 as reported by vG-pH measurement suggested a selective impairment in facilitation. We attempted to further validate this selective impairment in exocytosis using an imaging technique that directly quantifies glutamate release at various stimulation frequencies of paired-pulse stimulation as a complement to our vG-pH results that measured the exocytosis of synaptic vesicles. To this end, we used the ultrafast variant of the genetically encoded glutamate sensor (iGlusnFR S72T; \(K_d\) 600 \(\mu\)M and \(K_{off}\) of 468 s\textsuperscript{-1} for glutamate) previously validated in hippocampal slice (41). We found that this GlusnFR variant was rapid enough to resolve glutamate release at 50 Hz (Fig. 5 A–C). While control neurons exhibited a 31\% increase in the paired-pulse ratio of glutamate release when comparing stimulation at 50 to 10 Hz for control cells, there was no increase in the paired-pulse response of K\textsubscript{β}1 KD neurons, as shown in Fig. 5D, consistent with our vG-pH results. Thus the ability to exhibit frequency-dependent facilitation during paired-pulse stimulation was lost with knockdown of the K\textsubscript{β}1 subunit, but otherwise release seemed to be normal for lower frequencies.

Hippocampal neurons typically fire in short bursts of APs during physiological conditions (42), so we next examined the contribution of K\textsubscript{β}1-mediated K\textsubscript{β}1.1/1.2 inactivation during synaptic transmission consisting of 10 electrical pulses delivered at 4 or 50 Hz. We found that WT neurons displayed robust facilitation at 50-Hz stimulation (Fig. 6A). However, we observed no facilitation for K\textsubscript{β}1 KD neurons, despite nearly identical vesicle exocytosis at 4-Hz stimulation compared to WT neurons, suggesting a selective impairment in facilitation (Fig. 6B). Even under an extended protocol of 50 APs delivered at 50 Hz, no recovery was seen in exocytosis (SI Appendix, Fig. S4) in K\textsubscript{β}1 KD neurons. We found that frequency-dependent synaptic facilitation in neurons overexpressing (OE) hK\textsubscript{β}1 was no greater than that which we had observed in control neurons (Fig. 6 C and D). Critically, the magnitude of vesicle fusion elicited by a single AP in all conditions was not altered by the loss of K\textsubscript{β}1 nor was any impairment observed for stimulation of a train at 4 Hz. These results suggest that release probability and the vesicle fusion machinery are intact, but that the AP\textsubscript{syn} itself is a major modulator of facilitation or depression. While many factors could influence facilitation, we also looked more closely at inhibitory neurons, which typically failed to facilitate and are quite stable with regard to vesicle fusion per AP at any frequency.

Interestingly, hK\textsubscript{β}1 OE alone was able to switch inhibitory neurons into a facilitating state with an \(\sim 50\%\) increase in vesicle fusion in the OE inhibitory neurons when increasing the firing frequency from 4 to 50 Hz (Fig. 6 E–G). These results argue that the K\textsubscript{β}1 subunit alone can initiate facilitation without a need to alter downstream aspects of synaptic terminals such as Ca\textsuperscript{2+} sensing or vesicle fusion.

We also measured the change in the nFWHM of the AP waveform with minimal averaging (16 trials) for both control (WT; gray) and K\textsubscript{β}1 KD neurons (K\textsubscript{β}1 KD; orange) for brief trains of 10 AP stimulation at 4 and 50 Hz (Fig. 7 A and B). Here again we found a complete loss of AP\textsubscript{syn} broadening at 50-Hz stimulation for K\textsubscript{β}1 KD neurons and a significant narrowing of the AP\textsubscript{syn} at 4-Hz stimulation (Fig. 7C). While our evidence from paired-pulse stimulation experiments suggests small changes in Ca\textsuperscript{2+} that were mainly confined to the microdomain, we hypothesized that the loss of K\textsubscript{β}1-mediated broadening of AP\textsubscript{syn} could also lead to a cumulative overall increase in net Ca\textsuperscript{2+} entry during longer high-frequency stimulation. We tested this hypothesis using a cytosolic version of GCaMP6f and measured changes in presynaptic [Ca\textsuperscript{2+}] during stimulation with 10 APs at 50 Hz for WT (gray; Fig. 7D) and K\textsubscript{β}1 KD neurons (orange; Fig. 7E). Here we found that the presynaptic Ca\textsuperscript{2+} signal was strongly reduced (>50\%) during trains of stimulation at 50 Hz for K\textsubscript{β}1 KD neurons (Fig. 7F). As such, these results indicate that minor changes in AP\textsubscript{syn} broadening by loss of K\textsubscript{β}1-mediated K\textsubscript{β}1 inactivation likely have an immediate impact on the Ca\textsuperscript{2+} microdomain profile, but also can have a large cumulative impact on integrated [Ca\textsuperscript{2+}] and synaptic facilitation during physiological patterns of activity.
Discussion

Our central finding is that an important mechanism of synaptic facilitation in excitatory hippocampal neurons is AP$_{syn}$ broadening. We find that the surprisingly rapid frequency-dependent broadening of AP$_{syn}$ is enabled by a unique molecular combination of Kv1.1/1.2 channels with the Kv$\beta_1$ subunit. Indeed, this small broadening of the AP$_{syn}$ mediated by Kv$\beta_1$ has a tremendous impact on synaptic transmission as the loss of the Kv$\beta_1$ subunit blocks synaptic facilitation even during paired-pulse stimulation without altering initial vesicle fusion (Figs. 4–6). We believe that the conditions of AP$_{syn}$ broadening work to facilitate exocytosis through a host of additional molecular interactions that minimally include downstream Ca$^{2+}$ sensors and enzymes, but that Kv$\beta_1$ inactivation represents a critical initial step to enable facilitation.

Fig. 6. Synaptic facilitation is absent in Kv$\beta_1$ KD neurons. (A–C) Average traces of pHluorin measurements of exocytosis for WT (A), Kv$\beta_1$ KD (B), and human Kv$\beta_1$ overexpressed (C) excitatory neurons in responses to 10 APs delivered at 4 Hz (black) or 50 Hz (red) as measured with vG-pH. Bars at top of the graphs indicate the duration of each stimulation. (D) Normalization of average fusion induced by the 10th AP as a percentage of total vesicle fluorences measured by application of NH$_4$Cl. Neurons were stimulated with 10 AP at 4 Hz (black) or 50 Hz (red) (WT neurons, n = 16 cells; Kv$\beta_1$ KD neurons, n = 8 cells; hKv$\beta_1$ OE neurons, n = 6 cells; *P < 0.05, paired t-test). (E and F) Average traces of pHluorin measurements of exocytosis for WT (E) and hKv$\beta_1$ overexpressed (F) inhibitory neurons in responses to 10 APs delivered at 4 Hz (black) or 50 Hz (red) as measured with vG-pH. Bars at top of the graphs indicate the duration of each stimulation. (G) Normalization of average fusion induced by the 10th AP as a percentage of total vesicle fluorences measured by application of NH$_4$Cl. Neurons were stimulated with 10 AP at 4 Hz (black) or 50 Hz (red) (WT neurons, n = 7 cells; hKv$\beta_1$ OE neurons, n = 5 cells; *P < 0.05, paired t-test). Extracellular Ca$^{2+}$ concentration is 2 mM in all experiments.

Fig. 7. AP broadening and Ca$^{2+}$ accumulation are inhibited in Kv$\beta_1$ KD neurons. (A and B) Optical recording of APs in neurons expressing QuasAr stimulated with 10 AP at 4 Hz (A) and 50 Hz (B). nFWHMs of each AP across the stimulus train from WT (gray) and Kv$\beta_1$ KD (orange) cells are displayed (n = 16 trials per cell; WT = 9 cells; Kv$\beta_1$ KD = 7 cells). (C) Quantification of the averaged nFWHM of the 10th AP from WT and Kv$\beta_1$ KD (n = 9 cells for WT; n = 7 cells for Kv$\beta_1$ KD; *P < 0.05, Student’s t test). Error bars indicate mean ± SEM. (D and E) Ca$^{2+}$ influx was measured with GCaMP6f in control (D) and Kv$\beta_1$ KD (E) neurons. The light gray traces represent individual experiments with the averaged Ca$^{2+}$ influx depicted in dark gray (WT) or orange (Kv$\beta_1$ KD). (F) Quantification of the averaged GCaMP6f response of the 10th AP from WT and Kv$\beta_1$ KD neurons (n = 14 cells for WT; n = 11 cells for Kv$\beta_1$ KD; *P < 0.001, Student’s t test). Extracellular Ca$^{2+}$ concentration is 2 mM in all experiments.
This combination of Kv1 isoforms and subunits is not a ubiquitous system, and even in cultured hippocampal inhibitory and excitatory neurons demonstrated substantially different modulation of APsyn and presynaptic K, isoform enrichment (Figs. 1 and 6 and SI Appendix, Fig. S3). Interestingly, the expression of K, channel isoforms appeared to be one of the larger molecular differences between the synapses of these two neuronal cell types that display such opposite phenotypes in synaptic plasticity. This was demonstrated by simply overexpressing the Kv, subunit, which switched the inhibitory neurons from a depressing to a facilitating phenotype during high-frequency stimulation (Fig. 6 E–G). This result suggested a trafficking role for the Kv, subunit in addition to its role in Kv1 inactivation that modulates paired-pulse and frequency-dependent facilitation as we describe here. As such, we believe a detailed accounting of all of the K+ channels and subunits responsible for the APsyn shape across axons and associated terminals in various neural cell types could be an important contribution to our understanding of short-term synaptic plasticity and circuit dynamics. While many of the calcium sensors and vesicle release machinery seem to be expressed at nearly ubiquitous levels in these two types of cells, their response to the upstream voltage commands during stimulation could be a major deciding factor for dynamically altering neurotransmitter output during different stimulation frequencies. A central limitation of this study is that high-resolution optical measurement of the APsyn in hippocampal neurons is restricted to in vitro experiments. Nevertheless, we believe this well-studied model for presynaptic function, involving a channel enriched in hippocampal axons in vivo (Fig. 4 D and E), will further our understanding of how the APsyn is involved in short-term plasticity and learning and memory in vivo, as it has been previously reported in the K, knockout mouse (43). Furthermore, our noninvasive optical measurements of voltage did not impact normal release probability of the neurons studied, a drawback of the high-resolution whole-cell electrophysiology that was elegantly applied previously to the large mossy fiber boutons (2). It is unclear in those experiments if this alteration in synaptic properties is a consequence of the strong depolarization of the terminal membrane during electrode approach or a result of cytosolic washout of the terminal (2). Nevertheless, neither occur using rhodopsin-based voltage indicators, allowing rigorous measurements of the APsyn while perfectly preserving the properties of synaptic function to study aspects of synaptic plasticity.

Forms of synaptic enhancement, such as facilitation, augmentation, and posttetanic potentiation, are usually attributed to the effects of a residual elevation in presynaptic [Ca2+]i, acting on one or more molecular targets (44). Synaptotagmin 7 (Syt-7), a critical specialized high-affinity Ca2+ sensor (45), was identified as a requirement for facilitation in several regions of the brain including the hippocampus (42). However, subsequent studies found prominent levels of Syt-7 in several other neural cell types that exhibit synaptic depression (46–49). Taken together, these experiments suggest that Syt-7 produces facilitation in coordination with other molecular cascades, which remain to be determined. Our data suggest that part of this coordination inactivation is important for facilitation in excitatory hippocampus terminals as demonstrated by our measurements in inhibitory neurons (SI Appendix, Fig. S3). Interestingly, it appears that, without Kv, subunits, overall K, currents are activated during high-frequency stimulation. This lack of inactivation results in significant narrowing of APsyn that was not measurable during paired-pulse stimulation, which suggests a role for Kv, inactivation in coordination with other molecular cascades, which remain to be determined.

While we demonstrate that APsyn broadening mediated by Kv, inactivation is important for facilitation in excitatory hippocampal neurons, we do not believe it is conserved across all terminals as demonstrated by our measurements in inhibitory neurons (SI Appendix, Fig. S3). Interestingly, it appears that, without Kv, subunits, overall K, currents are activated during high-frequency stimulation. This lack of inactivation results in significant narrowing of APsyn that was clearly observed for paired-pulse stimulation in inhibitory neurons as well as in Kv, KD excitatory neurons. Presynaptic K, channel activation during high-frequency stimulation was previously reported in the Calyx of Held (56) and may indeed be the default mode for many presynaptic K, channels. This activation of K, channels could be a very useful property for neurons that typically exhibit high firing rates such as hippocampal parvalbumin-expressing inhibitory neurons to depress exocytosis and to maintain a supply of vesicles as well as to preserve the timing of neurotransmitter release. This also seemed to be the case in Purkinje cell terminals.
that exhibit frequency-dependent attenuation of APsyn at larger terminals synapsing with deep cerebellar nuclei (19). The mechanisms that activate Kᵥ channels are not fully resolved at present and may involve the recruitment of Ca²⁺-sensitive K+ channels such as SK and BK channels. What is clear is that K+ channel inactivation during physiological stimulation is not an inherent property of all neurons and that for some Kᵥ1 channels (Kᵥ1.1 and 1.2 heteromers) binding partners are required. Here we identified the Kᵥβ1 subunit as a powerful modulator of exocytosis and synaptic facilitation. Kᵥ1 channel inactivation by the Kᵥβ1 subunit is well conserved, with homologs (Shaker and Hyperkinetic) found in Drosophila which act in a similar manner (57). Additionally, impaired inactivation of Kᵥ1 channels was identified as a presynaptic channelopathy of ataxia, which, combined with our findings, suggests Kᵥ inactivation’s broad importance across several circuits in the brain (58). As previous behavioral experiments in Kᵥβ1 knockout mice have identified the Kᵥβ1 subunits as being critical for many memory tasks (43), we believe that our findings may be helpful for understanding the mechanism underlying this phenotype. Previous slice recordings in this knockout mouse did not show impaired facilitation. However, we point out that a critical difference between these measurements and ours was that the recordings in slice were performed at room temperature, while ours were performed at physiological temperatures (>34 °C). Additionally, while Kᵥβ1 subunits seem critical for frequency-dependent inactivation, other mechanisms or even subunits may also be involved through different analog voltage signals in the axon (17, 23). We believe our data provide evidence that the APsyn waveform is a critical modulator of synaptic facilitation in excitatory nerve terminals and that further study of presynaptic K+ channels is warranted across neuronal cell types.

Materials and Methods

Cell Culture and Transfection. Primary hippocampal neurons from postnatal day 1 Sprague–Dawley rats of either sex were cultured by dissociation from newborn rat pups. Briefly, hippocampal CA1-CA3 regions were digested with trypsin for 5 min at room temperature and dissociated into single cells. Cells were seeded inside a 6-mm-diameter cloning cylinder on polyornithine-coated coverslips. Plasmids were transfected into 5-to-6-d in vitro (DIV) neurons with the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol (25). All live-imaging experiments were set up as previously described (26, 60). Briefly, images were obtained using an Olympus microscope (IX-83) equipped with a 40× 1.35-numerical aperture (NA) oil immersion objective (UAPONX04X0340-2) and captured with an IXON Ultra 897 EMCCD (Andor). Coverslips were mounted in a laminar-flow perfusion and stimulation chamber on the stage of the microscope. Cells were perfused continuously at a rate of 400 µm/min in Tyrode solution containing the following (in mM): 119 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 25 Heps, and 30 glucose with 10 µM CNQX and 50 µM AP5 during experiments. All experiments were performed at 34 ± 3 °C with a custom-built objective heater.

For Kᵥ1 channel experiments, DTX was used at 100 nM concentrations. Cells were incubated with DTX in Tyrode solution for 1 min, followed by perfusion with normal Tyrode solution for an additional 1 min, and images were taken.

For measuring membrane potential, fluorescence of QuasAr was recorded with an exposure time of 980 µs, and images were acquired at 1 kHz using an Olympus Fluoview FV1000 microscope (Olympus) equipped with a high-speed CMOS sensor. Fluorescence of GCaMP6f and vG-pH were collected with an exposure time of 20 ms, and images were acquired at 50 Hz. Cells were illuminated by a 488-nm laser 6 to 8 mW (Coherent OBIS laser) with ZET635/20x, ET655lp5m, and ZT640drc filters, all obtained from Chroma. We repeated more than 100 trials to measure axonal AP waveforms and averaged the signals, except 10 AP stimulation results in Fig. 4 (16 trials). Timing of stimulation was delivered by counting frame numbers from a direct readout of the EMCCD rather than time itself for more exact synchronization using an Arduino board and software custom manufactured by Sensorstar.

For measuring Ca²⁺ influx and vesicle fusion, fluorosences of GCaMP6f and vG-pH were collected with an exposure time of 20 ms, and images were acquired at 50 Hz using an epifluorescence microscope (Olympus). Images were acquired using a S1000 microscope (Olympus) at 1 kHz. Image acquisition was performed using MatLab (The MathWorks) to synchronize the data acquisition.

For measuring glutamate release of single action potentials, fluorescence of GCaMP6f was collected with an exposure time of 2 ms, and images were acquired at 50 Hz using an epifluorescence microscope (Olympus). Images were acquired using a S1000 microscope (Olympus) at 1 kHz. Image acquisition was performed using MatLab (The MathWorks) to synchronize the data acquisition.

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fixed with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked by 5% goat serum in PBS for 30 min at room temperature. Cells were then incubated with the appropriate primary antibodies and visualized using Alexa Fluor-conjugated secondary antibodies in the PBS containing 5% goat serum. Images were obtained using a custom-made fluorescence microscope equipped with the 40x oil-immersion objectives and filters described above.

**Tissue Processing and Immunohistochemistry.** Mice (aged 4 to 12 wk) were anesthetized and perfused with 4% paraformaldehyde, and brains were postfixed overnight at 4 °C. Brain-tissue sections 50 μm thick were cut on a vibratome and processed for immunohistochemistry. Tissue sections were blocked in PBS containing 5% normal goat serum and 0.5% Triton-X-100 at 4 °C. For secondary antibodies, tissue sections were postfixed overnight at 4 °C. Brain-tissue sections 50 μm-diameter were vibratome and processed for immunohistochemistry. Tissue sections were blocked in PBS containing 5% goat serum and 0.5% Triton-X-100. The sections were incubated in primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. Specific antibodies used are listed in Antibodies and Reagents above.

**Image and Data Analysis.** Images were analyzed in ImageJ and Fiji using custom-written plugins (http://rsb.info.nih.gov/ij/plugins/time-series.html). To measure fluorescence of probes accurately, we selected 1.4-μm-diameter circular regions of interest (ROIs) from 3F images of each experiment, centering on the brightest pixel of the 3F image using an automated program free of bias by the selector. ROIs were selected on the basis of localized response of voltage, calcium, or vesicle fusion, rather than on morphology, to define a presynaptic terminal (even though en passant boutons can generally be recognized morphologically by a small swelling). All statistical data were presented as means +/- SEM (n = number of neurons), and all experiments were performed on more than three independent cultures. For examining AP broadening with QuasAr measurement, we used Origin version 9.1. To obtain the nFWHM of each peak, half maximum of the first peak was applied to every peak. For analyzing vesicle fusion of each condition, the delta response of pHluorin fluorescence was normalized to the change in fluorescence from the total number of vesicles in the synapse, measured by anion exchange in PBS at the end of the experiments. Conversion of GaMP6f fluorescence to a Ca2+ concentration was accomplished by inverting the Hill equation of fluorescence vs. [Ca2+] as previously described (35). In brief, to linearize the GaMP6f signal, the fluorescence signal was converted to the signal relative to that obtained with MgGreen using the equation. These linearized values were normalized to the average of the first stimulation to estimate the paired-pulse Ca2+ responses in excitatory neurons.

**Quantification and Statistical Analysis.** Statistical analyses were performed in Excel and Origin. We used paired two sample for means t test for paired results. Normally distributed data were processed with the Student’s t test for two independent distributions with a one-way ANOVA followed by Tukey’s post hoc comparison for comparing more than two groups to examine statistical significance. We specify the use of these tests and exact sample sizes in the figure legends for clarity.

**Data Availability.** All relevant data, associated protocols, and materials are provided in Materials and Methods and SI Appendix. Plasmids have been deposited in Addgene for distribution to all interested researchers under accession number 78223.

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