The Potassium Channel Subunit K β 1 is Required for Synaptic Facilitation

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Significance

Nerve terminals generally engage in two opposite and essential forms of synaptic plasticity (facilitation or depression) during high frequency stimulation that play critical roles in learning and memory. Measurements of the electrical impulses (action potentials) underlying these two forms of plasticity has been difficult in small nerve terminals due to their size. In this study we deployed a combination of optical measurements of vesicle fusion and membrane voltage to overcome this previous size barrier. Here, we found a unique molecular composition of Kv1 channel β -subunits that causes broadening of the presynaptic action potential essential to synaptic facilitation. Disruption of the K β 1 inactivation mechanism switches excitatory nerve terminals into a depressive state, without any disruption to initial probability of vesicle fusion.

Abstract

Analysis of the presynaptic action potential's (AP_{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^{2+} in cultured hippocampal neurons. These recordings revealed a critical and selective role for K_v1 channel inactivation in synaptic facilitation of excitatory hippocampal neurons. Presynaptic K_v1 channel inactivation was mediated by the $K_v\beta1$ subunit, and had a surprisingly rapid onset that was readily apparent even in brief physiological stimulation paradigms including paired-pulse stimulation. Genetic depletion of $K_v\beta1$ blocked all broadening of the AP_{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_v channels in synaptic facilitation at small presynaptic terminals of the hippocampal neurons upstream of exocytic machinery.

Introduction

The 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 rapidly change due to 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_{syn} at boutons, whose waveform can exhibit plasticity based on firing frequency. Repetitive firing may cause inactivation of axonal voltage-gated sodium channels (Na_v) and voltage-gated potassium (K_v) channels that control the depolarization and hyperpolarization of the waveform respectively. K_v inactivation primarily leads to an increase in AP width or broadening (2-9). The width of the AP_{syn} controls the fraction of time that Ca^{2+} channels open and the driving force of Ca^{2+} entry (10). The highly nonlinear influence of Ca^{2+} on exocytosis (11, 12) thus dictates that modest AP_{syn} broadening has the potential to critically impact synaptic facilitation (13-15). Indeed, AP_{syn} broadening during repetitive firing has been demonstrated to cause the facilitation of exocytosis in the pituitary nerve (3), dorsal root ganglion (16) and mossy fiber bouton (2), all due to K_v channel inactivation. However, the AP_{syn} waveform in Purkinje Cells has also been shown to undergo frequency-

dependent decreases in amplitude that substantially contributes to synaptic depression (17). Therefore, it is best to consider the AP_{syn} as a plastic signal that can powerfully modulate exocytosis bidirectionally, rather than a digital spike solely acting 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_{syn} waveform and its molecular regulators in the majority of brain regions.

Investigating the molecular regulation of AP_{syn} in the common *en passant* nerve terminals of the cortex and hippocampus remains elusive due to the small size of these structures ($<1\ \mu m$), which makes them inaccessible for whole-cell patch clamp recording. Our group has overcome this barrier by pioneering the use of genetically-encoded rhodopsin-based voltage indicators to measure AP_{syn} in *en passant* terminals of inhibitory and excitatory hippocampal neurons. Here we demonstrate a striking contrast between facilitating excitatory and depressing inhibitory nerve terminals in the hippocampus. We discovered that excitatory terminals are uniquely enriched with a combination of $K_v1.1/1.2$ heteromers and $K_v\beta1$ subunits. This combination of K_v subunits causes rapid AP_{syn} broadening during brief periods of high-frequency firing. This broadening was essential for enabling synaptic facilitation without altering initial exocytosis. Taken together, these results suggest that the molecular control of presynaptic K_v channel inactivation is an important modulator of synaptic facilitation upstream of vesicle release machinery.

Results

Previously, our measurements of the AP_{syn} in hippocampal neurons found a very high ratio of K_v to Na_v channels, with $K_v1.1/1.2$ channels dominating repolarization (18). We measured the sensitivity of exocytosis to changes in $K_v1.1/1.2$ conductance using an optical probe of exocytosis (vGLUT1-pHluorin; vG-pH)(12, 19). Blockade of $K_v1.1/1.2$ channels by application of dendrotoxin- κ (DTX) greatly enhanced exocytosis in excitatory hippocampal nerve terminals by $61\pm18\%$ when stimulated with 1 AP (**Figure 1A-B**). The sensitivity of exocytosis to DTX application was mirrored in the optical recordings of the AP_{syn} from neurons expressing the indicator QuasAr (20), with a characteristic broadening as measured by the full-width at half max (FWHM) of the waveform ($30\pm4\%$) (**Figure 1C-D**) in agreement with previous findings (18). 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 (**Figure 1E-F**). Furthermore, the AP_{syn} waveform displayed no changes in amplitude or width from DTX treatment (**Figure 1G-H**). We confirmed the identity of neurons after measuring exocytosis or AP_{syn} waveform as excitatory or inhibitory using a fluorescent antibody directed against the lumenal domain of the vesicular GABA transporter (vGAT) as previously

described (21) with example recordings for both cell types shown in **Figures 1I-J**. Taken together these experiments demonstrate a very selective enrichment of presynaptic enrichment of K_v1 channels in excitatory nerve terminals of hippocampal neurons.

We hypothesized that terminals enriched with $K_v1.1/1.2$ channels might exhibit AP_{syn} broadening during high frequency (>10 Hz) stimulation due to K_v1 channel inactivation. We observed a robust ($39 \pm 4\%$) broadening during a train of stimulation with 100 APs stimulated at 50 Hz (**Figure 2A**), with example binned recordings of the AP_{syn} shown in **Figure 2B** and the corresponding quantifications of the FWHM in **Figure 2C**. Next, we examined if AP_{syn} broadening also took place in short paired-pulse stimulation protocols associated with synaptic facilitation. We measured the FWHM of the AP_{syn} waveform during paired-pulse stimulation to compare how stable the shape of the waveform is across basal firing rates (4-10 Hz) and those typically associated with facilitation (50 Hz) (22, 23) (**Figure 2D-E**). The FWHM paired-pulse ratio is plotted as a function of the inter-spike interval in **Figure 2F** demonstrating that AP_{syn} broadening is reliably triggered by stimulation frequencies of 50 Hz. These results suggest that terminals enriched with $K_v1.1/1.2$ channels in presynaptic terminals undergo frequency-dependent broadening of the AP_{syn} even in minimal conditions of paired-pulse stimulation that could influence vesicle fusion.

While many changes in ionic conductances could underly the rapid broadening of AP_{syn} during stimulation, the most suggestive possibility from the previous experiments was that frequency-dependent $K_v1.1/1.2$ channel inactivation was responsible for broadening. The dominant mechanism of K_v1 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 (24-26) (**Figure 3A**). $K_v1.1/1.2$ channels are known to most prominently undergo inactivation when associated with cytosolic β subunits (27). As such, we investigated the role of the $K_v\beta1$ subunit for AP broadening using shRNA (**Figure 3B-C**). We combined shRNA targeting the $K_v\beta1$ subunits with QuasAr to determine the involvement of $K_v1.1/1.2$ 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 (**WT**; **Figure 3D**) or shRNA directed against $K_v\beta1$ (**$K_v\beta1$ KD**; **Figure 3E**). We also combined $K_v\beta1$ KD with expression of a human variant of $K_v\beta1$ to *rescue* KD expression levels and check for off-target effects of KD (**$hK_v\beta1$** ; **Figure 3F**). Genetic depletion of $K_v\beta1$ not only stopped AP broadening, but also caused a small amount of narrowing ($-7.0 \pm 2.6\%$ in the FWHM) compared to control and rescue terminals ($+10.4 \pm 4.1\%$ and $+8.9 \pm 2.6\%$ in the FWHM) (**Figure 3G**). This decrease in FWHM was accompanied by a more prominent hyperpolarization in KD neurons (**Figure 3D-F**) and an overall relative decrease ($\sim 11\%$) in presynaptic AP amplitude (**Figure 3H**), suggesting an enhancement of presynaptic K_v1 currents without expression of $K_v\beta1$.

present. We used vG-pH to investigate the consequences of these changes in AP_{syn} broadening on the facilitation or depression of exocytosis (**Figure 3I**). We found that control (WT) terminals displayed a 42±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_vβ1 KD neurons (-9±8%; **Figure 3J**). We were curious if the inhibitory nerve terminals that do not contain K_v1 channels underwent frequency dependent broadening of AP_{syn}. We found that inhibitory nerve terminals mimicked the excitatory K_vβ1 KD terminals and exhibited paired-pulse narrowing of the AP_{syn} (**Figures S1A-B**). Furthermore, when we found that this AP_{syn} narrowing was also accompanied by depression of neurotransmission during 50 Hz stimulation (vG-pH ratio < 1) akin to K_vβ1 KD excitatory neurons (**Figure S1C-D**). Taken together, these results indicate that K_vβ1 subunits play a critical role in AP broadening of excitatory nerve terminals during paired-pulse stimulation and suggest an important modulatory role for presynaptic K_v1.1/1.2 currents in facilitating glutamate release.

Hippocampal neurons typically fire in short bursts of APs during physiological conditions (28), so we next examined the contribution of K_vβ1-mediated K_v1.1/1.2 inactivation during synaptic transmission consisting of ten electrical pulses delivered at 4 Hz or 50 Hz. We found that WT neurons displayed robust facilitation at 50 Hz stimulation (**Figure 4A**). However, we observed no facilitation for K_vβ1 KD neurons, despite nearly identical vesicle release at 4 Hz stimulation compared to WT neurons suggesting a selective impairment in facilitation (**Figure 4B-C**). Even under an extended protocol of fifty APs delivered at 50 Hz, no recovery is seen in exocytosis (**Figure S2**) in K_vβ1 KD neurons. We also measured the change of the FWHM of the AP waveform with minimal averaging (16 trials) for both control (WT; gray) and K_vβ1 KD neurons (KD; orange) for brief trains of 10 AP stimulation at 4 and 50 Hz (**Figure 4D-E**). Here again we found a complete loss of AP_{syn} broadening at 50 Hz stimulation for K_vβ1 KD neurons and a significant narrowing of the AP_{syn} 4 Hz stimulation (**Figure 4F**). We hypothesized that the loss of K_vβ1-mediated broadening of AP_{syn} could lead to the opening of fewer presynaptic Ca²⁺ channels and impaired net Ca²⁺ entry during high frequency stimulation. We tested this hypothesis using a cytosolic version of GCaMP6f and measured changes in presynaptic [Ca²⁺]_i during stimulation with 10 APs at 50 Hz for control (**gray; Figure 4G**) and K_vβ1 KD (**orange; Figure 4H**). Here we found that the presynaptic Ca²⁺ signal was strongly reduced (>50%) during trains of stimulation at 50 Hz for K_vβ1 KD neurons (**Figure 4I**). We also created bicistronic expression vectors to measure voltage paired with Ca²⁺ or vesicle fusion in single nerve terminals. These measurements had limited signal to noise under such restrictions, but showed a clear correlation between AP_{syn} broadening and vesicle fusion during paired-pulse stimulation, but not total Ca²⁺ in these limited measurements (**Figure S3A-L**). Critically, the magnitude of vesicle fusion elicited by a single AP in all conditions was not altered by the loss of K_vβ1 or stimulation at 4 Hz frequency suggesting

that release probability and vesicle fusion machinery are intact, but that the AP_{syn} itself is a major modulator of facilitation or depression. As such, these results indicate that minor changes in AP_{syn} broadening by loss of $K_v\beta 1$ -mediated K_v1 inactivation had a large cumulative impact on integrated $[Ca^{2+}]_i$ and synaptic facilitation during physiological patterns of activity.

Discussion

Our central finding is that a necessary mechanism of synaptic facilitation in excitatory hippocampal neurons is AP_{syn} broadening. We find the surprisingly rapid frequency-dependent broadening of AP_{syn} is enabled by a unique molecular combination of $K_v1.1/1.2$ channels with the $K_v\beta 1$ subunit. Indeed, this small broadening of the AP_{syn} mediated by $K_v\beta 1$ has a tremendous impact on synaptic transmission as the loss of the $K_v\beta 1$ subunit blocks synaptic facilitation without altering initial vesicle fusion (**Figure 3-4**). We believe that the conditions of AP_{syn} broadening works to facilitate exocytosis through a host of additional molecular interactions that minimally include downstream Ca^{2+} sensors and enzymes, but that K_v1 inactivation represents a critical initial step to enable facilitation. This combination of K_v1 isoforms and subunits is not a ubiquitous system and even in cultured hippocampal neurons as inhibitory neurons even within the hippocampal culture demonstrated substantially different modulation of AP_{syn} and presynaptic K_v isoform enrichment (**Figure 2**). As such, we believe a detailed accounting of the channels responsible for the AP_{syn} 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. A central limitation of this study is that high-resolution optical measurement of AP_{syn} in hippocampal neurons are limited to *in vitro* measurements. Nevertheless, we believe this well-studied model for presynaptic function will translate into understanding how AP_{syn} relates to short-term plasticity and learning and memory *in vivo* such as those previously reported for the $K_v\beta 1$ knockout mouse (29).

Forms of synaptic enhancement, such as facilitation, augmentation, and post-tetanic potentiation, are usually attributed to effects of a residual elevation in presynaptic $[Ca^{2+}]_i$, acting on one or more molecular targets (Zucker 2002). In the latter regard, a critical specialized high-affinity Ca^{2+} sensor synaptotagmin 7 (Syt-7) (30) was identified to be required for facilitation in several regions of the brain including the hippocampus (28). However, subsequent studies have identified several other neural cell types that exhibit synaptic depression also have prominent levels of Syt-7 (31-34). Taken together, these experiments suggest Syt-7 produces facilitation in coordination with other molecular cascades that remain to be determined. Our data suggest that part of this coordination is upstream of Ca^{2+} sensing and vesicle release machinery. A widely accepted, but difficult-to-test model of facilitation attributes the enhancement of exocytosis to residual Ca^{2+} buildup during high frequency stimulation. *En passant* terminals like those in the cortex and hippocampus have been shown to have very efficient clearance of Ca^{2+} by diffusion thanks

to abundant adjacent axonal volume unlike larger single terminals such as the Calyx of Held (35). One identified mechanism for this enhancement of intracellular Ca^{2+} during high-frequency stimulation is activity dependent activation of Ca_v channels (36). However this does not seem to be relevant in hippocampal neurons at physiological temperatures (37) like the ones we used in our experiments. We measured Ca^{2+} in control and $\text{K}_v\beta 1$ KD neurons and found that substantial build up of global $[\text{Ca}^{2+}]_i$ is strongly enhanced by AP_{syn} broadening when measured during physiological trains of stimulation (**Figure 4**). We speculate that this is especially critical in light of efficient Ca^{2+} extrusion coupled with the low overshoot (+7 mV) of the excitatory AP_{syn} that will typically only open a fraction of available presynaptic Ca^{2+} channels, a phenomenon we have previously identified in excitatory terminals of the hippocampus (18). Thus AP_{syn} broadening may be a particularly efficient mechanism for facilitation in *en passant* boutons in general.

While we demonstrate that AP_{syn} broadening mediated by K_v1 inactivation is essential for facilitation in excitatory hippocampal neurons, we do not believe it is conserved across all terminals as demonstrated by our measurements in inhibitory neurons (**Figure 2**). Interestingly, it appears that without $\text{K}_v\beta 1$ subunits, overall K_v currents are activating during high frequency stimulation, resulting in significant narrowing of AP_{syn} clearly observed for paired-pulse stimulation in inhibitory neurons as well as $\text{K}_v\beta 1$ KD excitatory neurons. Presynaptic K_v channel activation during high frequency stimulation was previously reported in the Calyx of Held (38) and may indeed be the default mode for many presynaptic K_v channels. This activation of K_v channels could be a very useful property for neurons that typically exhibit high firing rates such as PV neurons to depress exocytosis and to maintain a supply of vesicles as well as the timing of neurotransmitter release. This also seemed to be the case in Purkinje cell terminals that show frequency-dependent attenuation of AP_{syn} at larger terminals synapsing with deep cerebellar nuclei (17). The mechanisms that activate K_v channels are not fully resolved at present and may involve the recruitment of Ca^{2+} sensitive K^+ channels such as SK and BK channels. What is clear is that K^+ channel inactivation during physiological stimulation is not inherent and in the case of some K_v1 channels ($\text{K}_v1.1$ and 1.2 heteromers) requires binding partners. Here we identified the $\text{K}_v\beta 1$ subunit as a powerful modulator of exocytosis and synaptic facilitation. K_v1 channel inactivation by the $\text{K}_v\beta$ subunit is well conserved with the respective fly homologues of *Shaker* and *Hyperkinetic* found in *Drosophila* to act in a similar manner (39). Additionally, impaired inactivation of K_v1 channels was also identified as a presynaptic channelopathy of ataxia, which combined with our findings could suggest a broad importance across several circuits in the brain (40). Previous behavioral experiments in mouse identified the $\text{K}_v\beta 1$ subunits as critical for many memory tasks in $\text{K}_v\beta 1$ knockout mice (29), we believe our findings may be mechanistically helpful to understanding this phenotype. 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 the temperature of the recordings in slice were performed at room temperature unlike what we report here at physiological

temperatures ($>34^{\circ}\text{C}$). We believe our data provide evidence that the AP_{syn} 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.

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Figure Legends

Figure 1. K_v1 channels were expressed exclusively in excitatory presynaptic terminals.

(A-D) Measurements of exocytosis using vG-pH (A-B) for both control (black) and DTX-treated (magenta) excitatory neurons; Arrow indicates when stimulation was applied; Example of recording of voltage using QuasAr (C) and corresponding FWHM (D);. Scale bar, 10 μ m. (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-F) in control (blue) and DTX-treated (orange) inhibitory neurons. Representative recording of voltage using Quasar (G) and the corresponding averaged FWHM (H). Arrows indicate when stimulation was applied. (vG-pH, n = 10 cells; QuasAr, n = 5 cells).

(I-J) Representative images of vGAT antibody live staining in excitatory terminals (I) and inhibitory terminals (J). Co-localization of vGAT staining signal with active synapses marked by vG-pH response indicated by the white arrows. Scale bar, 10 μ m.

Figure 2. Activity-dependent broadening of AP_{syn} in excitatory nerve terminals of hippocampal neurons.

(A) Example AP_{syn} 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 FWHM for four separate bins from excitatory cells. Black asterisk indicates significance relative to 1st AP bin in excitatory cells (n = 39 cells, *p < 0.05, ANOVA with Tukey's post hoc comparisons).

(D) Representative measurement of QuasAr with 2 AP at 4 Hz, 10 Hz 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 FWHM 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; *p < 0.05, Paired t-test). Error bars indicate mean $\pm$ SEM.

(F) Average FWHM ratio (2nd/1st AP) is plotted as a function of a stimulation interval (*p < 0.05, ANOVA with Tukey's post hoc comparisons).

Figure 3. $K_v\beta 1$ -induced $K_v 1$ inactivation is critical to AP_{syn} broadening with paired-pulse stimulation.

(A) A cartoon of $K_v\beta 1$ -induced inactivation of $K_v 1.1/1.2$ complexes.

(B) Immunofluorescence staining for GCaMP (anti-GFP antibody) and $K_v\beta 1$ in primary cultured hippocampal neurons co-transfected with GCaMP and $K_v\beta 1$ shRNA. Solid lines indicate the soma of transfected neuron and dashed lines indicate that of untransfected neurons. Scale bar, 20 μm .

(C) Quantification of relative expression of $K_v\beta 1$ in soma of $K_v\beta 1$ shRNA transfected neurons compared with control neurons ($n = 50$ for control; $n = 19$ cells for $K_v\beta 1$ shRNA transfected neurons; $*p < 0.001$, Student's t test).

(D-F) Average traces of AP waveforms in response to 2 AP at 50 Hz stimulation from control (D), $K_v\beta 1$ KD (E), and h $K_v\beta 1$ rescue (F) neurons. Insets provide a representative QuasAr ΔF image from each condition. Scale bar, 2 μm .

(G-H) Average FWHM (G) and amplitude (H) for the first (blue) and second (red) AP waveform as shown in D-F (Control, $n = 13$ cells; $K_v\beta 1$ KD, $n = 17$ cells; h $K_v\beta 1$ rescue, $n = 16$ cells; $*p < 0.05$, Student's t test for amplitude comparison between different conditions, Paired t-test for FWHM).

(I) Average traces of exocytosis for 1 AP (black) or 2 AP delivered at 50 Hz (cyan) from control and $K_v\beta 1$ KD neurons as measured with vG-pH.

(J) Average vG-pH ratio from control and $K_v\beta 1$ KD neurons (control, $n = 8$ cells; $K_v\beta 1$ KD, $n = 9$ cells; $*p < 0.01$, Student's t-test). Error bars indicate mean $\pm$ SEM.

Figure 4. Synaptic facilitation is absent in $K_v\beta 1$ KD neurons.

(A-B) Average traces of exocytosis for control (A) and $K_v\beta 1$ KD (B) in responses to 10 APs delivered at 4 Hz (black) or 50 Hz (red) as measured with vG-pH. Bars on top of the graphs indicate the duration of each stimulation.

(C) Average fusion induced by the 10th AP as a percentage of total vesicle fluorescences measured by application of NH_4Cl . Neurons were stimulated with 10 AP at 4 Hz (black) or 50 Hz (red) ($n = 11$ cells, ; $K_v\beta 1$ KD neurons, $n = 8$ cells; $*p < 0.001$, Paired-t test).

(D,E) Optical recording of APs in neurons expressing QuasAr stimulated with 10 AP at 4 Hz (D) and 50 Hz (E). FWHM of each AP across the stimulus train from WT (gray) and $K_v\beta 1$ KD (orange) cells are displayed ($n = 16$ trials per cell; WT = 9 cells; $K_v\beta 1$ KD = 7 cells).

(F) Quantification of the averaged FWHM of the 10th AP from WT and $K_v\beta 1$ KD ($n = 9$ cells for WT; $n = 7$ cells for $K_v\beta 1$ KD; $*p < 0.05$, Student's t test). Error bars indicate mean $\pm$ SEM.

(G-H) Ca^{2+} influx was measured with GCaMP6f in control (G) and $K_v\beta 1$ KD (H) neurons. The light gray traces represent individual experiments with the averaged Ca^{2+} influx depicted in dark gray.

305 (I) Quantification of the averaged GCaMP6f response of the 10th AP from WT and K_vβ1 KD (n = 14 cells
 306 for WT; n = 11 cells for K_vβ1 KD; *p < 0.001, Student's t test).
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Materials and Methods

Cell culture and transfection

Primary hippocampal neurons from postnatal day 1 Sprague Dawley rats of either sex were cultured as described previously (Cho 2017). 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 - 6 DIV (Days in Vitro) neurons with calcium-phosphate precipitants. All animal protocols were approved by Dartmouth College's Institutional Animal Care and Use Committee (IACUC protocol number 0002115).

Plasmids

QuasAr constructs and vGlut-pHluorin were obtained as described previously (41), respectively. To measure membrane potential and Ca^{2+} influx/vesicle fusion in the same cell, we designed a QuasAr-P2A-GCaMP/QuasAr-P2A-vGlut-pHluorin by inserting QuasAr fused to P2A peptide synthesized using GeneArt Gene Synthesis (Invitrogen) and GCaMP/vGlut-pHluorin under the human Synapsin1 promoter, inducing independent expression of two different proteins in neurons. We validated that QuasAr and GCaMP/vGlut-pHluorin were co-expressed in the same cells through immunostaining assay and many repetitive experiments. To knock-down endogenous $K_v\beta 1$ expression, shRNA plasmids were obtained from OriGene against the following mRNA target sequences: GCTTGGTCATCACAACTCTACTGG. For rescue experiments in $K_v\beta 1$ knockdown neurons, human $K_v\beta 1.1$ fused to P2A peptide synthesized using GeneArt Gene Synthesis was inserted into pFCK-QuasAr plasmid to induce independent expression of QuasAr and human $K_v\beta 1.1$ in neurons.

Antibodies and Reagents

Chicken polyclonal anti-GFP and rabbit polyclonal anti-mCherry were purchased from Invitrogen. Rabbit polyclonal anti-vGAT-Alexa 550 and mouse monoclonal anti-Synapsin1 were obtained from Synaptic Systems. Mouse monoclonal anti- $K_v\beta 1.1$ was purchased from Neuromab. Alexa Fluor 488-, 546-, and 647-conjugated goat anti-rabbit, anti-mouse, anti-Chicken and anti-guinea pig IgG were from Thermo Fisher Scientific. Dendrotoxin-k was purchased from Alomone (Israel).

Live cell imaging

All live imaging experiments were set up as previously described (42, 43). Briefly, images were obtained using an Olympus microscope (IX-83) equipped with a 40x 1.35 NA oil immersion objective (UApoN40XO340-2) 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 μ l/min in Tyrode solution containing the following (in mM): 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES, and 30 glucose with 10 μ M CNQX and 50 μ M AP5 during experiments. All experiments were performed at 34-35 °C with a custom-built objective heater.

For K_v1 channel experiments, dendrodotoxin (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 additional 1 min and images were taken.

For measuring membrane potential, fluorescence of QuasAr was recorded with an exposure time of 980 μ s and images were acquired at 1 kHz using an optomask (Cairn Research) to prevent light exposure of non-relevant pixels. Cells were illuminated by a 637 nm laser 70 - 120 mW (Coherent OBIS laser) with ZET635/20 $\times$, ET655lpm and ZT640rdc filters all obtained from Chroma. We repeated more than 100 trials to measure axonal AP waveform and averaged the signals, except 10 AP stimulation results in Figure 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 (Elkridge, MD).

For measuring Ca²⁺ influx and vesicle fusion, GCaMP6f and vGlut-pHluorin were collected with an exposure time of 20 ms and images were acquired at 50 Hz. Cells were illuminated by a 488 nm laser 6-8 mW (Coherent OBIS laser) with ET470/40 $\times$, ET525/50m, and T495lpxr filters (Chroma). We repeated more than 16 trials to measure single AP-induced responses and 6 trials to measure AP train stimulation-induced responses.

For differentiating inhibitory neurons from excitatory neurons, live vGAT antibody staining was performed using Alexa 550 pre-labeled vGAT antibody at the end of the experiments. Cells were stimulated by 1000 AP at 10 Hz with the antibody and followed by perfusion with normal Tyrode solution for 5 min. Fluorescence was captured using ET560/40 $\times$, ET630/75m, and T585lpxr filters (Chroma).

Immunofluorescence

To confirm the co-expression of QuasAr and GCaMP or vGlut-pHluorin in the neurons, 14-17 DIV neurons were fixed with 4 % paraformaldehyde and 4% sucrose in 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 40x oil-immersion objectives and filters described 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>). All statistical data are presented as means $\pm$ 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 FWHM of

each peak, half maximum of first peak was applied to every peak. For analyzing vesicle fusion of each condition, it was normalized to total number of vesicles measured by ammonium chloride treatment at the end of the experiments.

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 and with a one-way ANOVA followed by Tukey's post hoc comparison for comparing more than two groups to examine statistical significance. We specified the use of these tests and exact sample sizes in the figure legends for clarity.

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Supplementary Figure Legends

Supplementary Figure 1. Presynaptic terminal of inhibitory neurons do not exhibit activity dependent broadening of AP_{syn} and synaptic facilitation by paired-pulse stimulation.

(A) Representative measurement of voltage with QuasAr of 2 AP delivered at 50 Hz. Blue arrow indicates the first stimulation and red arrow indicates the second stimulation. Dashed red line represents the half maximum where the FWHM was measured.

(B) Average FWHM for the first (blue) and second (red) AP waveform in paired-pulse stimulation at 50 Hz of inhibitory neurons (n = 20 individual cells, *p < 0.05, Paired t-test).

(C) Average traces of vesicle fusion for 1 AP (black) or 2 AP delivered at 50 Hz (cyan) as measured with vG-pH.

(D) Average vG-pH ratio from excitatory (reproduced from Figure 3J) and inhibitory neurons (n = 8 individual cells for inhibitory neurons, *p < 0.01, Student's t-test).

Supplementary Figure 2. Impairment of synaptic facilitation during long trains of stimulation in K_vβ1 KD neurons.

(A) Average traces of vesicle fusion in responses to a 50 AP at 50 Hz stimulation as measured with vG-pH from control (black) and K_vβ1 KD (gray).

(B) Average max of % total vesicles (normalized to NH₄Cl alkalization) for control and K_vβ1 KD neurons (n = 22 for control; n = 9 for K_vβ1 KD; *p < 0.01, Student-t test).

Supplementary Figure 3. AP broadening by paired-pulse stimulation in excitatory neurons correlate with synaptic facilitation, but not with Ca²⁺ facilitation.

(A) Schematic of vector describing bicistronic expression of QuasAr and GCaMP separated by a P2A peptide that cleaves the two proteins for separate localization.

(B) Immunofluorescence of QuasAr-P2A-GCaMP expression in axons. GCaMP and QuasAr were stained with anti-GFP and anti-mCherry antibodies, respectively. QuasAr contains a mOrange-tag mutated to be non-fluorescent as to not have cross talk with the GFP signal. Synapsin1 staining was used for marking presynapses. Arrowheads mark presynaptic terminals. Scale bar, 10 μm.

(C) Schematic of vector describing bicistronic expression of QuasAr and vG-pH separated by a P2A peptide that cleaves the two proteins for separate localization.

(D) Immunofluorescence of QuasAr-P2A-vGpH expression in axons. vG-pH and QuasAr were stained with anti-GFP and anti-mCherry antibodies, respectively.

(E) Representative measurement of QuasAr from axon with *en passant* synapses with 2 AP at 50 Hz stimulation in excitatory neurons. Blue arrow indicates the first stimulation and red arrow indicates the second stimulation. Red dashed line represents the half maximum where the width was measured.

530 **(F)** Average FWHM for the first (blue) and second (red) AP waveform in paired-pulse stimulation of ex-
531 citatory neurons ($n = 19$, $*p < 0.001$, Paired t-test). Error bars indicate mean $\pm$ SEM.

532 **(G)** *Left*, Representative trace of GCaMP response to a single AP (blue) and paired-pulse stimulation (cyan).
533 *Right*, Representative QuasAr ΔF (red) and GCaMP ΔF images (green) during the stimulation. Scale bar, 2
534 μm .

535 **(H)** Average Ca^{2+} influx measured by GCaMP in paired-pulse stimulation ($n = 16$ individual cells, $*p <$
536 0.001 , Paired t-test).

537 **(I)** Correlation between 1st AP induced Ca^{2+} influx and FWHM of 1st AP. Linear fit is shown using a dashed
538 line.

539 **(J)** Correlation between paired-pulse ratio of GCaMP (PPR GCaMP) and FWHM (PPR FWHM) using a
540 linear fit.

541 **(K)** *Left*, Representative trace of vG-pH response to a single AP (black) and paired-pulse stimulation (cyan).
542 *Right*, Representative QuasAr ΔF (red) and vG-pH ΔF images (green) during the stimulation. Scale bar, 2
543 μm .

544 **(L)** Correlation using a linear fit between paired-pulse ratio of vG-pH (PPR vG-pH) and FWHM (PPR
545 FWHM). Gray dashed line is the fitted line ($n = 9$). Vertical red dashed line indicates where vesicle fusion
546 doubles in the second AP relative to the first AP.

547

Figure 1

Excitatory Presynaptic Terminals

Inhibitory Presynaptic Terminals

Figure 2

Figure 3

Figure 4

Supplementary Figure 1.

Supplementary Figure 2.

Supplementary Figure 3.