Synapse-Level Determination of Action Potential Duration by $K^+$ Channel Clustering in Axons

**Highlights**
- Spike duration varies between presynaptic boutons within an individual SC axon
- The varicose geometry of presynaptic boutons does not alter spike duration
- Fast-activating $K^+$ channels are clustered at boutons and restricted from shafts
- AP duration is determined by $K_v3$ channels immediately local to an individual bouton

**Authors**
Matthew J.M. Rowan, Gina DelCanto, Jianqing J. Yu, Naomi Kamasawa, Jason M. Christie

**Correspondence**
jason.christie@mpfi.org

**In Brief**
Rowan et al. find that APs are not uniformly represented across the axon arbor of SCs. Rather, spike duration is subject to local variation, determined at individual release sites, by clustering of fast-activating $K^+$ channels, thus contributing to release heterogeneity.
Synapse-Level Determination of Action Potential Duration by K⁺ Channel Clustering in Axons

Matthew J.M. Rowan, Gina DelCanto, Jianqing J. Yu, Naomi Kamasawa, and Jason M. Christie

INTRODUCTION

Spike width has a profound influence on neurotransmission, for the action potential (AP) is the command waveform directing Ca⁺ channel opening, and release probability is steeply dependent on Ca²⁺ concentration (Borst and Sakmann, 1999; Sabatini and Regehr, 1997). Recent work has shown that AP shape, determined in part by K⁺-mediated currents, may be adaptively modulated in response to changes in Ca²⁺ concentration (Borst and Sakmann, 1999; Sabatini and Regehr, 1997). Recent work has shown that AP width varies between presynaptic bouton sites, even within the same axon branch. The varicose geometry of SC boutons alone does not impose differences in spike duration. Rather, axonal patching revealed heterogeneous peak conductance densities of currents mediated mainly by fast-activating K₃.₃-type potassium channels, with clustered hotspots at boutons and restricted expression at adjoining shafts. Blockade of Kᵥ channels at individual boutons indicates that currents immediately local to a release site direct spike repolarization at that location. Thus, the clustered arrangement and variable expression density of K₃.₃ channels at boutons are key determinants underlying compartmentalized control of AP width in a near synapse-by-synapse manner, multiplying the signaling capacity of these structures.

SUMMARY

In axons, an action potential (AP) is thought to be broadcast as an unwavering binary pulse over its arm, driving neurotransmission uniformly at release sites. Yet by recording from axons of cerebellar stellate cells (SC) interneurons, we show that AP width varies between presynaptic bouton sites, even within the same axon branch. The varicose geometry of SC boutons alone does not impose differences in spike duration. Rather, axonal patching revealed heterogeneous peak conductance densities of currents mediated mainly by fast-activating K₃.₃-type potassium channels, with clustered hotspots at boutons and restricted expression at adjoining shafts. Blockade of Kᵥ channels at individual boutons indicates that currents immediately local to a release site direct spike repolarization at that location. Thus, the clustered arrangement and variable expression density of K₃.₃ channels at boutons are key determinants underlying compartmentalized control of AP width in a near synapse-by-synapse manner, multiplying the signaling capacity of these structures.

RESULTS

AP Width Varies between Axonal Boutons

We studied the functional properties of SC axons in acute cerebellar slices using targeted subcellular recording. To visualize axons, SCs were filled somatically during whole-cell recording with a fluorescent dye and imaged with two-photon (2P) microscopy. The boutons of labeled axons were optically targeted for loose-seal patch recording under continuous imaging using fluorescent pipettes (Sasaki et al., 2012) (Figure 1A). Soma-
elicited APs were resolved in the axon as rapid action currents with inward and outward components corresponding to the depolarizing and repolarizing phase of the underlying AP, respectively. The peak-to-peak timing of these components closely approximates AP half-width (Rowan et al., 2014; Sabatini and Regehr, 1997; Sasaki et al., 2011; Yang and Wang, 2006). Using two axonal electrodes, simultaneous recordings were obtained from boutons located on nearby branches (Figures 1A and 1B). Comparison of axonal action currents revealed considerable variability in duration between recording sites (Figures 1B and 1C), independent of bouton order or distance from the soma (Figure 1D), indicating that the rate of AP repolarization is not constant within an axon. Action current duration was unchanged during repetitive spiking (20 Hz; Figures S1A and S1B, available online), suggesting that differences persist despite ongoing suprathreshold activity. Differences in AP duration between recording sites were also observed at near-physiological temperature (32°C), in mature mice (>6 weeks old and 32°C; Figure 1C), and during spontaneous firing (16.2 ± 2.7 Hz; Figures S1C and S1D).

In a separate set of experiments, we used voltage-sensitive dye (VSD) imaging to record APs at several axon locations. After filling with the VSD di-2-AN(F)EPPTEA (Acker et al., 2011) during whole-cell recording (Figure 1E), somatically evoked APs were recorded with 2P VSD imaging from two boutons on the same axon (Figure 1F). Examples are shown from boutons separated by a branch point (left) or near neighbors on the same branch (right). (G) Summary data for multisite VSD imaging of boutons (different branch [≥1 branch between sites], mean distance 24.9 μm, range 19.9–52.5 μm; and same branch, mean distance 5.9 ± 0.8 μm, range 1.0–11.1 μm). Spike width varied to a greater extent between boutons located on separate branches (*p = 0.03; Mann-Whitney test).

(H) Relative to their adjoining bouton, APs in shafts were broader in duration (*p < 0.0001; paired t test; mean distance between recording locations 5.9 ± 0.8 μm).

(J) Orthodromic AP broadening in axon shafts depends on distance from the bouton. A monoexponential fit to grouped data (4 μm bins) yielded a length constant (λ) of 3.9 μm (*p < 0.05; one-way ANOVA with Dunnett’s multiple comparison test).

For all summary graphs, data are expressed as mean (±SEM) and shown in black with individual data points in gray. See also Figure S1.

Figure 1. Variable Spike Width in SC Axons

(A) 2P fluorescence image of an SC (Alexa 594; 60 μM) with simultaneous loose-seal recordings from two boutons on separate axon branches.

(B) Axonal action currents were elicited by somatically evoked APs. In the enlarged view, superimposed currents are aligned by the peaks of their inward components with the width difference indicated by the double-headed gray arrow.

(C) Group data summary of action current (AC) width comparisons for multisite axon recordings. Differences in width are expressed as a ratio between same-cell measurement locations. Conditions were not significantly different from one another (p > 0.05; one-way ANOVA with Tukey’s multiple comparison test).

(D) Action current widths are plotted for multisite axon recordings as a function of distance from the axon hillock. Same-cell measurements from a common axon are connected (mean distance between boutons 28.3 μm; range 9.2–46.8 μm; ≥1 branch between sites).

(E) Representative SC image with bouton VSD recording sites depicted in the inset.

(F) APs recorded with 2P VSD imaging from two boutons on the same axon. Examples are shown from boutons separated by a branch point (left) or near neighbors on the same branch (right).

(G) Summary data for multisite VSD imaging of boutons (different branch [≥1 branch between sites], mean distance 24.9 μm, range 19.9–52.5 μm; and same branch, mean distance 5.9 ± 0.8 μm, range 1.0–11.1 μm). Spike width varied to a greater extent between boutons located on separate branches (*p = 0.03; Mann-Whitney test).

(H) APs from a bouton as well as a site on the connecting shaft recorded using 2P VSD imaging.

(I) Relative to their adjoining bouton, APs in shafts were broader in duration (*p < 0.0001; paired t test; mean distance between recording locations 5.9 ± 0.8 μm).

(J) Orthodromic AP broadening in axon shafts depends on distance from the bouton. A monoexponential fit to grouped data (4 μm bins) yielded a length constant (λ) of 3.9 μm (*p < 0.05; one-way ANOVA with Dunnett’s multiple comparison test).

For all summary graphs, data are expressed as mean (±SEM) and shown in black with individual data points in gray. See also Figure S1.
resolved in axons at high temporal resolution from diffraction-limited points of interest with non-scanning 2P excitation (Rowan et al., 2014). Similar to electrophysiological measurements, spike width varied between boutons located on separate branches (Figures 1F and 1G), with the average absolute difference in AP duration comparable with both methods (0.21 ± 0.06 and 0.19 ± 0.04 ms; p = 0.94; unpaired t test; patch recording and VSD imaging, respectively). Unlike electrode recording, 2P VSD imaging allows for targeted subcellular recording without spatial constraint, including boutons within the same axon branch (Figure 1E). In paired measurements from within-branch boutons, variability in AP width was also apparent (Figures 1F and 1G), despite their close apposition. However, spike width was less variable relative to measurements from boutons belonging to different branches (Figure 1G), indicating increased regional homogeneity in AP repolarization rate within individual axon branches.

To assess the spatial extent by which spike width is locally determined within an axon branch, we used VSD imaging to record APs in both a bouton and its outwardly connecting shaft (Figure 1H). In this comparison, we biased recordings to relatively isolated bouton-shaft pairs where the close apposition of a bouton on the branch was >5 μm distal to the shaft recording location. Orthodromic APs were consistently broader in duration in shafts relative to boutons (Figure 1I), indicating that differences in AP width within an axon branch are not a simple reflection of random variability. As APs propagated from boutons into shafts, broadening occurred in a distance-dependent manner with a length constant (λ) of 3.9 μm (Figure 1J). Together, these results demonstrate that AP width within SC axons is not uniform. Rather, spike duration varies on an exquisitely fine spatial scale, indicating a high degree of compartmentalization in determination of AP repolarization rate.

**Axonal Geometry and AP Width**

If AP width is not uniform in an axon, then there must be local attributes that impose alteration of spike shape. As APs course through axons, they encounter complex geometric features, including frequent branch points and varicose enlargements at presynaptic boutons where discontinuities in impedance may alter spike propagation (Goldstein and Rall, 1974; Lüscher and Shiner, 1990). To assay how the relative geometric relationship of boutons and their adjoining shafts affects the width of a spike, we used compartmental modeling with simulated APs. To constrain our model, we quantified the structural details of SC axons using three-dimensional reconstructions from serial-section electron microscopy (EM) images (Figure S2). Notably, in these images there was no obvious oligodendrocyte ensheathment of shafts, indicating that SC axons are unmyelinated.

From these ultrastructural measurements, we developed a simplified compartmental model of an SC axon with reduced morphological complexity (Figure 2A); however, the diameters of shafts and boutons were constrained by mean values obtained from EM analysis (153 and 374 nm for shafts and boutons, respectively; Table S1). Within a test region containing a single bouton-shaft pair (Figure 2A), the geometric ratio (Segev and Schneidman, 1999) (GR) varied with bouton diameter (range 244–804 nm; e.g., GR = 3.8, mean bouton diameter). Axons were populated with uniform arrangements of fast-activating Na, and K, channels (Engel and Jonas, 2005; Lien and Jonas, 2003), resulting in APs with durations (802–857 μs) similar to our experimental measurements. Analysis of AP duration over a range of realistic GR values indicated that axonal geometry modestly contributes to local width changes of spikes in SC axons (Figures 2B and 2G). However, even the largest geometric mismatches between boutons and shafts (GR ≥ 12) failed to recapitulate our experimental observations.

We sought to experimentally assess whether AP duration is related to axonal geometry by comparing the spike widths of two boutons on the same branch, recorded using VSD imaging, to their relative volumetric difference. As structural features of SC axons approach the resolution limit of 2P microscopy, we used the fluorescence intensity of a chromatically compatible inert intracellular dye (Oregon Green 488) as measure of bouton volume (Svoboda, 2004). However, differences in AP width did not co-vary with bouton size (Figure 2D). As a second approach, we experimentally imposed an increase in GR between boutons and shafts and measured for an accompanying change in AP width. Perfusion of a hyposmotic extracellular solution induced rapid (~30 s) enlargements of axonal structures (Babila et al., 1990; Pullarkat et al., 2006) with a greater volume change in boutons relative to shafts (Figures 2E and 2F). Despite an apparent GR increase, AP width was unaffected (Figure 2G; 1.07% ± 0.04% of control, n = 8, p = 0.12; paired t test). Together, these results suggest that impedance mismatches alone, determined by the variable geometry of varicose boutons and their attached axon shafts, do not account for the local differences in AP width within axons of SCs.

**K,3 Channels Contribute to Variability of AP Width**

An emerging view suggests that axons are composed of discrete functional domains whose excitability is each determined by the unique complement of channels specific to that region (Debanne et al., 2011). In SCs, K,3-type channels direct spike repolarization at boutons, but not the AIS, indicating a regional influence of these channels in the control of spike signaling (Rowan et al., 2014). We examined the possibility that the select influence of K,3 channels at boutons imparts control of spike repolarization on a much finer spatial scale, thus contributing to differences in AP width within and between axon branches. As previously reported (Rowan et al., 2014), bath application of the K,3 channel modulator BDS-I (Yeung et al., 2005) or the blocker TEA (Coetzee et al., 1999; Ishikawa et al., 2003) broadened APs in boutons (Figures 3A and 3B) measured with VSD imaging. Surprisingly, APs were unaffected in axon shafts (Figures 3A and 3B), suggesting a bouton-specific role for K,3-mediated currents in determining spike repolarization. This location-dependent effect of TEA reduced the difference in AP width between boutons and connecting shafts (Figures 3C and 3D). The normalizing effect of TEA was not due to technical errors related to VSD imaging (Figure S3). Furthermore, AP width became more uniform between boutons in TEA when measured with either VSD imaging or loose-seal recording (Figures 3E–3H). Thus, in the absence of K,3-mediated currents, spike duration is less variable in SC axons, directly implicating these channels in subregional control of AP width.
Non-uniform K⁺ Conductance Density in Axons

The location-specific role of Kv3 channels in determining AP repolarization specifically at boutons is indicative of a high degree of K⁺ channel organization within SC axons. To functionally evaluate voltage-gated K⁺ channel activity, we measured pharmacologically isolated K⁺ currents in axons in the cell-attached recording configuration (membrane seal resistance $13.4 \pm 1.2 \ G\Omega; \ n = 98$) obtained using small open-tip diameter pipettes (Figures S4A and S4B). Cells were simultaneously voltage clamped at the soma ($80 \ mV$) during axonal recordings to mitigate changes in intracellular membrane potential induced by maximally activating voltage pulses elicited by the axon-attached pipette (Williams and Wozny, 2011). Depolarization of the axonal patch evoked fast K⁺ currents ($t < 2 \ ms$) in most boutons (13 of 19 patches; $p < 0.05$; one-sample z test; Figures 4A and 4C), whereas in shafts, patches with fast outward currents were rare (2 of 13 patches; $p > 0.05$; one-sample z test). Accordingly, the average activation rate ($\tau$) of K⁺ currents was slower in shafts compared to boutons (Figure 4C). A barely discernable conductance attributable to an unknown K⁺ channel type was apparent in many shaft recordings and in the few bouton patches lacking fast currents (Figures 4A–4C). To convert currents to conductance density ($g_K$), we determined patch area with capacitance measurements (Figures S4C and S4D). Peak $g_K$ was highly variable in boutons (coefficient of variation $[CV] = 144\%$), with $g_K$ exceeding $100 \ mS/cm^2$ in some measurements (Figure 4D). On average, $g_K$ was greater in boutons compared to shafts (Figures 4A and 4D).

In a subset of data presented above, we used a non-morphological method to demarcate putative release sites for targeted patching. Molecular layer interneurons, including SCs, were virally transduced in vivo with high specificity (Figure 4E) with the vesicular protein synaptophysin tagged with YFP (Syph-YFP; Figure 4F). Syph-YFP was segregated to small punctate areas of SC axons, usually corresponding to varicose bouton swellings (Figure 4F). When patch recordings were targeted to varicose axonal sites expressing Syph-YFP, fast-activating K⁺ currents were encountered in most patches ($\tau < 2 \ ms$) (Figures 4G and 4H). In patches lacking Syph-YFP sampled from non-varicose axon areas, fast-activating currents were seldom encountered ($\tau < 2 \ ms$, 1 of 5 patches; $p > 0.05$; one-sample z test) and $g_K$ was significantly less than at Syph-YFP sites (Figures 4G and 4H). Together, these observations indicate a non-uniformity of K⁺ currents in SC axons, with clusters of fast-activating channels at bouton release sites and their restriction from shafts.
Kv3-Mediated Currents at Boutons

For pinceau terminals of basket cell molecular layer interneurons, fast-activating K+ currents are likely mediated by a combination of both Kv1 and Kv3 channels (Southan and Robertson, 2000), similar to the calyx of Held and mossy fiber boutons in the hippocampus and cerebellum (Alle et al., 2011; Ishikawa et al., 2003; Ritzau-Jost et al., 2014). To study the potential contribution of Kv3 channels in SC boutons, we pharmacologically inhibited Kv1 channels by inclusion of dendrotoxin-I (DTX) (Alle et al., 2011) in the axonal patch pipette. Fast-activating currents were encountered in bouton patches during maximally activating depolarizing pulses, although infrequently (Figure 5A; 9 of 19; p > 0.05; one-sample z test), suggestive that pharmacological separation by channel type may reveal a more elaborate clustered arrangement of channels on the bouton membrane. For biophysical analyses, we used a selection criterion rejecting patches with slow activation kinetics (t > 2 ms), which correlated with negligible current density (Figure S5A). For pharmacologically isolated Kv3-mediated currents, activation was rapid for maximally activating pulses (Figure S5B; \( t = 0.59 \pm 0.07 \text{ ms} \); n = 9) with a midpoint potential similar to that of Kv3 currents in both inhibitory interneurons and hippocampal mossy fiber boutons (Alle et al., 2011; Lien and Jonas, 2003) (Figure 5B; \( V_{\text{half}} = -14.6 \text{ mV} \); n = 4).

In fast-spiking interneurons, Kv3 channels may result from the co-assembly of both Kv3.1 and Kv3.4 channels (Baranauskas et al., 2003), with the composite current activation profile reflecting the influence of both channel types. Using Kv3.1 KO mice (Ho et al., 1997), Kv3-mediated currents exhibited a negative shift in activation voltage compared to wild-type (WT) mice (Figure 5B; \( V_{\text{half}} = 38.0 \text{ mV} \); n = 4), in agreement with the observation that channels composed of Kv3.1 subunits activate at more positive voltages (Baranauskas et al., 2003). Correspondingly, in boutons of Kv3.4 KO mice, currents had a positive shift in the midpoint of activation (Figure 5B; \( V_{\text{half}} = 2.9 \text{ mV} \); n = 5), in keeping with the more negative activation range of Kv3.4 channels (Baranauskas et al., 2003). Additionally, when compared to WT mice, Kv3-mediated mean peak \( g_k \) (Figure 5D) was reduced in both Kv3.1 KO mice (59.9 ± 29.3 mS cm\(^{-2} \); n = 5; p = 0.03; Mann-Whitney test) and in Kv3.4 KO mice (64.5 ± 16.4 mS cm\(^{-2} \); n = 6; p = 0.03; Mann-Whitney test).

Having functionally identified constituent subunits of Kv3 channels, we sought to further examine for the possibility of clustered arrangements of these channels in SC axons. Therefore, we virally transduced SCs with YFP-tagged Kv3.4 subunits (Kv3.4-YFP). Despite overexpression, we observed punctate Kv3.4-YFP fluorescence along the axon localized at boutons (Figure S6A), the level of which was only weakly correlated.
with bouton volume (Figure S6B). Notably, the fluorescence intensity of puncta varied considerably between neighboring sites (Figure S5C), suggesting that subunit composition or abundance is not uniform across boutons.

**Kv3-Mediated Currents Are Predominant during APs**

Although a pure Kv3 channel blocker is unavailable, low-concentration TEA (1 mM) inhibits Kv3 with high selectivity while keeping Kv1 channels relatively unblocked (Coetzee et al., 1999; Ishikawa et al., 2003), including DTX-sensitive channels expressed in SCs (Rowan et al., 2014). Fast-activating currents were apparent in a minority of recordings with TEA (Figure 5A: 8 of 17 patches; p > 0.05; one-sample z test). Examining these putative Kv1 currents, mean peak $g_{K1}$ was significantly reduced compared to measurements in DTX, indicating that the Kv3-mediated conductance likely predominates at boutons (Figure 5D). Kv1 channels typically activate at lower voltages than Kv3 (Dodson and Forsythe, 2004). Accordingly, in TEA, currents were shifted to lower activation voltages (Figure 5B; V$_{half}$ = -32.1 mV; n = 5) with respect to Kv3 currents measured in DTX. Fast-activating K+ currents were absent when both DTX and TEA were included in the cell-attached pipette (Figure 5A: 8 of 8 patches). Thus, these results indicate that although both Kv1 and Kv3 channels are expressed at boutons, Kv3-mediated currents are the major voltage-activated K+ conductance at these presynaptic sites.

To determine the current contribution of these K+ channel types during an AP, we drove channel opening from a physiological membrane potential with an AP-like voltage waveform kinetically similar to an axonal spike measured with VSD imaging (+90 mV peak). Peak $g_{K1}$ was substantially greater for pharmacologically isolated Kv3 currents than for those mediated by putative Kv1 channels (Figures 5E and 5F), indicating that during spiking, the Kv3-mediated conductance is overwhelmingly predominant. The relative recruitment of available Kv1 and Kv3 channels by a spike, determined by comparing currents evoked by the AP-like waveform to those of a square pulse to a maximally activating potential (Baranauskas et al., 2003; Martina et al., 2007) (Figure 5G), was similar in both channel types (23.8% ± 7.5% and 16.6% ± 3.4%; Kv1 and Kv3, respectively; p = 0.94; Mann-Whitney test). Because Kv3 and Kv1 currents displayed similar activation kinetics at suprathreshold voltages (Figures S5B and S5C), the predominant recruitment of Kv3-mediated currents during an AP likely results from their greater relative availability.
Comparison of fast-activating K+ currents recorded in DTX in a WT, Kv3.1 KO, or Kv3.4 KO mouse, evoked by a depolarizing pulse to a maximally activating potential in an isolated axon. We returned to compartmental modeling to systematically explore how the distribution of K,3-mediated currents at boutons may affect local spike repolarization. We focused on width changes of propagating APs in the shaft due to the predictability of distance-dependent duration changes observed experimentally (Figure 1J). We found that a uniform K,3-mediated conductance at boutons resulted in little change in AP duration throughout the axon shaft test region (Figure S7A). However, increasing K,3 conductance density at the test bouton relative to surrounding boutons led to a more rapid AP that broadened as the spike propagated into the shaft (Figures S7A and S7C–S7E), a factor that multiplied the effect of K,3 conductance density on AP duration at the test site. Thus, the variable availability of K,3 current at boutons can impart local changes in AP width, an effect amplified by impedance mismatches dictated by differential axon geometry.

**Bouton-Specific Control of AP Duration by Clustered K,3 Organization**

Our findings suggest that the clustered arrangement of K,3 channels at boutons imparts local tuning of spike repolarization between release sites in SC axons. If so, AP duration should be set by K,3 channels immediately local to an individual bouton and resistant to alteration in channel availability at other presynaptic sites. To experimentally determine the spatial extent by which K,3 channels influence AP repolarization, we used 2P laser uncaging (2PLU) of the photolyzable K+ channel blocker RuBi-4AP (Zayat et al., 2003) to locally inhibit fast-activating K+ channels at individual boutons and probe for changes in spike duration using simultaneous 2P VSD imaging.

When laser pulses were directed near the periphery of targeted boutons, photo-liberated 4AP was sufficient to induce AP broadening (Figures 6A and 6B; also observed in mice >6 weeks; 32 °C; 129.0% ± 12.2% increase from matched control; n = 9; p = 0.03; paired t test). This effect began to reverse rapidly (Figure 6B), likely owing to the small focal volume of 2P excitation and diffusion. When uncaging points were repositioned slightly away from the target bouton, 2PLU failed to induce spike broadening (Figure 6C), indicating that the area of 4AP-mediated channel block was likely constrained to a region encompassing approximately a single bouton. These results confirm that K+ channels in the immediate vicinity of an individual bouton shape spike duration at that site. If the rate of AP repolarization is determined at an individual bouton, then it is expected that broadening in one bouton would not be reflected at nearby boutons. To address this question, we recorded APs in interleaved trials from two boutons located in close proximity on the same branch and used 2PLU of RuBi-4AP to induce a substantial increase in spike width (≥15% of control) at the first site (Figure 6D). Despite an alteration at the test location, we found that spike broadening was highly reduced in orthodromic APs measured at the second site (Figure 6E). This result could be recapitulated in our compartmental model by simulating the loss

---

**Figure 5. Fast-Activating K+ Channel Types at Boutons**

(A) Depolarization-evoked K+ currents, recorded in cell-attached patches from separate boutons, in the presence of DTX (200 nM), TEA (1 mM), or both DTX and TEA.

(B) Activation curves of fast-activating K+ currents recorded in bouton patches and TEA. In separate patches, currents were recorded either in DTX or TEA. To experimentally determine the spatial extent by which K,3 channels influence AP repolarization, we used 2P laser uncaging (2PLU) of the photolyzable K+ channel blocker RuBi-4AP (Zayat et al., 2003) to locally inhibit fast-activating K+ channels at individual boutons and probe for changes in spike duration using simultaneous 2P VSD imaging.

When laser pulses were directed near the periphery of targeted boutons, photo-liberated 4AP was sufficient to induce AP broadening (Figures 6A and 6B; also observed in mice >6 weeks; 32 °C; 129.0% ± 12.2% increase from matched control; n = 9; p = 0.03; paired t test). This effect began to reverse rapidly (Figure 6B), likely owing to the small focal volume of 2P excitation and diffusion. When uncaging points were repositioned slightly away from the target bouton, 2PLU failed to induce spike broadening (Figure 6C), indicating that the area of 4AP-mediated channel block was likely constrained to a region encompassing approximately a single bouton. These results confirm that K+ channels in the immediate vicinity of an individual bouton shape spike duration at that site. If the rate of AP repolarization is determined at an individual bouton, then it is expected that broadening in one bouton would not be reflected at nearby boutons. To address this question, we recorded APs in interleaved trials from two boutons located in close proximity on the same branch and used 2PLU of RuBi-4AP to induce a substantial increase in spike width (≥15% of control) at the first site (Figure 6D). Despite an alteration at the test location, we found that spike broadening was highly reduced in orthodromic APs measured at the second site (Figure 6E). This result could be recapitulated in our compartmental model by simulating the loss

**AP Duration Is Shaped by the Local Availability of K,3-Mediated Currents**

From our patch measurements, the peak K,3-mediated gK,3 varied considerably from bouton to bouton (Figure 5D) and was significantly greater in boutons with respect to interconnecting shaft regions (Figure 4D), suggesting that the relative availability of K,3 current may be an underlying factor in the local determination of AP duration. We focused on width changes of propagating APs in the shaft due to the predictability of distance-dependent duration changes observed experimentally (Figure 1J). We found that a uniform K,3-mediated conductance at boutons resulted in little change in AP duration throughout the axon shaft test region (Figure S7A). However, increasing K,3 conductance density at the test bouton relative to surrounding boutons led to a more rapid AP that broadened as the spike propagated into the shaft (Figures S7A and S7C–S7E), a factor that multiplied the effect of K,3 conductance density on AP duration at the test site. Thus, the variable availability of K,3 current at boutons can impart local changes in AP width, an effect amplified by impedance mismatches dictated by differential axon geometry.
of $g_K$, at the test bouton (Figure S7F), indicating that the local availability of $K_v$ channels is a critical determinant of this effect. Together, these results confirm that AP repolarization is shaped in a highly localized manner, thus allowing boutons to individually tune spike width independent of other release sites.

4AP inhibits both $K_v1$ and $K_v3$ channels (Coetzee et al., 1999), though $K_v3$ channels are particularly sensitive to 4AP block (Alle et al., 2011; Ishikawa et al., 2003; Southan and Robertson, 2000). To examine for the specific role of $K_v3$ channels in 2PLU-induced AP broadening, we repeated Rubi-4AP uncaging experiments including TEA in the bath solution to occlude $K_v3$ channels. In this condition, spike duration was relatively insensitive to Rubi-4AP uncaging (Figures 6F and 6G). A similar result was also observed in matched comparisons made at the same bouton, where spike broadening was induced by Rubi-4AP uncaging prior to TEA application (123% ± 6.0% and 107% ± 6.2% of control AP duration; control and TEA, respectively; n = 5; p < 0.05; paired t test). In contrast, Rubi-4AP uncaging continued to
induce spike broadening in DTX (Figures 6F and 6G). Thus, the local availability of Kv3 channels governs spike repolarization rate at individual boutons in SC axons, with Kv1 channels playing an insignificant role. Although we cannot rule out the possibility that low abundance of Kv1-mediated current was insufficient to impart local control of AP repolarization, we found that substituting slower delayed rectifier K+ channels for Kv3 in our model greatly increased the apparent length constant of spike broadening following the simulated uncaging condition (Figure S7G). This suggests that the biophysical features of Kv3 channels are particularly well-adapted for spatially restricted control of spike repolarization in SC axons.

**Spike Width Differences Contribute to Heterogeneity of Release Efficacy**

To examine how local determination of spike width at SC boutons contributes to control of neurotransmission, we measured AP-evoked GABA_A autoreceptor (pre-GABA_A) currents (Pouzat and Marty, 1999). In voltage-clamped cells, brief depolarizing pulses (0.5–1.0 ms; +10 mV) elicited uncamped spikes at the soma, followed immediately by a transient synaptic current mediated by the evoked release of GABA and subsequent binding to pre-GABA_ARs expressed on the axon (Figures 7A and 7B). Autoreceptor currents were also resolved in simultaneous axon-attached recordings from boutons, showing rapid kinetics and inhibition by the GABA_A antagonist GABAzine (Figures 7A and 7B). Following block of Kv3 channels with TEA (1 mM) to induce AP broadening, we measured an increase in the peak amplitude of the pre-GABA_A current at the axon recording site (136.3% ± 14.1% of control amplitude; n = 9; p = 0.03; Wilcoxon matched-pairs test) and an increase in the time to peak (143.7% ± 18.6% of control; n = 7; Wilcoxon matched-pairs tests; Figure 7E). Consistent with an alteration in strength of neurotransmission, uncaging also produced a change in PPR at the axon site (Figure 7F); however, the PPR of the somatic current remained unchanged (Figure 7F), likely due to sampling of abundant pre-GABA_A activity from non-target axon regions. Therefore, by locally setting spike duration, and hence the AP-evoked Ca^{2+} entry (Rowan et al., 2014), the availability of Kv3 channels at individual SC boutons must be a contributing factor to differences in release efficacy and timing among synapses on the same axon.

**DISCUSSION**

We show that in axons of cerebellar SCs, spike duration varies within the axon, including among neighboring boutons on the same branch. Local modulation of spike waveform results from a non-uniform organization of voltage-gated K+ channels, including clustering of fast-activating Kv3 channels at presynaptic sites. Such organization yields a powerful adaptive property allowing individual release sites to locally shape the duration of a propagating spike, dependent on the local abundance of Kv3 channels and separate of other sites. This feature allows for tuning of neurotransmission in a near synapse-by-synapse manner, thus contributing to the heterogeneity of release, an important factor permitting flexibility in neural circuit signaling.

**Local Determination of AP Duration in Boutons**

The perception that APs propagate as a static waveform throughout the entire axon has been continuously challenged in a variety of neuron types in both invertebrates (Lin, 2012; Nagano and Cooke, 1987) and vertebrates (Kawaguchi and Sakaba, 2015; Kole et al., 2007). Our results indicate that although spikes are reliably transmitted in SC axons, the duration of the AP frequently deviated from one presynaptic site to the next. This phenomenon may also occur in other neuron types. For example, whole-cell measurements from hippocampal mossy fiber boutons show considerable variation in spike duration within the sample population (i.e., 254–533 μs) (Geiger and Jonas, 2000), although direct observations from multiple sites on a single axon are lacking. In this report, we used multisite axon recording and found significant variability in spike duration within the axon of individual SCs, including nearby boutons on the same branch.

Control of spike signaling is often partitioned in axons and dependent on non-uniform distributions of voltage-gated ion channels, including K+ channels (Debanne et al., 2011; Trimmer, 2015). In SC axons, our functional mapping revealed fast-activating K+ channels at presynaptic boutons that were largely excluded from shafts, indicating a high degree of specificity in their subcellular organization. That the length constant of AP broadening in the shaft was less than that expected for purely
passive signals, including those with a high frequency content such as an AP, indicates that the organization of active currents is paramount for this effect.

APs are autoregenerative signals with repolarization largely determined by K+ currents. By clustering K+ channels at presynaptic locations in SCs, AP duration is shaped on a very local scale dependent on K+ currents immediately available at an individual bouton. In this way, spike broadening induced by focal block of K+ channels in a target bouton is highly reduced in the orthodromic spike measured at near-neighbor sites. Comparatively, focally induced AP broadening in axons of L5 pyramidal cells (Kole et al., 2007) and hippocampal CA3 neurons (Sasaki

Figure 7. Local Control of AP Width Contributes to Heterogeneity of Release

(A) Autocurrents recorded at the soma in voltage-clamp mode following brief depolarization as well as at the axon during simultaneous cell-attached recording in the loose-seal configuration. The unclamped spike elicited at the soma and action current at the axon were blanked for clarity.

(B) Autocurrent response measured at the axon in control and in gabazine (20 μM). Summary data show that the autoreceptor current, measured at either at the axon or soma, was GABAAR mediated.

(C) At the axon, TEA induced an increase in the peak-to-peak duration of the action current and a corresponding increase in the amplitude of the synaptic pre-GABAAR response. In the magnified view on the right, dashed lines show that the time-to-peak response of the pre-GABAAR current in TEA was also delayed relative to the control response.

(D) Multisite recording of pre-GABAAR currents from the same axon. The dotted line indicates the amplitude of the first response for paired stimuli.

(E) Targeted 2PLU of Rubi4AP (300 μM) over a small region of axon centered at the recording location (20–30 pulses; over ~15 μm of axon). Currents corresponding to the AP and pre-GABAAR response are shown for control and uncaging conditions.

(F) GABAAR responses following two closely timed APs in both control and uncaging conditions from the same recording location. Summary plots show uncaging induced changes in PPR at the axon recording site, but not at the soma (*p = 0.007; paired t test).

For all summary graphs, mean values (±SEM) are shown in black with data from individual patches in gray outline.
et al., 2011) may extend for much greater distances, perhaps owing to differences in the types and organization of K+ channels. Although, in L5 pyramidal cells regulation of AP repolarization by DTX-sensitive K,1 channels in axon collaterals may occur in a more localized manner (Foust et al., 2011), suggesting that local shaping of APs may be a generalizable feature of unmyelinated axon regions.

Kv3-Mediated Currents in Boutons
Following pharmacological block of Kv3, AP duration was much more uniform in the axons of SCs, indicating that the organization of this fast-activating channel type is a key parameter permitting heterogeneity of spike repolarization within individual axons. In cell-attached patches from SC boutons, we recorded K+ currents mediated by both K,1- and Kv3-type channels. The same current complement has also been reported for the principal axon specializations of cerebellar basket cells (Southan and Robertson, 2000), a closely related interneuron type in the molecular layer (Sultan and Bower, 1998). Although currents mediated by both K,1 and Kv3 channels contribute to AP repolarization in the boutons of some cell types (Hoppe et al., 2014; Ritzau-Jost et al., 2014), we found that the conductance carried by Kv3-mediated currents predominates when driven by an AP-like waveform from a physiologically resting membrane potential, likely explaining why AP repolarization is determined by this channel type rather than K,1 (Rowan et al., 2014). At hippocampal mossy fiber boutons, fast-opening Kv3 channels outpace other channel types to direct spike repolarization (Alle et al., 2011). However, we did not measure an appreciable difference in K,1 and Kv3 activation rates at suprathreshold potentials, suggesting that relative availability, rather than rapid and efficient opening, is the determinate factor guiding K+ channel participation in AP repolarization in SC axons. The reasons for these cell-type-specific differences are not clear; however, neurons may achieve state-dependent control of K+ channels by differential phosphorylation or assembly with accessory subunits to precisely determine excitability (Macica and Kaczmarek, 2001; Rettig et al., 1994; Ritter et al., 2012).

In myelinated axons, Kv3 channels are known to cluster in macromolecular complexes at nodes of Ranvier and, in the case of Kv3, are dependent on interactions with the membrane-bound proteins (Devaux et al., 2003). Our work points to differences in the current density of clustered Kv3 channels at release sites as the underlying factor contributing to variability in spike duration in SC axons. The trafficking, sequestration, and complexing of Kv3 channels in unmyelinated axons are, in general, poorly understood (Trimmer, 2015), and hence the mechanistic underpinnings resulting in the differential expression density of Kv3 at SC boutons remain unclear. It is important to note that variability in our patch measurements may stem from sampling random channel clusters on the membrane of individual boutons. In fact, fast-activating K+ currents weren’t apparent in many bouton patches, an observation that was more common following pharmacological isolation of K,1 and Kv3 currents, suggestive of partitioning of these channel types. However, micoclustering of Kv3 channels within a bouton would not necessarily prevent local shaping of APs, as the average conductance density across the bouton would still likely affect spike duration given the small and uniform nature of bouton morphology. Whether differences in Kv3 channel abundance at other synapses including mossy fiber boutons (Alle et al., 2011) are sufficient to impart a change in AP duration is unknown.

Kv3 Channel Types
Kv3 channel gating properties vary with subunit composition, including the voltage dependence of activation (Baranauskas et al., 2003). Using KO mice, we identify diminished Kv3-mediated current density in boutons as well as phenotypic alteration in activation voltage accompanying loss of either Kv3.1 or Kv3.4, indicating that these subunits are constituent components of Kv3 channels in SC axons. Kv3.4 channels have a hyperpolarized activation voltage that is amenable to efficient recruitment during an AP (Baranauskas et al., 2003), perhaps contributing to the high K+ flux required for local tuning of AP waveform, as indicated by our modeling. Interestingly, replacing Kv3 channels with a slower delayed rectifier type in our simulations increased the apparent length constant of AP broadening, indicating that the biophysical features of Kv3 channels are well-suited to support local control of AP shape. As Kv3 subunits can co-assemble to form functional channels with intermediate gating features (Coetze et al., 1999), it may be that the Kv3 subunit composition is differentially regulated across SC release sites, determined by development or plasticity, imparting differential control of local spike duration. However, we did not systematically explore the subunit stoichiometry of Kv3 channels in SC axons.

Axon Geometry and AP Duration
Ultrastructural inspection of SC axons revealed a compact electrotone structure amenable to local spike shaping. SC axons are thin, include frequent geometric swelling at presynaptic boutons, and are likely unmyelinated given the conspicuous absence of oligodendrocyte ensheathment. Though the varicoso morphology of boutons may impose a substantial electrical load, or impedance, to an invading AP (Goldstein and Rall, 1974; Lüscher and Shiner, 1990), our experimental and modeling data indicate that it is unlikely that geometry alone distorts the waveform to sufficiently account for the variability in AP duration we measured in individual SCs. Similar to the unmyelinated axons of CA3 pyramidal cells in the hippocampus (Shepherd and Harris, 1998), the geometric difference between boutons and their adjoining shafts in SCs is quite small. However, our modeling results indicate that bouton geometry may work in combination with the variable expression density of Kv3 channels to amplify changes in AP duration. Yet we found no predictable relationship between AP duration and bouton size, indicating that the organization of K+ channels may be regulated independent of morphological features.

Local Control of AP Duration and Neurotransmission
As shown previously in SC boutons, spike repolarization rate directly affects Ca2+ channel opening and Ca2+ influx, and therefore determines release efficacy (Rowan et al., 2014). In this report, using axon-targeted recording of pre-GABAergic mediated currents, we find intersynaptic variability of release probability that can be locally modified by the duration of the AP at a bouton release site. Release heterogeneity, even within individual axons of SCs,
depends on many structural and molecular properties, including the number of vesicle docking sites (Pulido et al., 2015; Trigo et al., 2012), whose attributes likely stem from the abundance of presynaptic Ca\textsuperscript{2+} channels that scale with bouton size, similar to other small central synapses (Holderith et al., 2012). We propose that variation in AP-evoked Ca\textsuperscript{2+} entry may not only reflect variability in Ca\textsubscript{v} channel number but also underlying differences in AP width, and may be set independent of bouton size (Emroyuk et al., 2012) if Ca\textsubscript{v} and K\textsubscript{v} channel abundance is set independently. However, adaptive plasticity may drive changes in AP duration through modification of presynaptic K\textsuperscript{v} channels in lockstep with the organization and abundance of Ca\textsubscript{v} channels to maintain release probability in a useful signaling range (Hoppa et al., 2012, 2014), indicating a concerted interplay between spike repolarization and Ca\textsuperscript{2+} channel availability and regulation. AP broadening is also well-suited to affect synaptic timing (Boudkikazi et al., 2011), as Ca\textsuperscript{2+} entry mainly occurs during spike repolarization (Sabatini and Regehr, 1996, 1997). Thus, local control of AP duration at individual release sites is likely an important feature contributing to heterogeneity in both the timing and strength of neurotransmission from sites on the same axon, representing an additional element contributing to the diverse coding range of synapses necessary for many computations. The absolute relationship between AP repolarization, Ca\textsuperscript{2+} entry, and effect on neurotransmission likely requires additional investigation, as GABA\textsubscript{R} receptor saturation at SC synapses will blunt the amplitude change of the synaptic response following an increase in release probability (Pulido et al., 2015). Additionally, a clustered organization of fast-activating K\textsuperscript{v} channels may be metabolically advantageous, as the energetic cost of AP signaling is thought to consume substantial amounts of ATP to maintain and restore ionic gradients (Alle et al., 2009; Carter and Bean, 2009). By limiting K\textsuperscript{v} channel activity to synapses, spike repolarization may be modulated in a more energy-efficient manner.

**EXPERIMENTAL PROCEDURES**

Experimental Procedures are described in detail in the Supplemental Experimental Procedures. All animal procedures were approved by the Max Planck Florida Institute for Neuroscience Animal Care and Use Committee.

**Presynaptic Recordings from SC Axons**

SC recordings were performed in cerebellar slices prepared from C57Bl/6 mice (post-natal day [P]15–P54) at 24°C or 32°C. Axonal loose-seal or channel recordings were guided with fluorescently labeled pipettes to axonal sites of interest. The voltage-sensitive dye di-2-AN-(F)EPPTEA (30 μM; L. Loew; University of Connecticut Health Center) was included in the intra-cellular solution for voltage imaging experiments. All imaging was performed with a 2P laser scanning microscope (Ultima; Bruker Corp.) using an Olympus upright microscope (BX51WI) and objective (60x, 1.0 NA), and excitation provided by a mode-locking Ti:sapphire laser (Chameleon Ultra II; Coherent Inc.).

**RuBi-4AP Uncaging**

The photolyzable voltage-gated K\textsuperscript{v} channel blocker bis(2,2’-Bipyridine-N,N’) bis(4-aminopyridine-N1) ruthenium (RuBi-4AP; Abcam) (Zayat et al., 2003) was recirculated in the bath solution and uncaged with rapid pulses (750 nm; 2 ms) from a second mode-locked laser (Chameleon Ultra II; Coherent Inc.). Pulses were modulated with a Pockels cell. Uncaging sites were directed within close proximity (~0.5 μm) of targeted boutons using a set of galvanometer mirrors independent of the imaging path, allowing for rapid positioning of the photolysis beam.

**Electron Microscopy and 3D Reconstructions**

Axons of SCs were ultrastructurally reconstructed from P19 mice using aldehyde-fixed cerebellar tissue. Utrathin serial sections (~70 nm) were imaged with a transmission electron microscope (Tecnai G2; FEI) at 80 kV. Image stacks were manually inspected to identify axon fragments of SC interneurons. Areas containing SC axons were cropped for reconstruction and analysis using RECONSTRUCT software (Fila, 2003).

**Compartmental Model and Simulations**

Simulations of AP propagation were performed using the NEURON environment (Hines and Carnevale, 2001) and analyzed using MATLAB (Mathworks). Compartmental model morphology was set to represent a simplified SC axon. Simulations included both voltage-gated Na\textsuperscript{+} and K\textsuperscript{+} channels implemented as Hodgkin-Huxley-type models.

**Intracranial Viral Injections**

Severoaxonal injections were performed on mice (P18–P28) under anesthesia. Viruses were injected into the cerebellar vermis. After allowing 14–42 days for expression, animals were sacrificed and the cerebellum used for acute slice preparation.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.05.035.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Chris McBain (NICHD), Yishai Elyada (Hebrew University), and the members of the J.M.C. lab for their helpful discussions and comments during the preparation of this manuscript. Synaptophysin plasmid DNA was generated by Hiroki Tanaguchi and Andre Steinecke (MPFI). Fluorescently tagged K\textsubscript{v}3.4 plasmid DNA was provided by Tatiana Tkatch (Lithuanian University of Health Sciences) and D. James Surmeier (Northwestern). Plasmids were prepared with help from the Max Planck Florida Molecular Core Facility. This work was supported by the Max Planck Society, the Max Planck Florida Institute for Neuroscience, NIH grant NS083127 (M.J.M.R.), and NIH grant NS083894 (J.M.C.).

Received: September 21, 2015

Revised: March 13, 2016

Accepted: May 23, 2016

Published: June 23, 2016

**REFERENCES**


Please cite this article in press as: Rowan et al., Synapse-Level Determination of Action Potential Duration by K+ Channel Clustering in Axons, Neuron (2016), http://dx.doi.org/10.1016/j.neuron.2016.05.035


Supplemental Information

Synapse-Level Determination of Action Potential Duration by K⁺ Channel Clustering in Axons

Matthew J.M. Rowan, Gina DelCanto, Jianqing J. Yu, Naomi Kamasawa, and Jason M. Christie
Figure S1

(A) To assess whether repetitive activity results in modulation of the AP waveform in SC boutons, a train of APs (20 Hz) were evoked at the soma by current injection and recorded simultaneously in a bouton-attached recording. In the enlarged view, axonal action currents from the first and tenth spike in the train are shown superimposed for comparison.

(B) Summary data from action current recordings during fixed (20 Hz) firing. The widths of the action currents were compared for both the 1st and 10th spikes. No change was observed (p > 0.05; paired t-test). Recordings were performed in mature (> 6 week old) animals at 32°C.

(C) Multisite axon recording of action currents from two boutons on separate axon branches during spontaneous firing. On the right, superimposed currents from two bouton sites, aligned by the peaks of their inward components, with width difference demarcated by double-headed arrow.

Figure S1, related to Figure 1.

Axonal AP width differences during repetitive firing
(D) Summary data for multisite axon recordings during spontaneous spiking. Differences in action current width are expressed as a ratio between measurement locations on the same cell. Recordings were performed in mature (> 6 week old) animals at 32°C.
Figure S2, related to Figure 2.

Ultrastructural analysis of SC axons

(A) A partial ultrastructural reconstruction of a SC with the axon membrane shown in blue. The axon is festooned with frequent varicose bouton swellings (in our analysis, > 50% increase in diameter compared to connecting shaft).

(B) Examples segments of axon shafts and boutons partitioned from larger axon fragments. Boutons (n = 40) always encompassed synaptic vesicles and, less frequently, mitochondria (67.5%). The scale bar applies directly to the bouton illustrated at the bottom right and approximately to the other segments as their 3D orientation yield modest perspective errors. In the reconstructions, vesicles are marked in red, mitochondrion in purple, and when apparent, the pre-SDs are in brown.
(C) Cumulative distributions of average diameter for segments of axon shafts and boutons. Data are fitted with Gaussian functions.

(D) In one SC axon fragment reconstructed from tissue prepared using a more conventional staining method (complete reconstruction not shown), we could identify unambiguous synaptic hallmarks in the majority of identified varicosities (5 of 8). Shown in the figure above, are serial micrographs from one bouton example. Unambiguous hallmarks of a release site including an electron dense pre- and post-SD (filled black triangles), nearby vesicle aggregates, and multi-vesicular structures. For the reconstruction, the pre-SDs is marked in brown, vesicles in red, and mitochondrion in purple. Thus, in SC axons, varicosities are typically morphological correlates of bouton release sites.
**Figure S3**

![Image of Figure S3](image)

**Figure S3**, related to Figure 3.

*Error analysis and resolution of the VSD imaging technique*

**(A)** Individual fluorescence traces recorded at the soma during two different AP-like voltage commands, each with different durations (half-width durations: 880 and 1100 μs, blue and red traces, respectively), are shown for comparison (80 trials each). The averaged response for each command waveform (± 2 SD) are displayed below.

**(B)** Summary data of trial-to-trial variability as a function of AP-command waveform duration (600, 880 and 1100 μs, for APs 1-3, respectively). The durations of the optical responses were measured for each individual trial and variability quantified using coefficient of variation (CV). CVs for each command waveform were determined for a number of cells, then averaged and group data compared with ANOVA followed by Tukey’s multiple comparison test. There were no differences (p > 0.05) between the groups. Thus, VSD signals are not subject to increased error over a physiologically-relevant range of AP widths. All values are plotted ± SD.

**(C)** APs recorded with VSD imaging from the same bouton were sampled at different rates.

**(D)** Relative to same site measurements at 10 kHz, no differences were found in AP duration when sampled at 20 kHz (both groups filtered at 1 kHz) or 50 kHz (filtered at 1 and 2 kHz respectively). Thus, AP width is accurately described when sampled at 10 kHz. In both plots, p > 0.05; wilcoxon matched-pairs test.
Figure S4, related to Figures 4 and 5.

**Properties of axonal patch pipettes**

(A) Scanning EM images of typical pipettes used for somatic whole-cell and axon patch recordings (open tip resistances of 6.9 and 34.0 MΩ, respectively). The axon pipette was fire-polished resulting in a thicker outer wall.

(B) Plot showing the relationship between open-tip pipette conductance (reciprocal of resistance) and inner diameter. Open-tip conductance was measured for each pipette following SEM imaging using standard solutions for cell-attached recordings. Dotted lines indicate the open-tip conductance range used for axon pipettes. Data were fit with a monoexponential function.

(C) Pipette with excised patch from a bouton pressed slightly into a silicone elastomer ball. The outside face of the vesicle was first ruptured by brief insertion into the silicone elastomer, resulting in the inside-out configuration. The difference current measured before and after insertion (<5 μm) of the patch-containing pipette into the elastomer ball is shown on the right. Currents were evoked by a -50 mV hyperpolarizing pulse (average of >40 trials each). Patch capacitance was determined by measuring the integral under the difference current (gray shaded region). Capacitance was converted to membrane area assuming a specific membrane capacitance of 0.9 μF/cm².
(D) In the summary graph, patch membrane area is plotted against pipette open-tip conductance with a linear regression fit to the dataset according to $A_{gp} = 6.458 \times g_p + 0.003$ where $A_{gp}$ is membrane patch area (μm$^2$) and $g_p$ is pipette conductance (μS). Accordingly, pipettes used for axon recordings sampled a membrane area corresponding to 0.12 - 0.54 μm$^2$. Thus the patch area sampled by axon-attached recording was normally much less than the area of a bouton (mean surface area 2.97 ± 1.97 μm$^2$; range 0.31-8.53 μm$^2$, as measured with EM [see Table S1]).
Figure S5, related to Figure 5.

Activation kinetics and conductance density of $K^+$ currents at boutons

(A) Summary plot of the relationship between $K^+$ current activation kinetics and conductance density in control conditions. Patches containing slowly activating ($\tau > 2$ ms) currents were correlated with negligible conductance densities. Dataset fit with a monoexponential showing a rapid decay in conductance density with increasing activation $\tau$.

(B) Monoexponential fits to fast-activating $K^+$ currents following a maximally activating depolarizing pulse, recorded in bouton patches including DTX (200 nM) and TEA (1 mM). A small remaining capacitive transient was blanked in the TEA trace. On the right, traces in DTX and TEA are normalized to their peak current for comparison.

(C) Summary voltage-activation plot of fast-activating $K^+$ currents from bouton patches in DTX and TEA. Time constants of activation ($\tau$) in DTX and TEA were similar for suprathreshold potentials. Significance (*$p < 0.05$) was determined with two-way ANOVA with Sidak’s multiple comparisons test.
Figure S6, related to Figures 4 and 5.

Clustered and variable expression of Kv3.4-YFP at boutons

(A) 2P image of a dye filled SC (Alexa 594; 60 µM) taken from a mouse injected with high titer AAV1.CAG.FLEX.Kv3.4-YFP and dilute AAV1.hSyn-Cre in the cerebellum. The open arrow shows the axon hillock.

(B) High-magnification image of a region of axon from a Kv3.4-YFP transduced SC. Bouton ROIs along the axon are demarcated with dotted circles. On the right, a plot of maximal fluorescence intensity for individual bouton ROIs from multiple cells.

(C) An axon segment from a Kv3.4-YFP transduced SC. On the right, the axonal region is shown straightened after fitting with a cubic spline curve. The corresponding fluorescence intensity profile for Kv3.4-YFP is shown below. Arrowheads mark puncta along the axon.
**Figure S7**

A non-uniform $K_v$-mediated conductance density imposes local changes in AP duration

(A) Simulated APs from the axon shaft are shown superimposed for three different model conditions. All simulations were performed using the simplified SC axon model modified to include a non-uniform density of $K_v$-mediated conductance ($g_{K_v}$) within its extent. Specifically, $K_v$-mediated conductances were clustered at boutons (36 mS·cm$^{-2}$; except in the test bouton where noted) and largely excluded from shaft regions (1 mS·cm$^{-2}$). On the left, the conductance density of $K_v$ matched all other boutons. In the middle, the conductance density of $K_v$ in the test bouton was increased seven-fold relative to other boutons. On the right, an increased density of $K_v$-mediated conductance is accompanied with an alteration in GR resulting from a larger diameter test bouton (GR = 12 and 3.8; test and surround boutons, respectively).
Simulated APs from the center of the bouton are shown with a shaft region for comparison. (C) Comparison of simulated APs in different conditions show that AP repolarization rate increases with $\bar{g}_{K^+}$.

Summary plot shows that, relative to the test bouton, the duration of the AP increases as the spike propagates into the shaft. This effect increases as $K_v$-mediated conductance density in the test bouton is increased relative to surrounding boutons.

In the summary plot, the GR of the test bouton was increased (GR = 12) relative to surrounding boutons (GR = 3.8) resulting in an enhancement of spike broadening induced by the non-uniform density of $K_v$-mediated conductance.

APs prior to and after loss of 90% of $g_{K^+}$ at the test bouton simulating the abrupt loss of channel availability following an uncaging-like condition. $K_v$-mediated conductances were clustered at boutons in this simulation, with seven time greater density relative to surrounding boutons and a larger GR (12).

AP broadening at the test bouton, induced by local loss of $K_v$ current, decayed over a short distance along the axon shaft. Substituting delayed rectifier $K^+$ channels for $K_v$ greatly increased the length constant of AP broadening.
Table S1

### Geometry of SC axon shafts and boutons

<table>
<thead>
<tr>
<th></th>
<th>Summary (mean +/- SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon shafts (N)</td>
<td>N=45</td>
<td></td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>0.153 ± 0.043</td>
<td>0.072 – 0.260</td>
</tr>
<tr>
<td>Depth (µm)</td>
<td>0.389 ± 0.138</td>
<td>0.191 – 0.736</td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>0.120 ± 0.093</td>
<td>0.014 – 0.318</td>
</tr>
<tr>
<td>Surface (µm²)</td>
<td>1.942 ± 1.305</td>
<td>0.293 – 4.575</td>
</tr>
<tr>
<td>Boutons (N)</td>
<td>N=40</td>
<td></td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>0.374 ± 0.138</td>
<td>0.157 – 0.868</td>
</tr>
<tr>
<td>Depth (µm)</td>
<td>0.646 ± 0.332</td>
<td>0.281 – 2.137</td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>0.318 ± 0.306</td>
<td>0.041 – 1.666</td>
</tr>
<tr>
<td>Surface (µm²)</td>
<td>2.974 ± 1.965</td>
<td>0.311 – 8.533</td>
</tr>
<tr>
<td>Vesicles (/bouton)</td>
<td>94 ± 147</td>
<td>1 – 847</td>
</tr>
</tbody>
</table>

Table S1, related to Figure 2.

Summary of quantitative analysis of bouton and shaft geometry from ultrastructural analysis.
Supplemental Experimental Procedures

Acute slice preparation

All animal procedures were approved by the Max Planck Florida Institute for Neuroscience Animal Care and Use Committee. Acute slices from cerebellar vermi were prepared from young or mature C57Bl/6 mice (postnatal day 15-21 or 42-65 respectively); in some experiments, we used Kv3.1\textsuperscript{-/-} [Kcnc1\textsuperscript{tm1Joho}](Ho et al., 1997); C57Bl/6) or Kv3.4\textsuperscript{-/-} (Taconic Biosciences [Kcnc4\textsuperscript{tm1Lex}; B6/129S) homozygous knockout (KO) mice. Mice were anesthetized under isoflurane, killed by decapitation and the cerebellum removed by dissection. Brain slices (200 μm) were sectioned in the parasagittal plane using a vibrating blade microtome (VT1200S, Leica Biosystems) in an ice-cold solution containing (in mM) 87 NaCl, 25 NaH₂PO₄, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 10 glucose, and 7 sucrose. Slices were transferred to an incubation chamber containing (in mM) 119 NaCl, 26.2 NaH₂PO₄, 2.5 KCl, 1 NaH₂PO₄, 1.5 CaCl₂, 1.5MgCl₂ and 11 glucose and maintained at 34°C for 30 min and then at room temperature (RT; 23-25°C) thereafter. During whole-cell recording, slices were continuously perfused with the same solution at 24.5 - 25.5°C (or 31.5-32.5°C where noted) with (in μM) 100 picrotoxin, 10 r-CPP, and 10 NBQX, respectively, to block synaptic transmission except when recording GABA\textsubscript{A} autoreceptor currents where picrotoxin was omitted. To induce hyposmotic swelling in axons, the extracellular solution was diluted slightly from 315 to 285 mOsm. All solutions were equilibrated with carbogen gas (95% O₂/5% CO₂) prior to use.

Electrophysiology

SC interneurons in the outer two-thirds of the molecular layer were targeted for somatic whole-cell recording using gradient-contrast video-microscopy. Axon recordings, guided by fluorescence imaging, were always directed to axon branches within the molecular layer. Borosilicate whole-cell patch pipettes were filled with an intracellular solution containing (in mM) 124 potassium gluconate, 2 KCl, 9 HEPES, 4 MgCl₂, 4 NaATP, 3 L-Ascorbic Acid and 0.5 NaGTP. To record GABA\textsubscript{A} autoreceptor currents, the internal solution was modified by including (in mM) 73 potassium gluconate, 70 KCl and 10 GABA. For experiments measuring AP-like command waveforms at the soma, the internal solution contained (in mM) 120 CsCl, 35CsF, 10 HEPES, 10 EGTA and 4 Mg₂ATP. All internal solutions were titrated to pH 7.25. The
VSD di-2-AN-(F)EPPTEA (30 μM; L. Loew; University of Connecticut Health Center) was included in the intracellular solution for voltage imaging whereas the morphological indicator Alexa 594 (60 μM; Life Technologies) was included for fluorescence-guided axonal patch recording. For VSD imaging experiments, pipettes were front-filled with a small amount of dye-free recording solution to limit inadvertent labeling of the surrounding neuropil during the patching procedure. Somatic pipette resistance was 4-6 MΩ for whole-cell somatic recordings. To record action currents, loose-seal axon recordings were obtained with a pipette resistance of ~20 MΩ (range: 7-35 MΩ) and contained (in mM) 119 NaCl, 2.5 KCl, 12 HEPES, 17 Dextrose, and 3 MgCl₂. To record K⁺ channel currents, tight-seal (≥ 1 GΩ) cell-attached patches were obtained from the axon with similar-sized pipettes (normally 25-35 MΩ) and the same solution with the addition of (in μM) 1 TTX, 10 ZD7288 (4-ethylphenylamo-1,2-dimethyl-6-methylaminopyrimidinium chloride), 10 XE991 (10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride), and 0.1 iberiotoxin to isolate voltage-gated K⁺ channel currents. Where noted, dendrotoxin-I (200nM) or TEA (1mM) was also included in the pipette. These pipettes were also coated with silicone elastomer (Sylgard 184, Dow Chemical), fire-polished, and fluorescently coated by briefly dipping the pipette in BSA-conjugated Alexa Fluor-594 (Life technologies; 0.02% w/v in 100 μM BSA) prior to use.

Electrophysiological recordings were obtained using Multiclamp 700B amplifiers (Molecular Devices). Signals were filtered at 2-6 kHz and sampled at 20-50 kHz using a Digidata 1440 (Molecular Devices) digitizer. Pipette capacitance was neutralized in all recordings and electrode series resistance compensated using bridge balance in current-clamp mode. Action potentials (APs) were initiated by somatic current injection (~250 pA, 5 ms).

Axonal action-currents were recorded in axons in the loose-seal configuration in voltage-clamp mode (input resistance 79.1 ± 9.3 MΩ; n = 36) and were aligned in tandem with somatically evoked spikes to reduce jitter and were averaged over many trials (typically, 40-180 trials). For spontaneous APs, the soma was depolarized above threshold for 500 ms epochs at 0.125 Hz to limit progressive changes in spike-frequency associated with Na⁺ channel inactivation. All simultaneous spikes recorded during the triple recording (typically 200 APs) were aligned to the somatic AP and averaged for analysis. For recording axonal autoreceptor signals, loose-seal axonal recordings began > 20 min after obtaining the somatic whole-cell
recording with the KCl based internal solution. Both spontaneous spiking and autoreceptor current experiments were performed at 32°C.

For K⁺ channel recordings, currents from axonal patches were leaked-subtracted using a scaled version of the command waveform. Membrane potentials are corrected from a measurement of the liquid junction potential (-10 mV). K⁺ channel activation curves, calculated using chord conductance (g) values from current peaks, were fit with a Boltzmann function:

\[
f = \frac{1}{1 + \exp\left(\frac{V_{\text{half}} - V}{k}\right)}
\]

where \(V\) is membrane potential, \(V_{\text{half}}\) is the midpoint potential and \(k\) the slope factor. The K⁺ reversal potential was calculated as -100 mV. Activation time constants were obtained by fitting the rising phase of the K⁺ current with a single exponential function. Square-wave pulses were 200 ms in duration; the maximally activating voltage pulse was +40 mV. The AP-like voltage command waveform was 800 μs in duration (half-width) with a peak of +30 mV. By convention, K⁺ currents were inverted for figure display and were the average of many trials.

To determine K⁺ conductance density, we used an estimate of membrane patch area derived from pipette conductance for each recording. For this approach, we first determined the linear relationship between patch area and pipette conductance. Using a subset of inside-out patches excised from either the soma or axon, we measured capacitive transients evoked by hyperpolarizing pulses (-50 to -100 mV) before and after insertion of the pipette into a silicone elastomer ball (Engel and Jonas, 2005; Sakmann and Neher, 1983; Sheng et al., 2012). Patch capacitance was calculated by integrating the difference current (average of 40-200 trials for each condition) and assuming a specific membrane capacitance of 0.9 μF/cm². Recordings were either targeted to clearly discernable varicosities (~ 1 - 2 μm in length; categorized as boutons), or to longer sections of non-varicose axon with uniform diameter (categorized as shafts). No developmental changes were found for bouton peak K⁺ conductance density (\(g_{K^+_{\text{young}}}, 168.8 \pm 94.67 \text{ mS} \cdot \text{cm}^{-2}; g_{K^+_{\text{mature}}}, 180.8 \pm 68.22 \text{ mS} \cdot \text{cm}^{-2}\)) or K⁺ current activation rate (\(\tau_{\text{young}}, 11.7 \pm 8.6 \text{ ms}; \tau_{\text{mature}}, 9.0 \pm 6.0 \text{ ms}\)). Similarly, we found no differences for shaft \(g_{K^+}\) or activation rate with development (\(g_{K^+_{\text{young}}}, 18.3 \pm 7.4 \text{ mS} \cdot \text{cm}^{-2}; g_{K^+_{\text{mature}}}, 16.7 \pm 6.6 \text{ mS} \cdot \text{cm}^{-2}\); \(\tau_{\text{young}}, 32.9 \pm \))
9.8 ms; \( \tau_{\text{mature}} = 20.0 \pm 5.4 \) ms). Comparisons were from \( n = 10 \) and \( n = 9 \) boutons, young and mature mice respectively; \( n = 8 \) and \( n = 5 \) shafts, young and mature respectively; all comparisons \( p > 0.05 \); Mann-Whitney tests. Thus, we pooled data from young and mature mice for control \( K^+ \) patch recordings. Notably, a subset of mature recordings were from Syph-YFP transduced mice.

Two-photon imaging

SCs were imaged with a two-photon (2P) laser scanning microscope (Ultima; Bruker Corp.) using an Olympus upright microscope, objective (60x, 1.0 NA), and oil immersion condenser (1.4 NA) with excitation provided by two mode-locking Ti:sapphire lasers (Chameleon Ultra II; Coherent Inc.); one for imaging, the second for uncaging. Epi- and transfluorescence emission was simultaneously collected using GaAsP photomultiplier modules (Hamamatsu) with chromatic separation using a t560lpxr dichroic and et640/120-2p and et510/80-2p bandpass filters (Chroma).

VSD imaging was performed as described previously (Rowan et al., 2014). Briefly, cells filled with the VSD (~0.5-1.0 hour to allow for diffusion) were excited (1040 nm) at diffraction-limited points under continuous illumination (i.e. non-scanning excitation). VSD fluorescence was sampled at 10 kHz and low-pass filtered offline (1 kHz) except where noted otherwise. To assess how our filter affected the overall shape of recorded APs, we compared VSD-recorded axonal APs both with and without the low-pass filter. The half-width of APs recorded after low pass filtering was unchanged (755 \( \pm \) 190 \( \mu \)s without filter, 746 \( \pm \) 185 \( \mu \)s with filtering; \( p > 0.05 \); \( n = 13 \) recordings tested). Prior to averaging (typically 25-80 trials per site for axons or somatic controls), optically recorded APs were aligned using simultaneously recorded somatic spikes to reduce jitter. AP duration was measured using the full-width at half-maximal amplitude (half-width) of the waveform. We avoided making absolute comparisons of VSD amplitude at different imaging locations, as non-responsive internal membranes (e.g., mitochondria) labeled by the VSD are non-homogeneously distributed within axons resulting in difference in baseline fluorescence. Thus, for display, APs were normalized to peak amplitude in comparisons. Unless specified, differences in AP duration are expressed as a ratio of half-width measurements from two boutons using the following:

\[
\frac{\text{Bouton}_2}{\text{Bouton}_1} = \max \left( \frac{\text{AP}_1}{\text{AP}_2}, \frac{\text{AP}_2}{\text{AP}_1} \right)
\]
where max is the largest value obtained for half-width measurements from two boutons ($AP_1$ and $AP_2$, respectively). Thus, bouton order is arbitrarily designated and does not imply a distance-dependence relationship from the soma.

In some experiments, we used the fluorescence of inert volume indicators (Oregon Green 488; 20μM or Alexa 594; 60 μM) to assess relative size differences of axonal elements, including both boutons and shafts. Oregon Green 488 is chromatically compatible with the VSD. In these analyses, we determined the average fluorescence from boutons or short regions of a shaft (~ 2μm stretch) from a maximum intensity projection as a proxy of volume. Boutons were categorized as such with line-plot profiles along the axon by their sharp gaussian-like increase (~2 μm in length) in fluorescence. ROIs for boutons and shafts were fit with circular and rectangular tools, respectively. For all maximum intensity projections ROIs, an equal area of background fluorescence was sampled by positioning the ROI slightly off-target, which was subtracted from the axonal ROI in our analysis.

**RuBi-4AP uncaging**

The caged voltage-gated K⁺ channel blocker Ruthenium bipyridine 4-aminopyradine (Zayat et al., 2003) (RuBi-4AP; Abcam) was continuously recirculated (7 ml volume; 300 μM) in the bath using a peristaltic pump with constant carbogen gas equilibration. 2P photolytic cleavage was induced (750 nm; 11.5 mW at the objective) using multiple uncaging sites directed within close proximity (~ 0.5 μm) of targeted boutons or axon regions. For uncaging trials with voltage imaging, APs were initiated 20 ms after laser pulses ($n = 6$ surrounding a single varicosity; 2-8 ms in duration; 1 ms between each pulse). For uncaging trials with axonal patch clamp, APs were initiated immediately after laser pulses (20-30 points around the perimeter of a ~10 μm axon region; 1.4 ms in duration; 1 ms between each pulse). Pulses were modulated with a Pockels cell. For uncaging experiments with voltage imaging, both control and uncaging trials were interleaved in bunched groups (5-10 trials per condition; trial interval 0.33 Hz) and repeated in this way throughout an experiment. For axonal patch experiments, uncaging was performed after control trials (typically 20-30 trials per condition) as 4-AP may be captured within the pipette following uncaging.
Electron microscopy and 3D reconstructions

Axons of SCs were ultrastructurally reconstructed using aldehyde-fixed cerebellar tissue stained by one of two methods. In the first method, slices were heavily stained by serial incubations with heavy metals to enhance membrane contrast (Wilke et al., 2013). An anesthetized mouse (p19) was flushed by aortal perfusion with a sodium cacodylate buffer (150 mM; pH 7.35) and subsequently fixed (12 min) using a solution containing paraformaldehyde (2%) and glutaraldehyde (2.5%) in sodium cacodylate buffer with the addition of 2 mM CaCl$_2$. The cerebellum was removed by dissection, post-fixed in the same solution (3 hr; 4°C) and kept in sodium cacodylate buffer overnight after repeated washing (3x). The cerebellum was cut into parasagittal slices (100 µm) and incubated in a series of heavy metal solutions: osmium tetroxide (2%) and potassium ferrocyanide in sodium cacodylate buffer (1 hr; 4°C), filtered aqueous thiocarbohydrazide (1%; 20 min, RT), aqueous osmium tetroxide (2%; 30 min, RT), aqueous uranyl acetate (1%; ~12 hr, 4°C), and finally a lead asparate en bloc staining solution containing lead nitrate (0.66% in 3 mM aspartic acid; 30 min, 60°C). Double distilled water was used to wash slices between incubations. Slices were then dehydrated with ice-cold ethanol (30, 50, 70, 90, 99.5% in series; 10 min each) followed by acetone and propylene oxide (10 min; RT).

We also used a more conventional method which, due to its less intense membrane staining, did not obscure fine ultrastructural features such as post-synaptic densities. An anesthetized mouse (p19) was flushed with phosphate-buffered saline followed by fixation with a phosphate buffer (0.1M, pH 7.3)-based solution containing paraformaldehyde (2%), glutaraldehyde (2.5%), and picric acid (15%). The cerebellum was post-fixed overnight (4°C), and then sectioned in the parasagittal plane (200 µm). Slices were incubated with osmium tetroxide (2%; 60 min, RT) in sodium cacodylate (100 mM, pH 7.4) and then stained en bloc with aqueous uranyl acetate (1%; RT). Slices were dehydrated with ethanol (50, 70, 90, and 95%) and acetone and propylene oxide.

After staining, cerebellar slices were embedded in Durcupan ACM resin and polymerized at 60°C. Sample blocks were trimmed and consecutive ultrathin serial sections (~70 nm) were cut and collected on Fombar-coated copper slot grids (130 and 36 sections for each method, respectively). Each grid was carbon-coated (2~3 nm) by vacuum film deposition followed by electron beam pre-exposure (2~3 nm) at low magnification to minimized specimen deformation. The samples were imaged with a transmission electron microscope (Tecnai G2; FEI) at 80 kV.
large field of the molecular layer (~120 x 90 μm) was captured using montage imaging (SA 4800x; ≥120 tiles per ultrathin section; Eagle CCD; FEI). For conventionally-stained tissue, regions of interest containing identified SC axons were reimaged at higher magnification (SA60,000x; Veleta CCD; Olympus). Images were post-processed and stitched using Photoshop and FIJI software (Preibisch et al., 2009; Schindelin et al., 2012). Image resolution was calibrated using a standard grid (4.46 and 0.79 nm/pixel; at SA4800x and SA60,000x, respectively). Section thickness, calibrated using mitochondria diameter measurements (Fiala and Harris, 2001), was 67.4 ± 2.4 nm (n = 30) and 57.6 ± 2.4 (n = 14) for heavy-metal and conventional staining methods, respectively.

Image stacks were manually inspected to identify axon fragments of SC interneurons. Fragments (n = 3) were identified in fixed tissue based on their characteristic anatomy including multiple branched elements (≥ 2) projecting parallel to the parasagittal plane and their location in the outer half of the molecular layer (Palay and Chan-Palay, 1974); only one fragment was traced back to the soma. Areas containing SC axons were cropped for reconstruction and analysis using RECONSTRUCT software (Fiala, 2005). Prior to geometrical measurements, subregions containing individual boutons and axonal shafts were recropped and finely aligned to minimize error. Reconstructions were evaluated by a second experimenter to reduce bias with ambiguities resulting in rejection.

Compartmental model and simulations

Simulations of AP propagation were performed using the NEURON (Hines and Carnevale, 1997) environment and analyzed using MATLAB (Mathworks). Compartmental model morphology was set to represent a simplified SC axon including a soma (10 μm diameter) followed by a stretch of axon (250 μm; >λDC) after which a series of boutons en passant were localized (n =10). Boutons and shafts were of regular length (1.5 and 2.1 μm) except after the sixth bouton, which served as the test bouton throughout our simulations, where the connecting axon shaft was 15 μm. Axon shaft diameter was fixed (153 nm); bouton diameter varied within a range informed by anatomical measurements (range 244 - 804 nm). An additional segment of axon (100 μm) was included at the distal end the compartmental model to reduce sealed-end effects. For simplicity, branch points were omitted. Segment length for each compartment was set to satisfy the d-lambda rule (Hines and Carnevale, 2001) (soma 0.5 μm; axon 0.1 μm). The
integration time step was fixed at 5 μs. Passive electrical properties including specific capacitance (0.9 μF/cm²), membrane resistivity (Rm; 20 kΩcm²), and internal resistance (Ri; 150 Ωcm) were assumed to be uniform and matched experimentally inferred values obtained from SC recordings (Abrahamsson et al., 2012). The resting membrane potential was -80 mV; reversal potential (E_{Leak}) was -81 mV. APs were evoked in the soma by short current injections (200 pA; 2 ms duration).

Simulations included both voltage-gated Na⁺ and K⁺ channels implemented as Hodgkin-Huxley type models. The Na_v channel model was experimentally derived from presynaptic recordings of hippocampal mossy fiber boutons (Engel and Jonas, 2005). The reversal potential (E_{Na}) was set to +50 mV, with conductance densities (\(g_{Na}^+\)) of 10 and 50 mS·cm² in the soma and axon, respectively. K_{V3} channels expressed in axons of MLIs, including SCs, mediate fast-activating currents that determine AP repolarization in these structures (Laube et al., 1996; Rowan et al., 2014; Southan and Robertson, 2000). Therefore in our model, axonal K⁺ conductances were uniformly mediated by this channel type unless stated otherwise. Although in many patch recordings, pharmacologically-isolated K_{V3} currents inactivated, this was not always the case. Thus, we choose to modify the K_{V3} channel model, initially derived from hippocampal CA1 interneurons (Lien and Jonas, 2003; Lien et al., 2002), to include steady-state inactivation (h_{V3}) parameters resembling a fast delayed rectifier (\(V_{half} = -40.6\) mV, slope = 7.8 mV) instead of A-type K⁺ channels (Lien et al., 2002) with a fixed inactivation time constant (\(\tau = 1000\) ms) and an non-inactivating component (8%). E_{Kv3} was -85 mV. We also used a previously published slow delayed rectifier model (KDR) (Engel and Jonas, 2005) where noted. The K_{V3}-mediated \(g_{Kv3}^-\) was set to 36 mS·cm² in the uniform model (Engel and Jonas, 2005). In the clustered model, \(g_{Kv3}^-\) was reduced to 1 mS·cm² in the shaft and remained at 36 mS·cm² in boutons except at the test bouton where the K_{V3}-mediated conductance density was systematically varied across a physiological range. The voltage and time dependence of Na⁺ and K⁺ conductances are described in by the following equations:

\[
I_{Na} = g_{Na}m^3h(V - E_{Na}) \\
I_{Kv3} = g_{Kv3}ni(V - E_{Kv3}) \\
I_{KDR} = g_{KDR}p^4(V - E_{KDR}) \\
I_{Leak} = g_{Leak}(V - E_{Leak})
\]
\[
\frac{dx}{dt} = -\frac{x - x_\infty}{\tau_x}, \tau_x = \frac{1}{\alpha_x + \beta_x}, x_\infty = \frac{\alpha_x}{\alpha_x + \beta_x}
\]

where \( x = m, h, n, i \) or \( p \)

\[
\alpha_m(V) = -93.8285 \times \frac{(V - 105.023)}{e^{-(V-105.023)/17.7094} - 1}
\]

\[
\beta_m(V) = 0.16839 \times e^{-V/23.2707}
\]

\[
\alpha_h(V) = 0.000354 \times e^{-V/18.706}
\]

\[
\beta_h(V) = 6.62694 \times \frac{1}{e^{-(V+17.6769)/13.3097} + 1}
\]

\[
\alpha_n(V) = 0.01893 \times \frac{-(V - 4.184)}{e^{-(V-4.184)/6.426} + 1}
\]

\[
\beta_n(V) = 0.01586 \times e^{-V/25.48}
\]

\[
\alpha_i(V) = 0.92 \times \frac{1}{e^{-(V+40.6)/7.8} + 1} + 0.08
\]

\[
\beta_i(V) = 0.92 \times \frac{e^{-(V+40.6)/7.8}}{e^{-(V+40.6)/7.8} + 1}
\]

\[
\alpha_p(V) = 0.01 \times \frac{-(V + 55)}{e^{-(V+55)/10} - 1}
\]

\[
\beta_p(V) = 0.125 \times (e^{-(V+45.6599)/2.30235} + 1)
\]

**Intracranial viral injections**

Mice (postnatal day 18-28) were head-fixed with earbars in a stereotactic frame (David Kopf Instruments) under isoflurane anesthesia (1.8-4.0%) with thermoregulation provided by a heating plate with biofeedback using a rectal thermocouple. The scalp above the cerebellum was opened using a lidocaine/bupivacaine cocktail as a local anesthetic and a small craniotomy was cut in the skull (~ 1 mm) above the vermis. A glass microinjection pipette was advanced into the brain just below the surface (~ 0.25-1.00 mm). A viral solution (0.75-1 μL) containing AAV1.hSyn.synaptophysin-eYFP.WPRE.bGH (packaged at the University of Pennsylvania Vector Core Facility) was injected with slight pneumatic pressure over a period of 5 minutes. For injections of AAV1.CAG.FLEX.Kₐ₃.4-YFP (packaged at the University of Pennsylvania Vector Core Facility) we also included dilute AAV1.hSyn.Cre (1:1000) to allow for expression in a sparse population of molecular layer interneurons. Following all injections, the pipette was held in place for 5 minutes before withdrawal. The scalp was closed by surgical adhesive (Vetbond)
and the animal recovered under analgesia (carprofen and buprenorphine SR LAB, 5 mg/kg and 0.35 mg/kg, respectively). After allowing 14-35 days for expression, animals were sacrificed and the cerebellum used for acute slice preparation and experimentation.

*Data and statistical analysis*

Axograph X, Graphpad Prism (Graphpad Software) ImageJ and Excel (Microsoft) were used for analysis with values in text and figures reported as mean ± s.e.m unless noted otherwise. Statistical differences were deemed significant with α values of \( p < 0.05 \). Paired t-tests were used for matched parametric datasets. The Mann-Whitney test was used for comparison between unmatched nonparametric datasets. Where appropriate, group data were compared with 1 or 2-way ANOVA and significance between groups was determined with Dunnett’s, Tukey’s or Sidak’s multiple post-hoc comparison tests. 1-sample proportionality z-tests were used to test the likelihood of encountering K_v channels in axonal patches. Normality was determined using D’Agostino & Pearson omnibus or Shapiro-Wilk tests.
Supplemental References


