Conduction Velocities of Excitatory Axons Innervating Parvalbumin Interneurons in Primary Somatosensory Cortex

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Abstract

Timing plays a critical role in signaling throughout the nervous system. Signaling times vary widely depending on distance and speed, and timing delays influence circuit computations such as coincidence detection, synaptic integration, entrainment, temporal summation, and plasticity. Parvalbumin (PV) interneurons are a major type of inhibitory neuron with critical roles throughout the brain. Their rapid-spiking characteristics enable them to control the dynamics of neural circuits across a range of time scales. However, little is known about the factors that determine the timing of their synaptic inputs. Here, we measured the conduction velocity (CV) of excitatory axons that target PV interneurons in mouse primary somatosensory cortex. Using a genetically-encoded hybrid voltage sensor (hVOS) targeted to PV interneurons, we clocked the arrival of excitatory synaptic inputs with sub-millisecond precision in large numbers of cells across multiple layers. Arrival times varied with distance, allowing us to determine the CV of the excitatory axons. Values ranged from 28–241 μm/msec across layers 2-5, and interlaminar CV was about twice as fast as intralaminar CV. This will enable more rapid computation within columns, while integration between columns will be slower. hVOS imaging from specific cell types offers a unique opportunity to investigate conduction in axons defined by their targets.

Introduction

Axonal conduction velocity (CV) influences neural computations by controlling the timing of synaptic inputs. CV impacts processes such as coincidence detection, temporal summation, entrainment, oscillations, synaptic integration, and synaptic plasticity (Bucher & Goaillard, 2011; Chomiak, Peters, & Hu, 2008; Fields, 2015; Pajevic, Basser, & Fields, 2014; Salami, Itami, Tsumoto, & Kimura, 2003). Small variations in CV can have a profound impact on synchrony and oscillation frequency (Ivanov, Polykretis, & Michmizos, 2019; Pajevic et al., 2014), and CV alterations have been linked to a variety of diseases including diabetes (Candy & Szatkowski, 2000), schizophrenia (Corcoba et al., 2015), and Down Syndrome (Olmos-Serrano et al., 2016). Demyelination associated with degenerative diseases such as multiple sclerosis has a major impact on CV (Compston & Coles, 2002; Nave, 2010; Pedroni, Minh do, Mallamaci, & Cherubini, 2014), and slower CV caused by decreased erbB signaling has been linked to bipolar disorder and schizophrenia (Roy et al., 2007).

CV is determined by axonal diameter and other geometric properties, as well as the capacitance and intrinsic excitability of the axonal membrane. CV varies widely between different axons and functional pathways. Axons projecting from ventral temporal cortex to the inferior colliculus conduct approximately three times faster than those projecting to caudate putamen (Chomiak et al., 2008), and hippocampal dentate granule cell (mossy fiber) axons conduct more rapidly than the axons of hilar mossy cells (Ma, Bayguinov, & Jackson, 2017). CV can also differ within the axons of the same cell. For a given ventral temporal cortical neuron, axonal branches with contralateral targets have significantly slower CV compared to axonal branches with ipsilateral targets (Chomiak et al., 2008). CV may also vary based on postsynaptic cell type. As a result of differences in CV, stimulating the same thalamic neuron activates

61 inhibitory targets before excitatory targets in somatosensory barrel cortex (BC), thus setting a
62 very narrow time window for excitation (Kimura et al., 2010). Additionally, cell type can
63 influence CV variability. Martinotti cells in layer 5 (L5) BC have more variable axonal CVs
64 compared to pyramidal cells in the same layer (Shlosberg, Abu-Ghanem, & Amitai, 2008). These
65 many variations in CV determine not only computation speed, but by influencing timing and
66 delays, determine how circuits encode and process information.

67 Previous measurements of CV have been hindered by technical constraints arising from
68 the difficulty of recording directly from axons, as well as low throughput, and lack of spatial and
69 temporal resolution. Voltage imaging has provided a powerful general method for the
70 measurement of axonal CV (Grinvald, Ross, & Farber, 1981; Hamada, Popovic, & Kole, 2017;
71 Popovic, Foust, McCormick, & Zecevic, 2011; Sakai et al., 1991), and genetically-encoded
72 voltage indicators have extended the approach to these problems through the addition of
73 targeting specificity (Ma, Bayguinov, & Jackson, 2019; Panzera & Hoppa, 2019). The hybrid
74 voltage sensor (hVOS) has sub-millisecond temporal resolution, single-cell spatial resolution
75 (Chanda et al., 2005; Ghitani, Bayguinov, Ma, & Jackson, 2015; Ma et al., 2017), and can be
76 targeted to specific cell populations (Bayguinov, Ma, Gao, Zhao, & Jackson, 2017; Chanda et al.,
77 2005; Ghitani, Bayguinov, Ma, & Jackson, 2015; Ma et al., 2017). hVOS reveals voltage
78 changes from large numbers of neurons simultaneously, and when targeted directly to axons, can
79 resolve differences in CV based on cell type (Ma et al., 2017). In this study, we used hVOS to
80 measure CV along excitatory axons targeting inhibitory parvalbumin (PV) interneurons in mouse
81 BC. Because they fire rapidly and have a short membrane time constant, PV interneurons play a
82 critical role in controlling the temporal integration window of their targets (Cardin, 2018;
83 Ferguson & Gao, 2018; Galarreta & Hestrin, 2002; Pouille & Scanziani, 2001). They are also

essential for the generation of gamma oscillations, rhythmic brain waves between 30 and 80 Hz which affect sensory integration, working memory, social cognition, and other functions (Gandal, Edgar, Klook, & Siegel, 2012; Gloveli et al., 2005; Gonzalez-Burgos, Cho, & Lewis, 2015; Pouille & Scanziani, 2001). Both PV interneurons and gamma oscillations have been implicated in a variety of psychiatric and neurological disorders including schizophrenia, bipolar disorder, and autism spectrum disorder (Gonzalez-Burgos et al., 2015; Lauber, Filice, & Schwaller, 2018; T. Y. Liu et al., 2012). The fast-spiking behavior of PV interneurons suggests that they are adapted to rapid computations. Thus, the speed of their activation by excitatory inputs is likely to be important to their function.

While PV interneurons are present throughout L2-6, their density and circuitry vary widely within the cortex (Staiger & Petersen, 2021). Properties such as excitation/inhibition balance and gamma oscillation frequency, both linked to PV interneuron function, differ based on cortical layer (Adesnik, 2018; Xu et al., 2016). We used the response latencies of PV interneurons to determine CV within and between different cortical layers. CV varied widely from 44 ± 15 μm/msec (mean ± SD) for L2/3 intralaminar CV to up to 241 μm/msec for L5 → L4 CV. The mean interlaminar CV (111 ± 49 μm/msec) was about twice as fast as the mean intralaminar CV (57 ± 41 μm/msec). These differences between inter- and intralaminar CV will influence processing within the BC to make computations within columns faster than computations between columns.

105 **Results**

106 *PV interneuron responses to stimulation in L2/3, L4, and L5*

107 In mice from PV-Cre crosses with hVOS Cre reporter mice (see Methods), hVOS probe
108 has been shown to be expressed in 83% of PV interneurons with 99.2% specificity (Bayguinov et
109 al., 2017). In slices from these mice, BC cortical layers and barrels are visible in gradient
110 contrast optics (Fig. 3A) and fluorescence (Fig. 3B; note that the images were taken with
111 different cameras, so the fields of view do not align precisely). Layers were identified by cell
112 density and cell size (Feldmeyer, 2012; Woolsey & Van der Loos, 1970), and boundaries
113 between layers are marked with dashed lines (Figs. 1, 3, 4, Supp. Fig. 1). Fields of view
114 generally contained L2/3 through L5 (Fig. 1). Barrels were separated into faint "hollows"
115 (Feldmeyer, 2012; Woolsey & Van der Loos, 1970) as well as stronger fluorescence in L4, and
116 boundaries between columns marked by dotted lines (Fig. 3, 4, Supp. Fig. 1). Electrical
117 stimulation in L2/3, L4, and L5 elicited voltage changes in PV interneurons distributed through
118 L2-5, as shown in the maximum amplitude heatmaps in Figs. 1A (L2/3), 1B (L4), and 1C (L5).
119 Warmer colors correspond to greater changes in fluorescence and indicate the presence of
120 responsive PV interneurons. Traces from locations (indicated by number and color) reveal
121 corresponding variations in the magnitude of PV interneuron depolarization (Fig. 1D-F). Dark
122 blue regions of the heatmap indicate the absence of responsive PV interneurons, and traces from
123 those locations show no discernable stimulus-evoked fluorescence change.

124

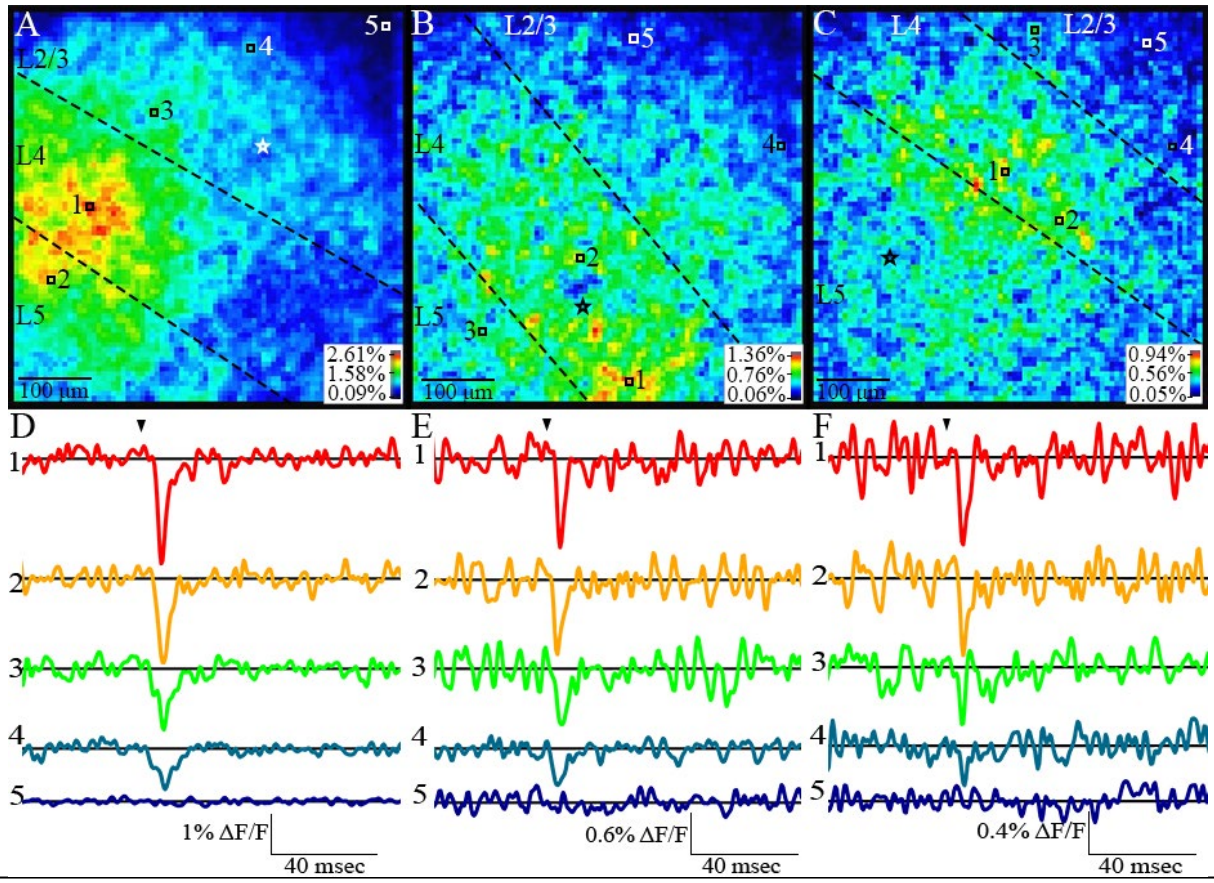

Figure 1. PV interneurons throughout a slice respond to stimulation in L2/3 (A), L4 (B), L5 (C) (stimulation sites indicated by white or black stars). In maximum amplitude heatmaps, warmer colors indicate greater changes in fluorescence. These differences in amplitude are visible in selected traces of fluorescence versus time (D-F) from the corresponding heatmaps above. Locations of traces are indicated with small white or black squares and numbers in each map. Arrowheads indicate time of stimulation.

Stronger stimulation elicited responses in more cells over greater distances. Heatmaps of signal-to-noise ratio (SNR) revealed that stimulation with 10 µA rarely elicited detectable responses (Fig. 2A). Increasing the stimulus current to 20 µA (Fig. 2B) and 60 µA (Fig. 2C) elicited responses in more neurons over greater distances, and increasing to 100 µA depolarized still more neurons over a broader range of distances encompassing nearly the entire field of view (Figs. 1, 2D). Responses depended on synaptic excitation, as they were blocked by 10 µM NBQX (Figs. 2E-2F). Patterns of response spread varied but often included intralaminar responses across multiple barrel columns (Video 1) and/or interlaminar responses within and between barrel columns (Videos 2, 3). These excitatory responses spread away from the stimulus

electrode as action potentials propagated along excitatory efferents; the speed of this spread reflects the CV along these axons.

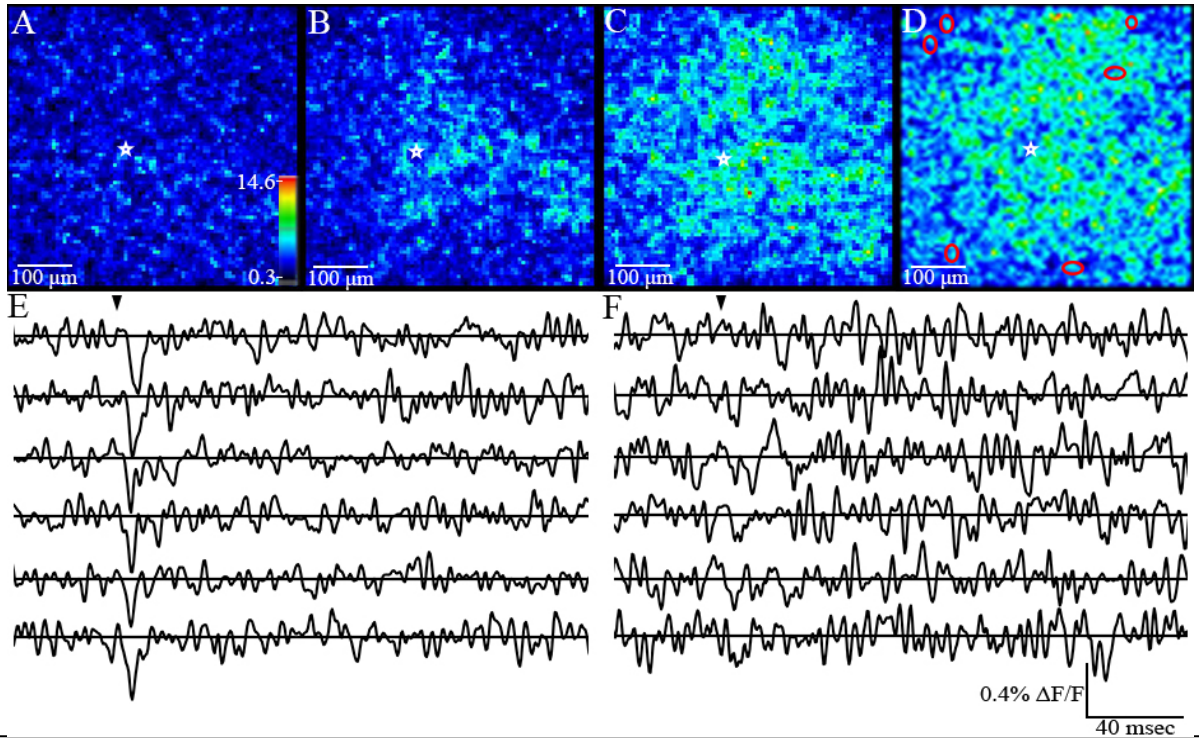

Figure 2. PV interneuron responses to different stimulation currents. Heatmaps of SNR following stimulations of 10 µA (A), 20 µA (B), 60 µA (C), and 100 µA (D). Site of stimulation is marked with a white star in A-D. Heatmap scale in (A) also applies to (B-D). A method of analysis was developed to identify individual, non-overlapping cells with clear responses (see Methods for criteria). E-F. Traces from 6 clearly identified PV interneurons (circled in red in D) show fluorescence versus time before (E) and after (F) the addition of 10 µM NBQX. Blockade by NBQX indicates that responses are glutamatergic.

Identification of single PV interneurons

Determining CV requires the observation of responses in significant numbers of clearly identified neurons with well-resolved locations and accurately measured latencies. Fluorescence images of slices (Fig. 3B) display diffusely distributed fluorescence due to the dense arborization of PV interneurons (Fukuda & Kosaka, 2003). This makes it difficult to locate individual cells precisely. This, together with the large numbers of responsive cells, made manual analysis extremely time-consuming. We therefore developed a semi-automated method to identify responsive PV interneurons (see Methods and Supp. Fig. 1). Briefly, pixels above a baseline SNR cutoff (Supp. Fig. 1A) were divided into groups using k-means clustering of the SNR maps

145 (Supp. Fig. 1B). Putative somata were identified as contiguous groups of pixels in the same k-
146 means cluster based on the reasoning that pixels from the same cell body are expected to have a
147 similar SNR. A pixel group was considered to be a PV interneuron soma if its amplitude and
148 SNR were above the cutoffs (0.1% for amplitude, 5 for SNR), did not share a face with another
149 pixel group, and was < 20 μm across (consistent with the size of a PV interneuron soma
150 (Kooijmans, Sierhuis, Self, & Roelfsema, 2020; Selby, Zhang, & Sun, 2007; Y. Wang, Gupta,
151 Toledo-Rodriguez, Wu, & Markram, 2002)).

152 This process provided a reproducible, robust method of identifying individual PV
153 interneurons free of subjective choices. The procedure is illustrated with a slice from which a
154 gradient contrast image is shown in Fig. 3A and a resting fluorescence image is shown in Fig.
155 3B. The electrode, visible in L2/3 in both images, was used to apply a stimulation of 100 μA. A
156 SNR heatmap revealed activated cells concentrated in L4, but cells in L2/3 and L5 also
157 responded (Fig. 3C). This heatmap highlights 56 PV interneurons identified with our clustering
158 method. Responsive neurons were outlined in black, or in red for locations selected for display of
159 fluorescence versus time (Fig. 3D). The 8 selected traces show clear depolarizing responses
160 following stimulation.

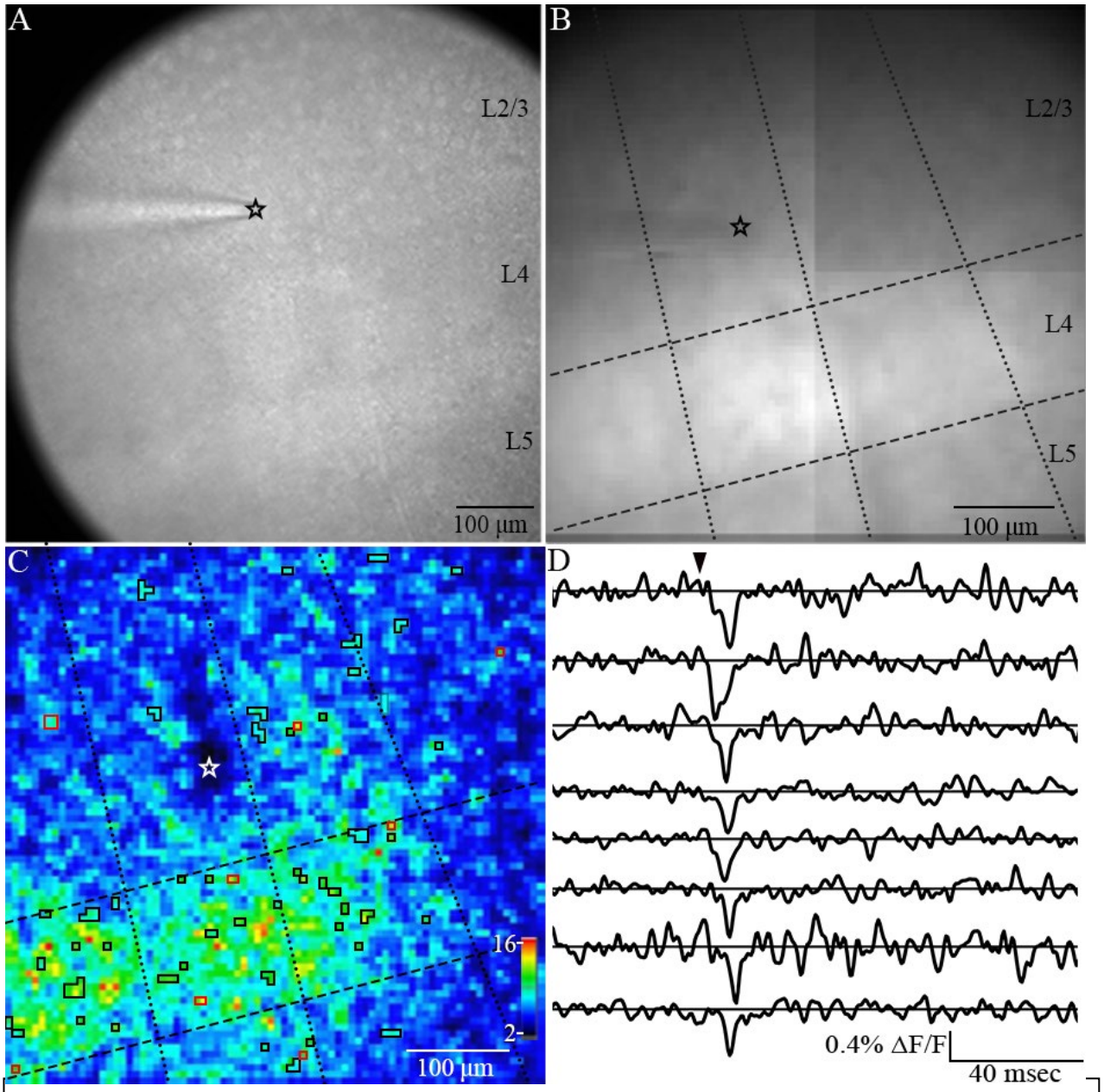

Figure 3. Identification of responsive PV interneurons. A. Gradient contrast image (taken with the Kiralux camera), and B. fluorescence image (taken with the CCD-SMQ camera) of a BC somatosensory cortical slice containing L2/3, L4 and L5. The stimulating electrode is visible in L2/3 (star at the tip, black in A and B, white in C). Layer boundaries are indicated by dashed lines, and barrel column borders are indicated with dotted lines in B-C. Following 100 µA stimulation, responsive PV interneurons were identified based on K-means clustering of SNR and other criteria (see Methods and Supp. Fig. 1). C. A SNR heatmap shows that responsive PV interneurons span the field of view and are present in L2-5. We identified 56 responsive PV interneurons outlined in black or red. D. Traces of fluorescence versus time for the 8 PV interneurons outlined in red in C. Arrowhead above indicates stimulation time. All displayed traces show clear decreases in fluorescence following stimulation, indicating depolarization of the highlighted PV interneurons.

Returning to Fig. 2, this method identified 12, 96, 176, and 115 responsive PV interneurons for 10, 20, 60, and 100 µA stimulation, respectively. The decrease in number of

163 identifiable cells with 100 μA reflects the higher proportion of overlapping cells that could not be
164 disambiguated. The best balance between extensive spread with large numbers of readily
165 resolved individual cells versus too much overlap was seen with 100 μA, so this stimulus
166 strength was used for experiments on CV. The broad patterns of propagation are clearly evident
167 in unprocessed data as illustrated in the heatmaps and supplemental videos described above.
168 However, by employing method we were able to focus on somata and improve the precision of
169 latency determination. We were thus able to analyze large numbers of cells over a broad area
170 with a wide range of propagation distances. This analysis thus provided a robust identification of
171 responsive neurons and, together with our choice of experimental conditions, optimized the
172 determination of CV.

173 *Intra- and interlaminar conduction velocity*

174 As an action potential propagates along the axon of an excitatory neuron, PV
175 interneurons closer to the site of initiation respond first and those farther away respond later (Fig.
176 4A). A sequence of frames taken at one msec intervals from 2 msec to 6 msec after stimulation
177 illustrate the spatiotemporal pattern of response spread (Fig. 4B). Note that these frames
178 represent the SNR at successive time points, compared to the maps in Figs. 1-3 showing the
179 maximum value within the entire post-stimulus measure window. The frames in Fig. 4B show
180 that PV interneuron responses occur first mostly nearer the stimulating electrode in L4 (2-3
181 msec), spread vertically both ways to L2/3 and L5 (4-5 msec), and then spread both vertically
182 and horizontally through L2/3 and L5 (5-6 msec). Thus, a single experiment can capture
183 propagation along different populations of axons, and because fields of view contained large
184 numbers of responsive neurons (typically ~50-100), we were generally able to identify 8 or more
185 neurons along distinct trajectories to track propagation. Fig. 4C-D presents resting fluorescence

186 and maximum SNR maps, respectively, showing the locations of responsive cells along one
187 selected trajectory (L4 → L2/3 CV). A plot of latency versus distance fitted to a line gave us the
188 CV as inverse of the slope (Fig. 4E). By constructing plots along different trajectories, we
189 determined the CV of different subpopulations of axons that target PV interneurons. Focusing on
190 PV interneurons in the same layer as the stimulation electrode (between dashed lines in Fig. 4B-
191 D) provided a determination of intralaminar (horizontal) CV, and focusing on PV interneurons in
192 the same barrel column as the stimulating electrode (between dotted lines in Fig. 4B-D) provided
193 a determination of interlaminar (vertical) CV. Interlaminar CV often included an additional
194 neighboring barrel column. This is consistent with results from rat BC indicating that excitatory
195 axons tend to project within the same and/or neighboring columns but usually not to columns
196 beyond the nearest neighbor (Narayanan et al., 2015).

197 In 22 out of 59 slices, plots of latency versus distance had statistically significant
198 correlations. Among the remaining slices, negative correlations occurred only rarely (4 out of 59
199 slices). The highest CV we were able to measure was 241 μm/msec (19 PV interneurons), and
200 nearly half (15) of the experiments without significant positive correlations yielded CV values
201 above the highest measurable value. In these cases, the spread within the field of view was too
202 fast to detect differences in latency. In most cases of such rapid propagation, latencies varied by
203 less than 0.5 msec (average root-mean-square error = 0.427). These results indicate that in many
204 slices, the CV was faster than can be measured over the distances accessible within our field of
205 view. In the remainder of this work, we will focus primarily on CV measurements with
206 significant distance-latency correlations, unless stated otherwise, and recognize that this biases
207 our analysis uniformly toward slower conduction.

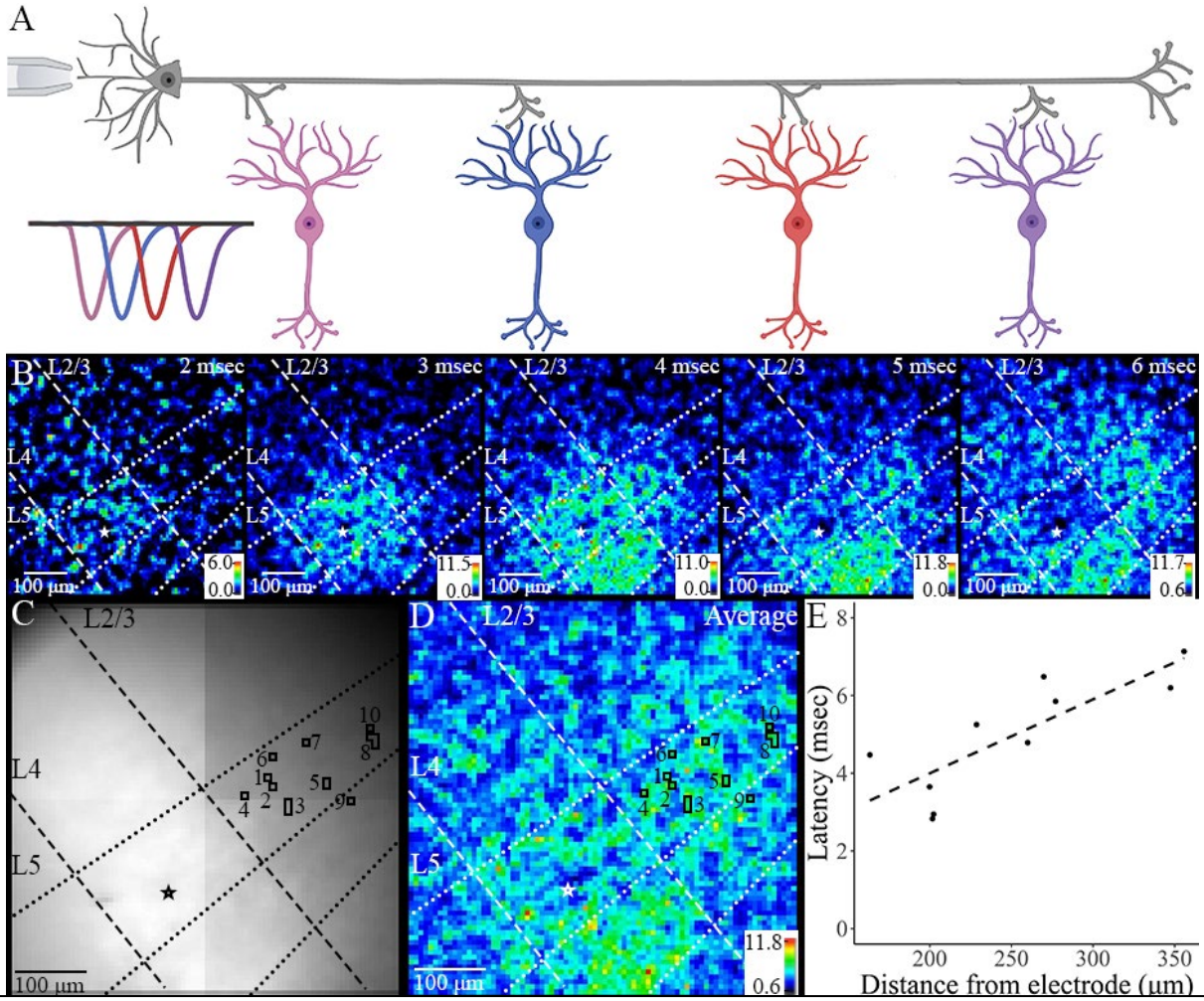

Figure 4. A. Stimulation excites principal cells, their axons, or both (gray). As an action potential propagates along an excitatory axon, synapses on PV neurons evoke sequential responses (pink, blue, red, purple). (Created with BioRender; https://biorender.com). B. Sequential responses can be observed in a sequence of SNR heatmaps at one msec intervals (times after stimulation indicated in the top right corner of each frame). PV interneuron responses occur first mostly near the stimulating electrode in L4 (2-3 msec), and spread vertically and horizontally through the slice. A white start indicates the site of stimulation in all frames (See videos in supplemental data). Resting fluorescence image (C) and average SNR response heatmap within a 20 msec measure window (D). Black (C) or white (D) stars indicate site of stimulation. Dashed lines mark layer boundaries (B-D). Dotted black (C) or white (B, D) lines perpendicular to layer boundaries outline the stimulated and neighboring barrel. Ten responsive PV interneurons identified by our grouping method along an L4 → L2/3 CV trajectory are outlined in black in C and D, with numbers indicating response order. E. Latency plotted versus distance for these 10 cells. The relationship between latency and distance was significant (t = 4.255, p-value = 0.004), and the slope yielded a CV of 51 µm/msec.

Stimulation in L2/3, L4, and L5 elicited responses that spread within those layers, and latency versus distance plots yielded intralaminar CV values in L2/3 and L5. In L4 the lack of correlation prevented us from determining the CV (N=3), and this may indicate that intralaminar

211 conduction is faster in this layer. L2/3 intralaminar CV measurements were generally slow with
212 low variance (44 ± 15 μm/msec, N = 52 PV interneurons from 4 slices). The two measured L5
213 intralaminar CV values were very different (28 μm/msec and 136 μm/msec, N = 18 PV
214 interneurons from 2 slices), potentially reflecting differences between L5 pyramidal cell
215 subtypes, which are known to vary in target projection layers and morphology (Larsen &
216 Callaway, 2006). Fig. 5A shows a plot of latency versus distance with an intralaminar CV in
217 L2/3 of 37 μm/msec (t = 4.86, p = 0.0009).

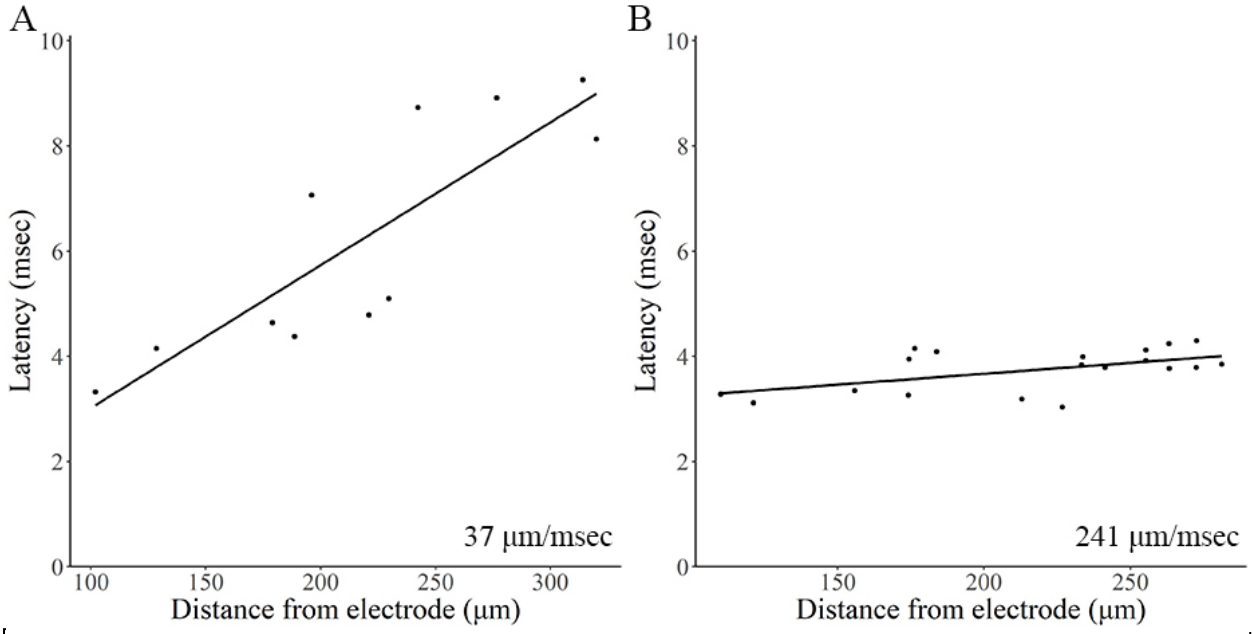

Figure 5. Latency versus intralaminar (A) or interlaminar (B) distance for two representative experiments. The steeper slope in A compared to B indicates that the L2/3 intralaminar CV represented in A (37 μm/msec) is slower than the L5 → L4 interlaminar CV shown in B (241 μm/msec).

218 Correlated latency-distance plots for interlaminar propagation were obtained for all pairs
219 of layers with the exception of L5 → L2/3. Fig. 5B shows a plot for L5 → L4 conduction
220 yielding a CV of 241 μm/msec (t = 2.63, p = 0.018). For three other pairs of layers CV values
221 were similar to one another, with L4 → L2/3 giving CV = 94 ± 37 μm/msec (N = 62 PV
222 interneurons, 3 slices), L5 → L2/3 giving CV = 107 ± 48 μm/msec (N = 57 PV interneurons, 3
223 slices), and L2/3 → L4 giving CV = 81 ± 36 μm/msec (N = 93 PV interneurons, 5 slices). Faster

CV values were obtained for L4 → L5 (144 μm/msec, N = 8 PV interneurons, 1 slice) and L5 → L4 (154 ± 58 μm/msec, N = 63 PV interneurons, 4 slices), including our fastest CV of 241 μm/msec (Fig. 5B). The plot of latency versus distance for L5 → L4 in Fig. 5B is noticeably less steep than the plot of L2/3 intralaminar CV in Fig. 5A, supporting the view that the L5 → L4 CV is faster than the intralaminar CV.

Interlaminar CVs (111 ± 49 μm/msec, N = 16 slices) were about twice as fast as intralaminar CVs (57 ± 41 μm/msec, N = 6 slices), and this difference was statistically significant ($t = -2.587$, $p = 0.026$, Fig. 6A). This difference remained significant even excluding the single particularly fast interlaminar CV value of 241 μm/msec ($t = -2.352$, $p = 0.046$). ANOVA followed by post-hoc testing revealed that intralaminar CV is about 2.7 times slower than L5 → L4 interlaminar CV (Fig. 6B, ANOVA: $F = 3.085$, $p = 0.046$; Tukey's honestly significant differences: L5 → L4 CV versus intralaminar CV: $p = 0.025$). Including values for slices with CVs too fast to produce significant correlations further supports the assertion that interlaminar CV is faster than intralaminar CV. This increased the number of intralaminar CV measurements by 50% (N = 3 additional slices) but increased the number of interlaminar CV measurements by 75% (N = 12 additional slices). Interestingly, one of these three additional intralaminar CV values was from L4, possibly suggesting faster intralaminar CV in L4 than L2/3 or L5. Because a higher proportion of interlaminar CV values were too fast to measure, our focus on experiments with statistically significant correlations underestimates the difference between interlaminar and intralaminar CV.

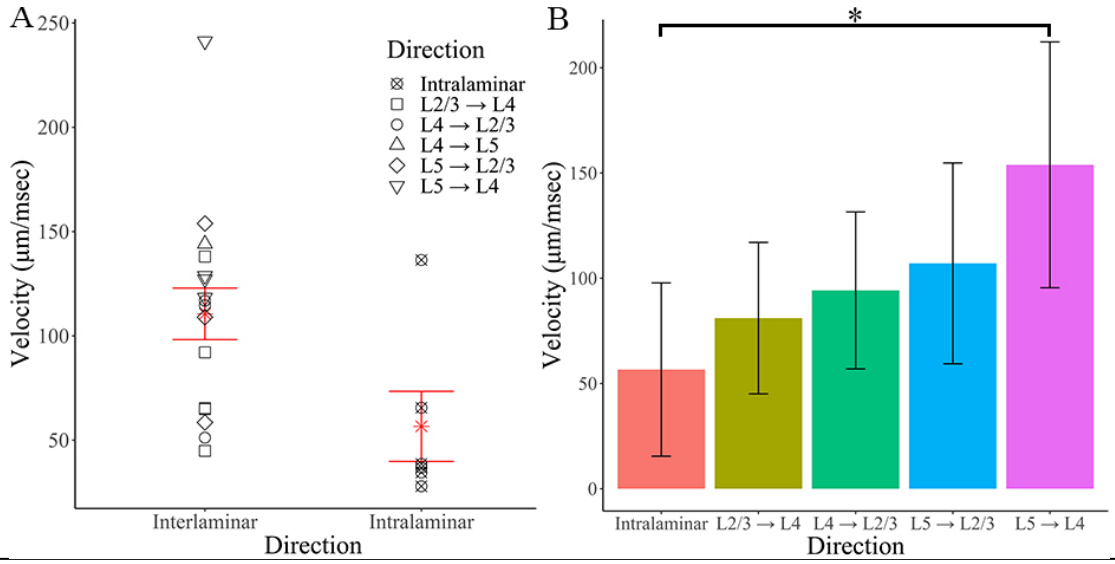

Figure 6. Inter- and intralaminar CV. A. Interlaminar CV (111 ± 49 µm/msec) is approximately twice as fast as intralaminar CV (57 ± 41 µm/msec; t = -2.587, p = 0.026). Symbol shapes indicate CV direction (in legend), and group mean ± SD are shown in red. B. Intralaminar (70 PV interneurons, 6 slices), L2/3 → L4 (93 PV interneurons, 5 slices), L4 → L2/3 (62 PV interneurons, 3 slices), L5 → L2/3 (57 PV interneurons , 3 slices), and L5 → L4 (63 PV interneurons, 4 slices) CV. Error bars indicate SD. CV varies significantly based on direction (F = 3.085, p = 0.046), and intralaminar CV is significantly slower than L5 → L4 CV (p= 0.025).

Discussion

Action potential conduction influences virtually all aspects of neural circuit function, and many approaches have been taken to measure its velocity. Antidromic activation (Chomiak et al., 2008; Palmer & Stuart, 2006; Salami, Itami, et al., 2003; Shlosberg et al., 2008), whole-cell recording (Chomiak et al., 2008; Helmstaedter, Staiger, Sakmann, & Feldmeyer, 2008; Kim, Renden, & von Gersdorff, 2013; Kimura et al., 2010; McDougall et al., 2018; Salami, Itami, et al., 2003; Salami, Kimura, & Tsumoto, 2003; Shlosberg et al., 2008; Shu, Duque, Yu, Haider, & McCormick, 2007) and voltage-sensitive dye imaging (Grinvald et al., 1981; Hamada et al., 2017; Popovic et al., 2011; Sakai et al., 1991) provide accurate measurements of CV but are limited by their low-throughput and lack of specificity. Other methods lack spatial resolution and are poorly suited to investigations of single-cell responses (Candy & Szatkowski, 2000; Drakesmith et al., 2019; Emmenegger, Obien, Franke, & Hierlemann, 2019; Kress & Mennerick, 2009; Radivojevic et al., 2017; Salami, Kimura, et al., 2003). The present study used the

259 genetically-encoded voltage sensor hVOS to measure CV of excitatory axons targeting PV
260 interneurons in mouse BC. Because it can be genetically targeted, hVOS provides a unique
261 opportunity to measure CV along axons innervating specific cell types. hVOS imaging has a
262 rapid response time that is well-suited for measuring the sub-millisecond differences in latency
263 required for the determination of CV. Although DPA increases membrane capacitance and thus
264 slows propagation, this effect is small as increasing DPA from 2 to 4 μM reduced CV by only
265 15% in mossy fibers (Ma et al., 2017). Furthermore, this uniform effect will reduce CV
266 proportionally in different populations of axons. In the present study it was difficult to measure
267 faster CV values, but this could be remedied by reducing magnification to track conduction over
268 longer distances. Our analysis has incorporated the assumption that axons follow a direct path to
269 target PV interneurons at different distances. This adds to the biases that reduce our estimates of
270 CV. This is probably more important for interlaminar than intralaminar conduction, as
271 interlaminar axons sometimes extend vertically through cortical layers before branching
272 horizontally (Larsen & Callaway, 2006; Schubert, Kotter, Luhmann, & Staiger, 2006).

273 CV values reported here are somewhat slower than in previous studies, which can
274 probably be attributed to our exclusion of experiments without a significant correlation between
275 latency and distance. Including these experiments yielded a value of 304 ± 501 μm/msec (N =
276 670 PV interneurons from 37 slices), which trends toward the higher end of the range from
277 previous reports. Prior studies reported CV values of 140-160 μm/msec in mouse anterior
278 cingulate cortex (Lee, Miao, Chen, Li, & Zhuo, 2021), 150-550 μm/msec in rat visual cortex
279 (Murakoshi, Guo, & Ichinose, 1993), 161-240 μm/msec in rat CA3 (Soleng, Raastad, &
280 Andersen, 2003), about 200-300 μm/msec in mouse BC (Shlosberg et al., 2008), 200 μm/msec in
281 rat BC (Helmstaedter et al., 2008), and 340 μm/msec in rat primary somatosensory cortex

282 (Telfeian & Connors, 2003). While some of the variations in these studies can be attributed to
283 methodological differences such as temperature, this wide range of CV values probably also
284 reflects the diversity of cortical axons. Axonal diameter, varicosity geometry, and myelination
285 vary widely and all have a major impact on CV. Ion channel expression also varies widely
286 between cell types and compartments (Liu, Wang, Pitt, & Liu, 2022; Vacher, Mohapatra, &
287 Trimmer, 2008), and by controlling the intrinsic excitability of the axonal membrane will have
288 an impact on CV. Our measurements of CV values for different projections in the same
289 preparation offer a perspective to the variations between prior reports, and support the view that
290 this quantity can vary between axons in the same brain region.

291 PV interneuron microcircuitry, density, and excitation/inhibition input ratios differ
292 between cortical layers (Adesnik, 2018; Staiger & Petersen, 2021; Xu et al., 2016), and the CV
293 of axons innervating different PV interneurons will impact their function in cortical
294 microcircuits. The canonical cortical circuit for information flow begins with thalamic inputs to
295 L4 followed by transmission to L2/3 and then L5. We could follow this flow to measure a CV of
296 94 ± 37 μm/msec along L4 excitatory axons targeting L2/3 PV interneurons. We also report CV
297 along excitatory axons following noncanonical circuits. For example, L2/3 receives input from
298 L5 (Narayanan et al., 2015; Staiger & Petersen, 2021; Thomson, West, Wang, & Bannister,
299 2002; Vecchia et al., 2020), and for this projection we measured a CV of 107 ± 48 μm/msec,
300 which was similar to the L4 → L2/3 CV. We also measured CV along axons from L2/3 and L5
301 to L4 PV interneurons. Although most L2/3 excitatory cells project to infragranular or
302 supragranular cortical layers, a small portion target L4 PV interneurons (Thomson et al., 2002),
303 including a subset of L2/3 pyramidal cells with somata near the L3/4 border (Larsen & Callaway,
304 2006). Our L2/3 → L4 CV of 81 ± 36 μm/msec was slightly slower than other interlaminar CVs.

305 There is also evidence for monosynaptic L5 excitatory cell coupling to L4 fast-spiking
306 interneurons (Vecchia et al., 2020), and our CV in this direction of 154 ± 58 μm/msec was
307 particularly rapid. These non-canonical pathways targeting L4 PV interneurons may influence
308 feedback to L4 and therefore impact integration of intracortical and incoming ventral
309 posteromedial thalamic input. Intralaminar spread was observed in L2/3, L4, and L5. The very
310 slow L2/3 intralaminar CV value reported here of 44 ± 15 μm/msec is consistent with reported
311 values of 33-60 μm/msec for intralaminar spread of population excitatory responses in this layer
312 (Petersen, Grinvald, & Sakmann, 2003).

313 Interestingly, we found the average intralaminar CV (of L2/3 and L5) to be about twice
314 as slow as the average interlaminar CV (Fig. 6A) and approximately 2.7 times slower than the
315 interlaminar L5 → L4 CV (Fig. 6B). These differences have important implications for cortical
316 function. Long, horizontal L2/3 pyramidal cell axons have been previously observed in multiple
317 cortical areas across species (Bruno, Hahn, Wallace, de Kock, & Sakmann, 2009; Gilbert, 1992;
318 Gottlieb & Keller, 1997; Keller & Asanuma, 1993; McGuire, Gilbert, Rivlin, & Wiesel, 1991).
319 These projections are thought to connect cortical areas with similar functions, such as visual
320 regions with similar orientation selectivity (Gilbert, 1992). In BC, L2/3 pyramidal cell
321 intralaminar axons tend to extend across barrels corresponding to whisker rows rather than arcs,
322 possibly reflecting a directional preference (Feldmeyer, 2012). Both regular-spiking and fast-
323 spiking units, putative pyramidal cells and PV interneurons, respectively, show selectivity to
324 whisking direction in BC L2/3 (Andermann & Moore, 2006; Kremer, Leger, Goodman, Brette,
325 & Bourdieu, 2011). Our measurement of L2/3 intralaminar CV may reflect the targeting of the
326 PV interneurons of these domains. Murine L5 intralaminar excitatory projections connect
327 multiple barrel columns (Schubert et al., 2006; Schubert et al., 2001), and intralaminar excitatory

328 inputs to L5 pyramidal cells usually extend across one and a half barrel columns, so L5
329 pyramidal cells may integrate information from three whiskers. Thus, the L5 intralaminar CV is
330 likely to impact multi-whisker integration. While L5 → L4 axonal projections are uncommon
331 (Staiger & Petersen, 2021), optogenetic stimulation of L5 pyramidal cells elicits firing in L4 fast-
332 spiking interneurons, and inhibition of L5 pyramidal cells lengthens the reaction time in a texture
333 discrimination test (Vecchia et al., 2020). L5 → L4 CV may therefore impact temporal sharpness
334 of responses to sensory stimuli. Together, these results suggest that interlaminar cortical circuits
335 process more rapidly than intralaminar circuits. Thus, precise timing of inputs to PV interneurons
336 is likely to play an important role in computations performed within a cortical column and to be
337 less critical in the collective computations performed by multiple columns. The higher CV for
338 interlaminar conduction than intralaminar conduction found in the present study will thus meet
339 distinct demands of different forms of cortical computation.

340 hVOS imaging offers a new and powerful approach to the study of CV, not only in the
341 axons of defined cell types (Ma et al., 2017), but also in the axons defined by their targeted cell
342 types. An important example of such specificity is that thalamocortical projections to inhibitory
343 neurons have a faster CV than projections to excitatory cells, allowing interlaminar inhibition to
344 activate L4 targets prior to L2/3 targets (Kimura et al., 2010). Targeting hVOS probes to
345 different types of neurons has the potential to reveal many additional forms of axon
346 specialization adapted to different forms of neuronal computation.

347

348 **Materials and Methods**

349 *Animals*

350 Ai35-hVOS1.5 (C57BL/6-*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}hVOS1.5)Mbja}$/J, JAX 031102) Cre reporter
351 mice (Bayguinov et al., 2017) were bred with PV Cre driver mice (B6.129P2-Pvalb$^{tm1(cre)Arbr}$/J,
352 JAX 017320) to create animals with PV interneuron-specific hVOS probe expression. All animal
353 procedures were approved by the Animal Care and Use Committee of the University of
354 Wisconsin-Madison School of Medicine and Public Health (ACUC protocol: M005952).

355 *Hybrid voltage sensor (hVOS)*

356 The hVOS probe used here harbors a cerulean fluorescent protein (CeFP) tethered to the
357 inner cell membrane by a truncated h-ras motif (Wang, Zhang, Chanda, & Jackson, 2010). Slices
358 are perfused with 4 μM dipicrylamine (DPA), a small anion which partitions into the cell
359 membrane. Membrane depolarization drives DPA towards the CeFP to quench fluorescence
360 through Förster resonance energy transfer. Repolarization drives the DPA away allowing
361 fluorescence to return to baseline (Chanda et al., 2005; Wang et al., 2010). Fluorescence thus
362 reports voltage changes selectively from PV interneurons because these cells express the probe
363 with very high specificity (Bayguinov et al., 2017). DPA has a time resolution < 0.5 msec
364 (Bradley, Luo, Otis, & DiGregorio, 2009; Chanda et al., 2005) and tracks action potentials with
365 excellent temporal fidelity (Ghitani et al., 2015; Ma et al., 2019). Parameters such as latency,
366 amplitude, and half-width were determined from traces of fluorescence versus time (Fig. 7).
367 Amplitude was taken as the peak change in fluorescence from the pre-stimulus baseline, and
368 half-width as the time between rise and fall at half-maximal response. Latency is the time from
369 stimulation to half-maximal response.

370 *Slice preparation*

371 Four- to ten-week-old male and female mice were deeply anesthetized with isoflurane
372 and sacrificed via cervical dislocation. Brains were dissected and placed into ice-cold cutting
373 solution (in mM: 10 glucose, 125 NaCl, 4 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 6 $MgSO_4$, 1 $CaCl_2$)
374 bubbled with 95% O_2/5% CO_2. Coronal slices (300 µm) were prepared using a Leica VT1200S
375 vibratome and placed into artificial cerebrospinal fluid (ACSF, same as cutting solution except
376 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 4 µM DPA) and bubbled with 95% O_2/5% CO_2 for at least
377 one hour.

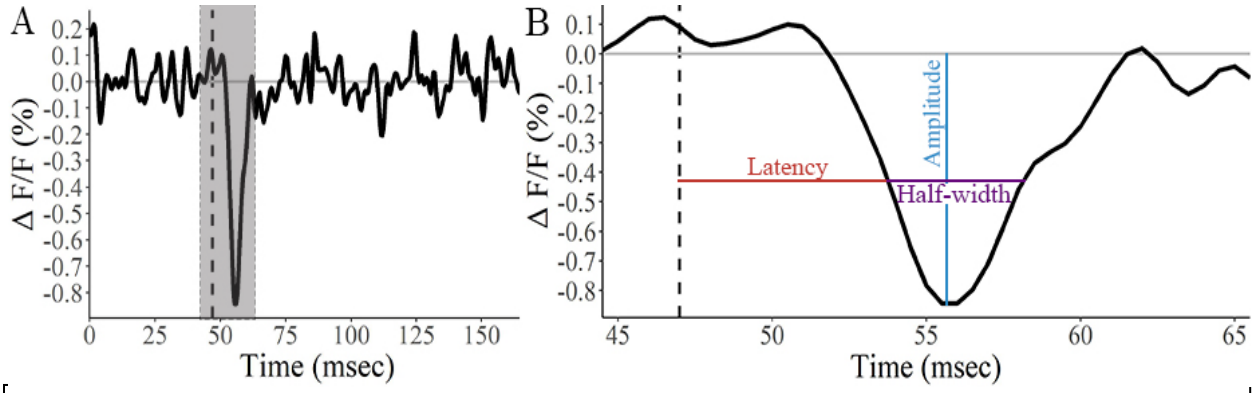

Figure 7. PV interneuron electrical activity was recorded as fluorescence using a genetically-encoded hybrid voltage sensor (hVOS). A. PV interneuron response parameters are extracted from traces of fluorescence versus time. Gray shading indicates the 20 msec measurement window expanded in B. B. Amplitude is the maximum change in fluorescence from baseline. Half-width is the time between half-maximal change in fluorescence during the rising and decay phases. Latency is the time from stimulation to half-maximal change in fluorescence of the rising phase.

378 *Voltage imaging*

379 Slices were continuously perfused with ACSF at room temperature and viewed using a
380 BX51 Olympus microscope. Layer and barrel boundaries were visually identified based on both
381 fluorescence and gradient contrast images (Feldmeyer, 2012; Woolsey & Van der Loos, 1970).
382 Stimulus pulses (100 µA, 180-µsec, except Fig. 2A-C which used 10-60 µA) were generated by a
383 stimulus isolator (World Precision Instruments, Sarasota, Florida) and applied to various
384 locations within BC with fire-polished, ACSF-filled KG-33 glass electrodes (King Precision
385 Glass, Claremont, California) with tip diameter ~6-8 µm. Traces of fluorescence versus time
386 were averages of 15-30 trials. Slices were illuminated with 435 nm light using an LED light

387 source (Prizmatix, Holon, Israel). Gradient contrast images were acquired with a Kiralux
388 (Thorlabs, Newton, New Jersey) CMOS camera; fluorescent images were acquired with this
389 camera for better visualization, but for voltage imaging we used a CCD-SMQ camera (RedShirt
390 Imaging, Decatur, Georgia) at a framerate of 2000 Hz and 80x80 spatial resolution. Data were
391 acquired with a custom data acquisition and analysis program that controlled stimulation and
392 illumination (Chang, 2006).

393 *Data processing and analysis*

394 Fluorescence divided by resting light intensity ($\Delta F/F$) was passed through a nine-point
395 binomial temporal filter and a spatial filter with $\sigma = 1$. A polynomial baseline correction was
396 calculated using fluorescence outside of a 20 msec measurement window from 2 msec before to
397 18 msec after the stimulus. Parameters including amplitude, half-width, and latency were
398 calculated from traces of fluorescence versus time. Heatmaps showing SNR are each normalized
399 to the maximum value within the field of view (with the exception of Fig. 2A-D, as explained in
400 the figure legend). Heatmaps showing maximum amplitude and associated traces of fluorescence
401 over time (Fig. 1) are normalized but not divided by resting light intensity.

402 *Responsive neuron identification*

403 We developed a semi-automated method for objective, reproducible identification of
404 responsive PV interneurons. Supp. Fig. 1 is an extension of Fig. 3 and illustrates this process.
405 The peak fluorescence change from baseline in the 20 msec post-stimulus time window (Fig. 7B)
406 was calculated for each pixel and divided by the pre-stimulus root-mean-square fluorescence
407 during a 20 msec pre-stimulus interval to give the SNR. A SNR cutoff was determined using the
408 pre-stimulus root-mean-square values, and pixels with a response SNR < cutoff were discarded
409 (gray pixels, Supp. Fig. 1A). k-means clustering was then performed on the SNR of the

410 remaining pixels (Supp. Fig. 1B). This served two main purposes. First, k-means clustering
411 divides pixels into groups likely to contain responsive cells, using the criterion that only pixels
412 with SNR > 5 represent responsive cell bodies (corresponding to yellow, light blue, and medium
413 blue clusters, Supp. Fig. 1B). Second, if multiple pixels represent one cell body, they are likely to
414 have similar SNR values. Because each k-means cluster contains pixels with similar SNRs, two
415 pixels in the same cluster sharing a face are likely to represent the same cell body. This method
416 basically compared each pixel to its neighbors and grouped them based on the likelihood they
417 represent the same cell. One or more contiguous pixels in the same SNR cluster were grouped as
418 one cell if they were no greater than three pixels across, because each pixel measures 6.68 μm,
419 and murine PV interneuron somata are approximately 20 μm in diameter (Kooijmans et al.,
420 2020; Selby et al., 2007; Y. Wang et al., 2002). These criteria excluded some larger clusters of
421 pixels as potentially representing more than one cell even though they had a high SNR. For
422 example, some yellow and orange pixels in Fig. 3C and Supp. Fig. 1A, which correspond with
423 the highest SNR k-means cluster (yellow) in Supp. Fig. 1B, form groups which are larger than 3
424 pixels across and therefore must contain more than one cell. Likewise, Fig. 2D had fewer
425 identifiable responsive cells that Fig. 2C because Fig 2D had greater overlap. Pixel groups with
426 SNR < 5 or amplitude < 0.001 were excluded and not used to select individual responsive
427 neurons. We also excluded groups of pixels sharing a face to ensure that a single group did not
428 represent multiple cells. The rationale for this grouping procedure is that pixels draw from an
429 area that is smaller than a PV interneuron cell body, and we expect SNR to be uniform over the
430 soma of one neuron. Pixels at a cell edge will not be included due to their lower SNR. Thus, this
431 criterion is conservative in focusing on pixels containing signals arising from distinct, spatially
432 separated neurons.

433 *Experimental design and statistical tests*

434 Further analysis of responses from groups of pixels identified as described above was
435 performed using R and Python. Responses with latency < 1 msec were removed to exclude the
436 effect of direct stimulation; these were generally very close to the stimulation electrode.
437 Responses with latency or time to peak > 13 msec were removed to exclude polysynaptic
438 responses. Disynaptic responses, defined as those occurring more than 2 msec after responses
439 from neighboring cells (< ~ 20 μm) were also excluded. Potential outliers, defined as responses
440 with amplitude, half-width, or latency outside the first or third quartile ± 1.5 * interquartile
441 range, were iteratively identified and visually verified or excluded as appropriate.

442 Each CV was based on a plot of latency versus distance with at least 8 responsive cells ≥
443 100 μm from the stimulating electrode, spanning a range of distances ≥ 100 μm. Analyses
444 included 855 PV interneurons from 59 slices from 13 animals (6 female and 7 male).
445 Relationships between latency and distance were evaluated with linear regression, and p-values
446 were corrected for multiple tests using the false-discovery rate. Euclidean distance was
447 determined between pixels on the 80x80 grid of the CCD-SMQ camera. To determine the
448 appropriate statistical tests, normality was evaluated with a Shapiro-Wilk test, and differences in
449 group variances were evaluated with Levene's tests. CV was normally distributed ($W = 0.9115$,
450 $p = 0.051$). Variance did not differ significantly for male and female animals ($F = 1.2505$, $p =$
451 0.277), for inter- and intralaminar CV ($F = 0.516$, $p = 0.481$), or for CV directions (intralaminar,
452 L2/3 → L4, L4 → L2/3, L5 → L2/3, L5 → L4; $F = 0.0519$, $p = 0.994$). Therefore, interlaminar
453 versus intralaminar CV, and CV for male versus female animals were compared with t-tests. CV
454 did not differ significantly based on sex ($t = 0.289$, $p = 0.778$). The effect of direction on CV was

evaluated with ANOVA followed by post-hoc comparisons using Tukey's honestly significant differences.

Code accessibility

Custom software, R code, and Python code available on request.

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