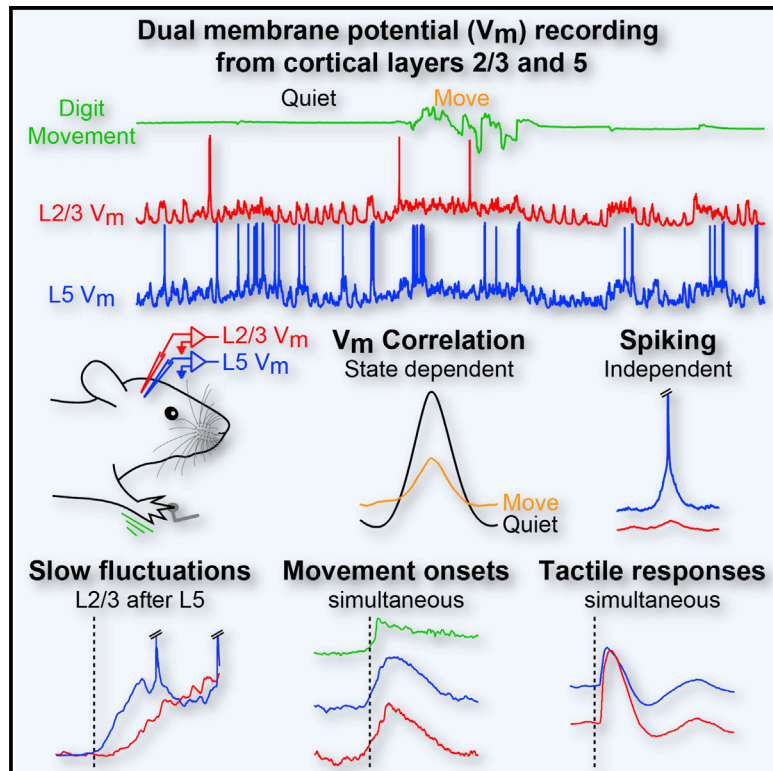


Translaminar Cortical Membrane Potential Synchrony in Behaving Mice

Graphical Abstract



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In Brief

Zhao et al. use dual whole-cell recordings from primary somatosensory cortical excitatory neurons in layers 2/3 and 5 in awake mice to identify layer-specific cellular properties and firing rates, and they show that translaminar membrane potential synchrony is dependent both on behavioral state and the source of the synaptic input.

Highlights

- We made dual whole-cell recordings from L2/3 and L5 cortical neurons in behaving mice
- Layer-specific membrane properties determine higher mean firing rates of L5 neurons
- Synchrony of translaminar synaptic activity is determined by the origin of input
- L5 neurons signal spontaneous and sensory-triggered movements



Translaminar Cortical Membrane Potential Synchrony in Behaving Mice

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SUMMARY

The synchronized activity of six layers of cortical neurons is critical for sensory perception and the control of voluntary behavior, but little is known about the synaptic mechanisms of cortical synchrony across layers in behaving animals. We made single and dual whole-cell recordings from the primary somatosensory forepaw cortex in awake mice and show that L2/3 and L5 excitatory neurons have layer-specific intrinsic properties and membrane potential dynamics that shape laminar-specific firing rates and subthreshold synchrony. First, while sensory and movement-evoked synaptic input was tightly correlated across layers, spontaneous action potentials and slow spontaneous subthreshold fluctuations had laminar-specific timing; second, longer duration forepaw movement was associated with a decorrelation of subthreshold activity; third, spontaneous and sensory-evoked forepaw movements were signaled more strongly by L5 than L2/3 neurons. Together, our data suggest that the degree of translaminar synchrony is dependent upon the origin (sensory, spontaneous, and movement) of the synaptic input.

INTRODUCTION

Primary sensory cortex is composed of six layers of interconnected microcircuits. Gain- and loss-of-function experiments have shown laminar-specific effects on local cortical processing (Beltramo et al., 2013; Olsen et al., 2012), but how the layers work together remains unclear. The synchrony of action potential (AP) firing across cortical layers is thought to be a fundamental aspect of translaminar processing and is determined by the strength, sign and timing of the underlying synaptic input. Here, we investigate the synaptic mechanisms of cortical synchrony between excitatory neurons in layers 2/3 and 5 in behaving mice.

Measuring translaminar membrane potential (V_m) synchrony and linking it to sensory processing and behavior require simul-

taneous V_m recordings from different layers in awake animals. However, the vast majority of V_m recordings of cortical neurons in behaving animals have been made from superficial layers (Bennett et al., 2013; Crochet and Petersen, 2006; Gentet et al., 2010; Polack et al., 2013; Poulet and Petersen, 2008; Poulet et al., 2012; Reimer et al., 2014; Zhou et al., 2014). These studies have shown that internally generated, spontaneous network activity dominates the V_m of cortical neurons across cortical regions and is correlated with the behavioral and arousal state. Large-amplitude, slow fluctuations are highly correlated between neighboring layer 2/3 (L2/3) neurons in resting animals but are abolished during movement, resulting in a desynchronized or “active” cortical state (Harris and Thiele, 2011; Poulet and Petersen, 2008). The active state may result from arousal-related effects associated with movement and has been linked to a modulation in sensory responsiveness (Crochet and Petersen, 2006; Otazu et al., 2009; Pinto et al., 2013; Polack et al., 2013; Reimer et al., 2014; Schneider et al., 2014; Vinck et al., 2015; Zhou et al., 2014), adaptation (Castro-Alamancos, 2004), and even perception itself (Bennett et al., 2013; McGinley et al., 2015).

Few studies have examined the V_m activity of deeper layer cortical neurons in behaving animals (McGinley et al., 2015; Schieman et al., 2015). Extracellular recordings, however, have shown higher spontaneous and sensory-evoked firing rates in deeper layer neurons (de Kock et al., 2007; O'Connor et al., 2010) and, intriguingly, that sensory-evoked and spontaneous spiking have different temporal structures across layers (Sakata and Harris, 2009).

The rodent forepaw somatosensory system is a relevant and accessible model system to investigate cortical sensory processing during behavior. The forepaw has five digits (Figure 1A) that can be used to grasp and manipulate objects as well as discriminate somatosensory stimuli (Milenkovic et al., 2014). We made whole-cell recordings from primary forepaw somatosensory cortex L2/3 and L5 excitatory neurons in awake mice to compare the synchrony and integration of external (sensory) and internal (movement-evoked and spontaneous) synaptic input. Our data highlight layer-specific membrane properties that underlie differences in AP firing and show that translaminar V_m synchrony is dependent both on the behavioral state and the source of synaptic input.



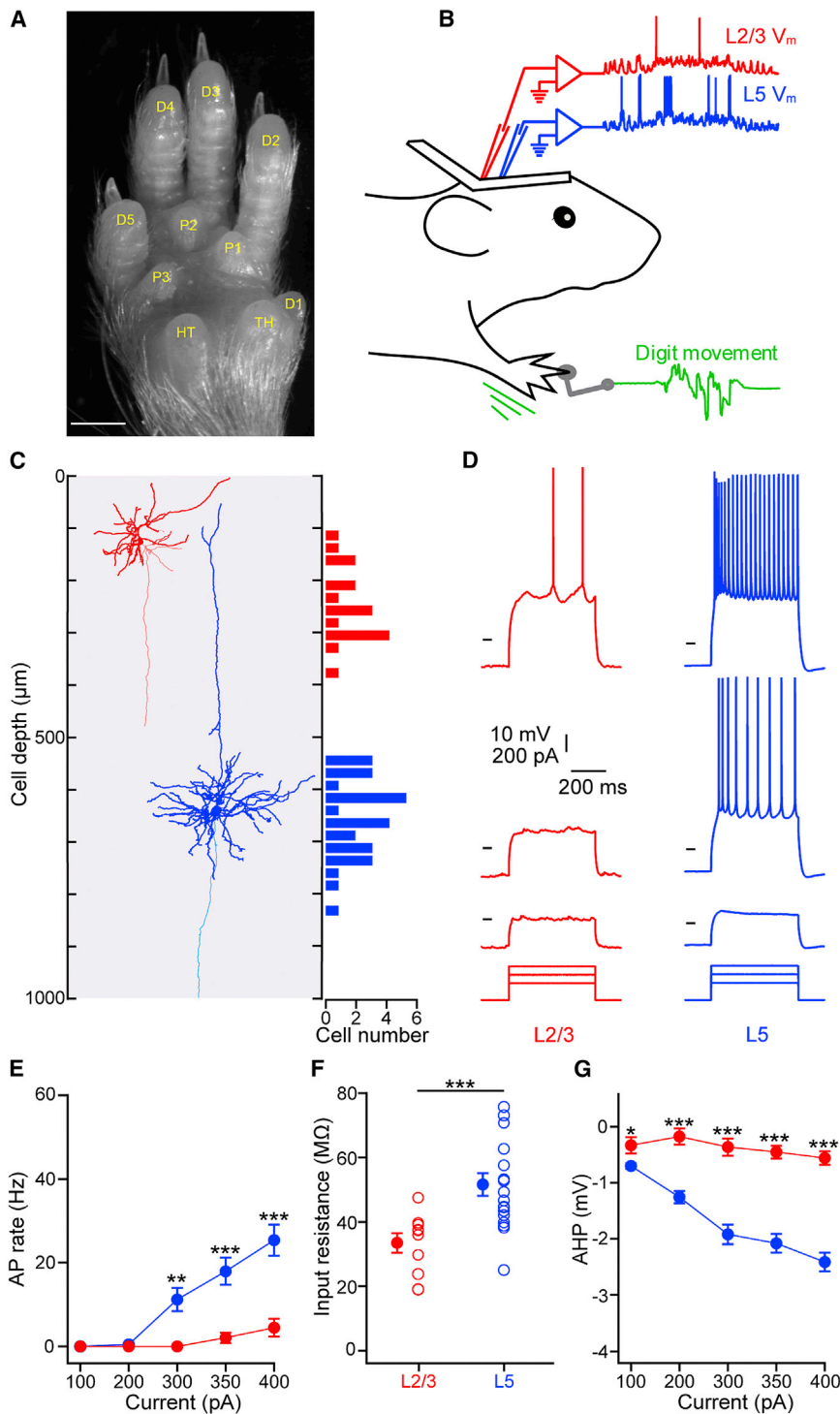


Figure 1. Distinct Membrane Properties of L2/3 and L5 Cortical Pyramidal Neurons

(A) Image of the glabrous skin of the right forepaw showing five digits (D1–D5), three central pads (P1–P3), the thenar pad (TH), and the hypothenar pad (HT) (Waters et al., 1995). Scale bar, 1 mm.

(B) Cartoon schematic showing head-fixed awake mouse with recording electrodes in red (L2/3) and blue (L5), with forepaw digit movement (green) monitored by the sensing arm (gray) that was also used for tactile stimulation.

(C) Biocytin reconstructions of L2/3 (red) and L5 (blue) neurons, with axons in lighter color, next to a histogram showing the depths of all recorded L2/3 and L5 neurons (n = 17 L2/3 neurons and n = 28 L5 neurons) based on micromanipulator reading and biocytin staining.

(D) Three single trial responses of a L2/3 (red) and a L5 (blue) pyramidal neuron to intracellular current injection with different amplitudes (from top to bottom: +400 pA, +300 pA, and +200 pA). The L2/3 example corresponds to the reconstructed L2/3 neuron in (C). Horizontal lines indicate –60 mV for L2/3 and L5.

(E) Plotting the evoked spike rate as a function of current injection amplitude reveals that L5 neurons are more excitable than L2/3 neurons. Filled circles with error bars show mean ± SEM.

(F) L5 neurons have a significantly larger input resistance than L2/3 neurons during hyperpolarizing current injection. Open circles show individual cells.

(G) L5 neurons show a significantly larger amplitude hyperpolarization following positive current injection (afterhyperpolarization [AHP]) than L2/3 neurons at all current amplitudes tested.

For all panels, *p < 0.05, **p < 0.01, and ***p < 0.001.

cordings targeted to the digit 3 cortical representation in primary somatosensory forepaw cortex (S1) in awake mice. Mice were head-restrained and had their right forepaw tethered to the platform (Figure 1B). The tips of the digits 2 and 4 overhanging the edge of the platform while the tip of digit 3 was positioned on a flat, circular head of a combined movement sensor and force-feedback tactile stimulating arm. The stimulating arm head was held in contact with the glabrous skin of digit 3 throughout all recordings. Whole-cell recordings from 17 L2/3 neurons

were made at subpial depths between 121 and 384 μm (245.30 ± 17.91 μm) and from 28 L5 neurons between 538 and 823 μm (649.43 ± 14.28 μm) (Figure 1C). All neurons had evoked regular-spiking firing patterns during current injection, and a subset (L2/3 = 4/17 and L5 = 15/28 neurons) was confirmed by post hoc biocytin staining to be excitatory pyramidal neurons.

RESULTS

Distinct Intrinsic Membrane Properties of L2/3 and L5 Primary Somatosensory Forepaw Cortical Excitatory Neurons

To investigate the intrinsic membrane properties of L2/3 and L5 excitatory neurons, we made blind whole-cell patch clamp re-

We first examined the intrinsic membrane properties soon after break-in during quiet wakefulness. L5 neurons generated more APs in response to equivalent current injection amplitudes than L2/3 neurons (Figures 1D and 1E). The increased excitability may be due to the higher resting V_m value of L5 neurons during quiet wakefulness ($L2/3_{Mean} = -56.92 \pm 1.21$ mV, $n = 12$ cells versus $L5_{Mean} = -50.70 \pm 0.65$ mV, $n = 19$ cells; $p < 0.001$) and their higher input resistance (Figure 1F; $L2/3_{IR} = 33.46 \pm 2.80$ M Ω , $n = 11$ cells versus $L5 = 51.66 \pm 3.35$ M Ω , $n = 18$ cells; $p < 0.001$). Interestingly, following the termination of a depolarizing current step, L5 neurons showed a larger-amplitude afterhyperpolarization than L2/3 neurons (Figure 1G; at 400 pA: $L2/3_{AHP} = 0.56 \pm 0.12$ mV, $n = 10$ cells versus $L5_{AHP} = 2.42 \pm 0.17$ mV, $n = 22$ cells; $p < 0.001$). Overall, our data show distinct intrinsic membrane properties of forepaw S1 L5 and L2/3 neurons.

Layer-Specific V_m Dynamics during Forepaw Behavior

We next compared the V_m properties and dynamics of L2/3 and L5 neurons during forepaw behavior. Spontaneous digit movements were monitored by the sensing arm and used to define periods of quiet wakefulness (Q) and digit movement (M) (see Experimental Procedures). Q was characterized by large-amplitude, low-frequency V_m fluctuations observed in all recordings from neurons in both layers (Figure 2A). Slow fluctuations had a similar mean duration across layers ($L2/3$ 329.06 ± 27.55 ms, $n = 13$ cells versus $L5$ 266.94 ± 11.15 ms, $n = 23$ cells) but lower frequency in L2/3 ($L2/3$ 2.03 ± 0.08 Hz, $n = 13$ cells versus $L5$ 2.29 ± 0.04 Hz, $n = 23$ cell; $p = 0.005$) (Figure S1). In both layers, M was accompanied by a reduction in the amplitude of low-frequency fluctuations, with fast Fourier transform analysis highlighting a reduction in the power in the 1 to 5 Hz range (Figures 2B and 2C), and a reduction in the SD of the V_m (Figure 2D). Mean AP firing rates were higher in L5 than L2/3 neurons during Q (Figure 2E; $L2/3_{FR} = 0.32 \pm 0.10$ Hz, $n = 12$ cells versus $L5_{FR} = 2.86 \pm 0.60$ Hz, $n = 19$ cells; $p < 0.001$). Moreover, a significant increase in mean firing rates during M was observed in L5 neurons ($L5_{FR} = 2.86 \pm 0.60$ Hz versus $M = 6.14 \pm 1.16$ Hz, $n = 19$ cells, $p = 0.005$), but not L2/3 ($L2/3_{FR} = 0.32 \pm 0.10$ Hz versus $M = 0.45 \pm 0.18$ Hz, $n = 12$ cells; $p = 0.851$). Inter-spike intervals (ISIs) showed a skewed distribution in both layers, with $\sim 22\%$ of L2/3 APs and $\sim 30\%$ of L5 APs having an ISI of < 25 ms (Figure S2). We next analyzed AP bursting and observed similar burst durations across layers but overall more bursts in L5 neurons. No differences were observed between Q and M periods (burst frequency $L2/3_{BF} = 0.02 \pm 0.004$ Hz, $M = 0.03 \pm 0.01$ Hz, $n = 13$ cells versus $L5_{BF} = 0.14 \pm 0.05$ Hz, $M = 0.25 \pm 0.12$ Hz, $n = 23$ cells, $L2/3$ versus $L5$ Q, $p = 0.019$; Figure S2).

To investigate what drove the laminar differences in mean AP rates, we next examined the AP threshold. AP threshold in L2/3 and L5 neurons varies dependent on the speed of pre-spike depolarization in V_m , with faster depolarizing ramps evoking APs at lower threshold (Figure S3). There was, however, no overall difference in AP threshold between L2/3 and L5 neurons in Q or M periods (Figure 2F). Measurement of the mean V_m showed a depolarization during M in both L2/3 (Figure 2G; $L2/3_{Mean} = -56.92 \pm 1.21$ mV versus $M = -51.45 \pm 1.73$ mV, $n = 12$ cells; $p < 0.001$) and L5 neurons ($L5_{Mean} = -50.70 \pm 0.65$ mV versus $M = -45.89 \pm 0.80$ mV, $n = 19$ cells; $p < 0.001$), with L5 neurons significantly more depolarized in both behavioral states (Q $L2/3_{Mean}$ versus $L5_{Mean}$ $p < 0.001$; $M = L2/3_{Mean}$ versus $L5_{Mean}$ $p = 0.006$).

Notably, plotting the mean spontaneous AP rates as a function of the difference between AP threshold and the mean value of the top 10% of the V_m distribution (max V_m) revealed an exponential decay (Figure 2H). L5 neurons were distributed on the falling slope and L2/3 on the tail of the slope (Figure 2H), suggesting that the simplest explanation for higher firing rates in L5 neurons is their more depolarized V_m . Most likely, this works in combination with the higher input resistance of L5 neurons (Figure 1) to push excitatory postsynaptic potentials over AP threshold and trigger more APs in L5 than L2/3 neurons in behaving mice.

V_m Synchrony across Cortical Layers Is Regulated by Behavioral State

Synchrony of cortical activity across layers is thought to be an important feature of cortical coding. To examine translaminar subthreshold and spiking synchrony, we next inserted two pipettes through neighboring craniotomies to target recording sites in the same vertical axis and made nine simultaneous, dual whole-cell recordings from L2/3 and L5 neurons (Figure 3A). We went on to measure V_m synchrony at different timescales using cross-correlation, coherence analysis, and spike-triggered averaging.

Visual inspection of dual V_m recordings and cross-correlation analysis shows that large-amplitude slow fluctuations in resting mice are highly correlated across layers (Figures 3A–3E). As mice went from Q to M, however, the correlation of the V_m between L2/3 and L5 neurons was reduced in all pairs of cells (Figures 3D and 3E). Unexpectedly, the peak time of the cross-correlation showed a significant time lag, indicating that L5 subthreshold activity preceded that in L2/3 by 8.04 ± 1.40 ms in resting mice and by 4.35 ± 1.85 ms during M (Figure 3F). Coherence measurements suggested that the drop in correlation during M was due mostly to the reduction in low-frequency (1–5 Hz) coherence (Figures 3G and 3H). Thus, dual whole-cell recordings showed that subthreshold activity of cortical neurons in awake animals is under dynamic control, with cortical layers becoming more independent during active brain states.

To quantify AP synchrony from dual recordings, we made spike-triggered peri-stimulus time histograms (PSTHs) of spontaneous APs. The chance of observing an AP in a L5 neuron in a 10-ms window around a L2/3 AP is 6.1% during Q and 5.6% during M, and the chance of observing an AP in a L2/3 neuron around a L5 AP is 1.0% during Q and 0.4% during M. Thus, while the chance of observing an AP is dependent upon the firing rate, AP firing appears asynchronous at fast timescales across cortical L2/3 and L5 in awake mice. We next examined the synaptic input driving spontaneous APs. In both layers, APs were triggered by large-amplitude and cell-specific depolarizing synaptic input (Figures 3I–3K). At the time of the AP, a slow, small-amplitude depolarization was observed in the simultaneously recorded cell in resting mice, necessarily induced by the slow network fluctuations. A similar picture was present during movement: APs were triggered by large-amplitude, depolarizing inputs in the spiking cell that were absent in the simultaneously recorded neuron. These observations provide a synaptic basis for independent laminar spontaneous firing and suggest that

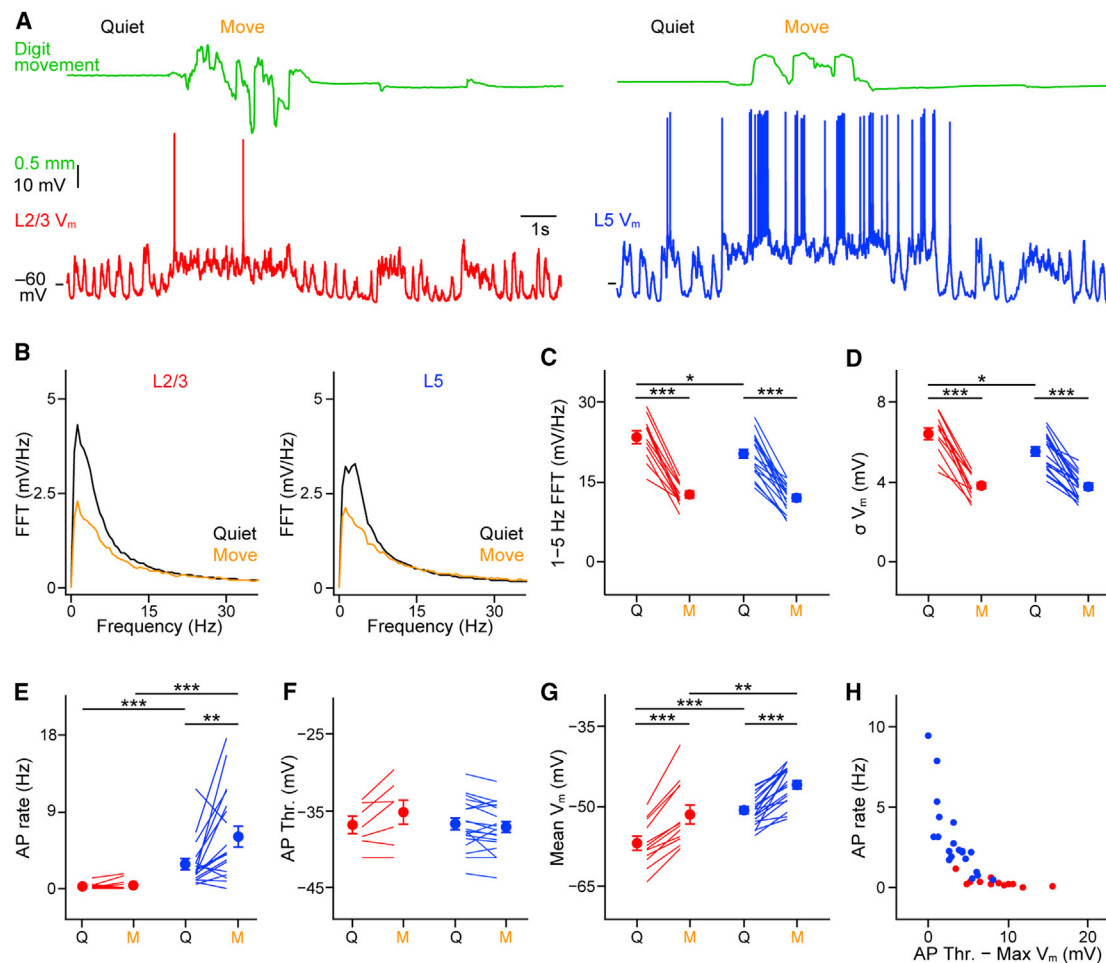


Figure 2. Laminar-Specific V_m Dynamics of L2/3 and L5 Neurons during Forepaw Behavior

(A) Example whole-cell recordings from a L2/3 neuron (red) and L5 neuron (blue) with the digit movement (green) measured by the stimulator/sensing arm in contact with the glabrous skin of forepaw digit 3.

(B) Population average fast Fourier transforms (FFTs) of the V_m of L2/3 and L5 neurons during quiet wakefulness (black) and digit movement (orange).

(C) Power of low-frequency activity (1–5 Hz) in the quiet (Q) and moving (M) state shows a significant reduction during digit movement in both L2/3 and L5 neurons. Filled circles with error bars show mean \pm SEM, and lines represent individual neurons.

(D) SD of the V_m was significantly reduced during forepaw movement in both L2/3 and L5 neurons.

(E) L5 neurons showed an overall higher AP firing rate than L2/3 neurons in quiet and moving mice and a significant increase in AP firing rate during movement.

(F) AP threshold was not significantly different at rest and during movement in L2/3 and L5 neurons.

(G) Both L2/3 and L5 neurons depolarize during digit movement, but L2/3 neurons are more hyperpolarized than L5 neurons in both behavioral states.

(H) The mean firing rate (Q and M periods) of L2/3 and L5 neurons plotted as a function of the distance between AP threshold and the mean value of the maximum 10% of the V_m (Max V_m). Filled circles show the mean value for one cell.

For all panels, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. See also [Figures S1–S3](#).

spontaneous AP firing in L2/3 and L5 excitatory neurons is driven by sparsely connected excitatory networks.

L5 Neurons Show an Earlier Onset of Slow Spontaneous Fluctuations

The time lag of the cross-correlation analysis and independent synaptic input during spiking suggested that there might be laminar differences in the fine temporal structure of spontaneous subthreshold fluctuations. We therefore first compared the onset timing of the slow depolarizing fluctuations in resting mice from dual L2/3 and L5 recordings ([Figures 4A and 4B](#)). We defined a

slow depolarizing event (SDE) as depolarizing epochs in Q periods whose average V_m level between onset and offset was $>60\%$ of the V_m range between the most hyperpolarized and depolarized values for at least 100 ms. Plotting the duration and V_m of isolated SDEs across seven dual recordings as a heatmap ([Figures 4B and S4](#)) revealed a range of durations with a mean of ~ 300 ms ([Figure S1B](#) for single and dual recordings, L2/3 = 329.06 ± 27.55 ms, $n = 13$ cells, L5 = 266.94 ± 11.15 ms, $n = 23$ cells, $p = 0.086$). Normally a SDE in one neuron was accompanied by a SDE in the simultaneously recorded neuron, but a minority of small-amplitude, short-duration SDEs were observed in one layer only

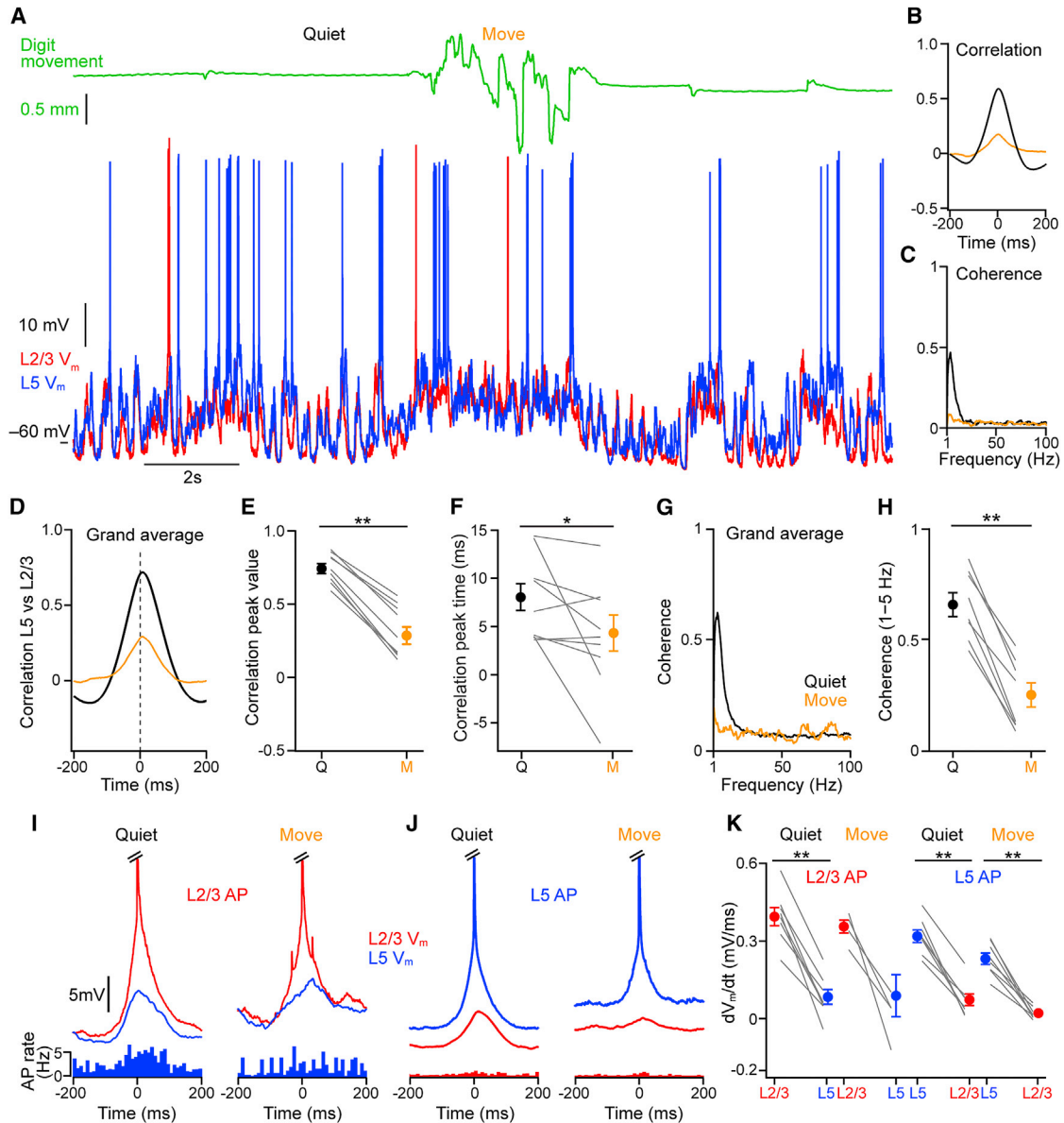


Figure 3. V_m Synchrony between L2/3 and L5 Neurons Is Dependent on Behavioral State

(A) Example dual whole-cell recording from a L2/3 (red) and a L5 (blue) cortical neuron during digit movement (green).
 (B) Mean cross-correlation for the example recording shown in (A), taking L5 as the reference, shows a higher correlation during quiet (Q) than digit movement (M).
 (C) Example mean coherence spectrum from the example recording shown in (A) from Q and M periods.
 (D) Population mean cross-correlation (n = 9 pairs) during Q and M periods.
 (E) Significant reduction in the peak cross-correlation value in M compared to Q periods. Filled circles with error bars show mean \pm SEM, lines show data from individual pairs.
 (F) The peak time of the cross-correlation shows a positive lag indicating that L5 neurons are active before L2/3 neurons in Q and M periods.
 (G) Population mean average of coherence spectrum during Q and M periods (n = 8 pairs).
 (H) A significant reduction in coherence from Q to M periods in frequency band 1–5 Hz.
 (I) Population V_m average of L2/3 (red) and L5 neurons (blue) centered on APs in L2/3 neurons during quiet (left, n = 8 pairs) and moving (right, n = 4 pairs) periods. Bottom, corresponding population L5 spike-time PSTHs.
 (J) Same as (I) but for L5 spike-triggered averages with L2/3 PSTH below (n = 8 pairs).
 (K) Quantification of the V_m rise time in L2/3 and L5 neurons between -22 ms and -2 ms before a (left) L2/3 AP and (right) L5 AP in quiet and moving periods. Filled circles show population mean with error bars showing mean \pm SEM. Gray lines show values from individual cells.
 For all panels, *p < 0.05, **p < 0.01, and ***p < 0.001.

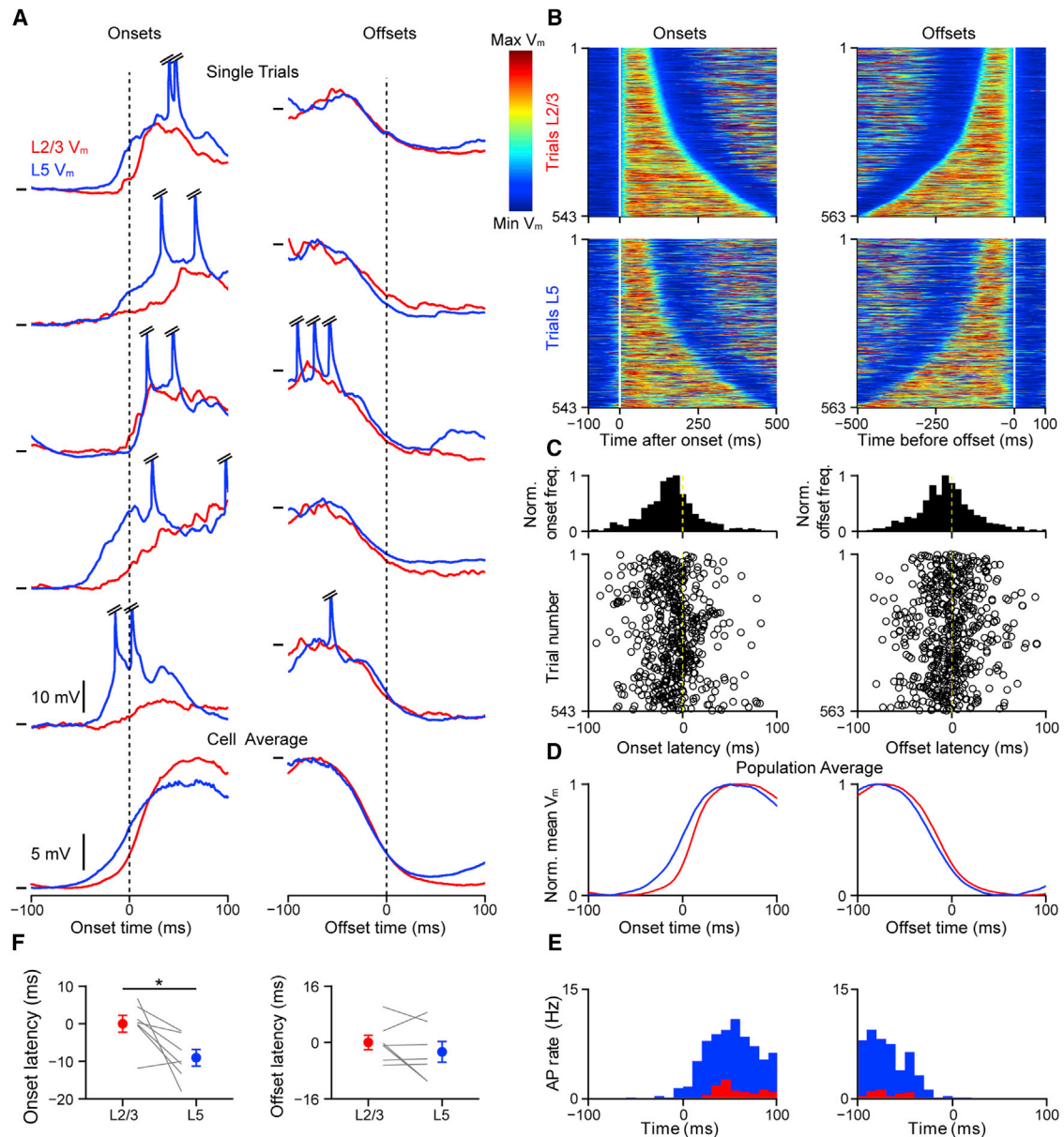


Figure 4. Slow Depolarizing Fluctuations during Quiet Wakefulness Have an Earlier Onset in L5 Neurons

(A) Example slow depolarizing events (SDEs) from a dual L2/3 (red) and L5 (blue) whole-cell recording in an awake, resting mouse. V_m traces are aligned to the onset (left) and offset (right) of the SDE in the L2/3 neuron, and bottom traces show V_m averages. Note that L5 leads at the onset, but not the offset, of the SDEs. Horizontal lines indicate V_m (mV) for L2/3 and L5 (trial 1 onset, $-69.3/-63.1$ mV and offset, $-47.7/-44.7$ mV; trial 2 onset, $-59.3/-59.1$ mV and offset, $-40.0/-37.7$ mV; trial 3 onset, $-60.9/-57.0$ mV and offset, $-31.8/-31.7$ mV; trial 4 onset, $-59.7/-56.4$ mV and offset, $-42.0/-37.7$ mV; and trial 5 onset, $-58.3/-55.7$ mV and offset, $-40.0/-30.1$ mV). Average onset, $-60.0/-52.6$ mV and offset, $-41.9/-37.0$ mV. APs have been truncated.

(B) Plots of selected SDEs from seven dual whole-cell recordings. SDEs were aligned to threshold crossing at the onset (left) and offset (right) of the SDE in the L2/3 neuron and arranged by duration. Top boxes show L2/3 data, and bottom boxes show L5 with colors corresponding to the normalized V_m from minimum (blue) to maximum (red) values.

(C) Population distribution (top) and trial-by-trial measurements (bottom) of the subthreshold onset (left) and offset (right) times in L5 neurons relative to the onset and offset times in L2/3, respectively ($n = 7$ dual recordings). Onset and offset times were estimated by the 5% level of a sigmoidal fit to the V_m at onset and offset (see [Supplemental Experimental Procedures](#) for details).

(D) Population average of the normalized V_m SDEs in L2/3 and L5 relative to the threshold-crossing at onset and offset of the L2/3 neuron.

(E) Population peri-SDE time histogram of AP times from the dataset used in (D).

(F) Population analysis of onset and offset times relative to the L2/3 SDE shows a significantly earlier onset in L5 but similar offset times. To calculate the onset/offset timing difference, we first measured the time of SDE onset/offset relative to the time of threshold crossing of the L2/3 SDE. Then, we subtracted the population mean L2/3 onset/offset time from all values. Filled circles with error bars show mean \pm SEM, and lines show data from individual pairs.

For all panels, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. See also [Figures S4](#) and [S5](#).

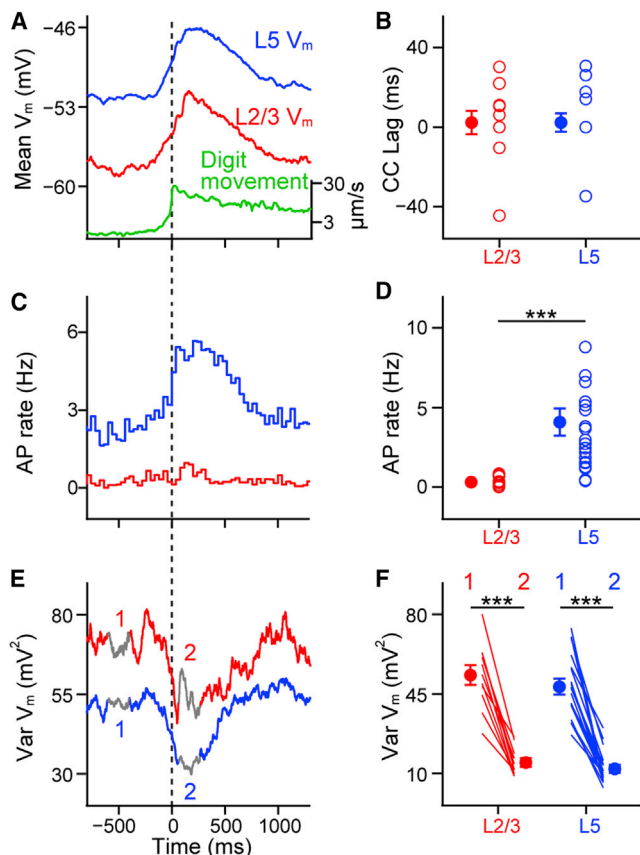


Figure 5. Movement Onset Synchronizes Synaptic Input across Layers and Results in an Increase in L5 AP Rate

(A) Population V_m average responses of L2/3 (red) and L5 (blue) neurons to onset of spontaneous digit movement (green shows the rectified first derivative of the digit movement (digit_{FD}); $n = 11$ L2/3 cells and $n = 19$ L5 cells, from single and dual recording experiments). Movement onsets (dashed vertical line) were detected via thresholding of digit_{FD} (see [Experimental Procedures](#)). (B) Peak cross-correlation between the digit_{FD} and the V_m shows no significant time lag between layers, indicating synchronous depolarization. Filled circles with error bars show mean \pm SEM, and open circles show individual cells. (C) Mean population AP rates over time with respect to movement onset from L2/3 and L5 neurons. (D) L5 neurons show a significantly higher AP firing rate after movement onset (between 0 and 1 s) than L2/3 neurons. (E) Population average of the variance of the V_m over time with respect to movement; gray sections and numbers show time points for analysis in (F). (F) L2/3 and L5 neurons show a significant reduction in the V_m variance following movement onset; the two measurement time windows (1 and 2) are indicated in (E).
For all panels, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

([Figure S5](#)), likely accounting for the slightly shorter durations of SDEs in L5. In those SDEs with a measurable V_m onset in both layers, plotting the trial-by-trial onset times ([Figure 4C](#); [Supplemental Experimental Procedures](#)), the population averaged V_m ([Figure 4D](#)) and population spiking rates ([Figure 4E](#)) revealed that L5 SDEs started earlier than those in L2/3 ([Figure 4F](#); L5 leading L2/3 = 9.07 ± 2.19 ms, $n = 7$ pairs, $p = 0.031$). In contrast, the hyperpolarizing offset time was not significantly different across layers when SDEs were triggered on L2/3 onset ([Figures 4A](#) and [4F](#)).

SDEs thus have an earlier onset in higher-firing L5 neurons, supporting the proposal that L5 neurons are important drivers of supragranular slow network activity ([Beltramo et al., 2013](#); [Sanchez-Vives and McCormick, 2000](#)).

Movement Onset Triggers Synchronous Input across Layers

Is the laminar-specific synaptic input observed during AP firing ([Figure 3](#)) and spontaneous activity ([Figure 4](#)) a general feature of subthreshold processing in cortical neurons, or are there other sources of more synchronized synaptic input? Movement triggers an active, desynchronized cortical state, which can be driven by thalamic input ([Poulet et al., 2012](#)). To examine the fast dynamics of movement related synaptic input across layers, we next analyzed the V_m dynamics at M onset (detected by thresholding the first derivative of the digit movement signal; [Supplemental Experimental Procedures](#)). Averaging the V_m showed that M onset is accompanied by simultaneous depolarizing synaptic input to L2/3 and L5 neurons ([Figures 5A](#) and [5B](#)). Close inspection of the log-scale digit movement trace indicated that the depolarization in both layers was tightly coupled to the tiny initial movements of the digits and did not appear before movement. To quantify the timing of the depolarization, we performed cross-correlation analysis between the averaged digit movement and the averaged V_m response at M onset. The peak of the cross-correlation showed no significant time lag ([Figure 5B](#)), suggesting an internal, non-sensory origin for synaptic input at movement onset.

Analysis of 2-s epochs of V_m activity showed an overall reduction in the SD of the V_m during extended M periods ([Figure 2](#)), but how fast this occurs was unclear. We therefore quantified the variance of the L2/3 and L5 subthreshold activity around all recorded M onsets, including short-duration movements ([Figures 5E](#) and [5F](#)). In all cells across both layers, we observed a rapid and robust reduction in variance within the first 200 ms of movement onset. Thus, early movement-evoked input acts to reduce subthreshold variability simultaneously across cortical layers. The reduction in variance, however, does not result in synchronous AP firing. Instead, L5 neurons showed higher firing rates after movement onset than L2/3 neurons ([Figures 5C](#) and [5D](#)). Thus, unlike the laminar-specific V_m dynamics during SDEs and spontaneous APs, movement onset triggers simultaneous V_m depolarization across layers, which results in an increase in firing only in L5 neurons.

Tactile Stimulation Evokes Correlated Synaptic Input across Layers

Another major source of synaptic input to S1 is sensory-evoked thalamic input. Thalamo-cortical axons are unequally distributed across the cortical layers, with some L5 neurons receiving direct thalamic input ([Bureau et al., 2006](#); [Constantinople and Bruno, 2013](#)). We therefore next examined whether there is laminar specificity in sensory processing across layers in behaving animals. We delivered brief (2 ms), light (10 mN) tactile stimuli via the stimulating/sensing arm to digit 3 during single and dual whole-cell recordings. The sensing arm was in constant contact with the glabrous skin of digit 3. Tactile stimulation evoked a subthreshold response in all cells. While the first spike latency was similar for both behavioral conditions and layers

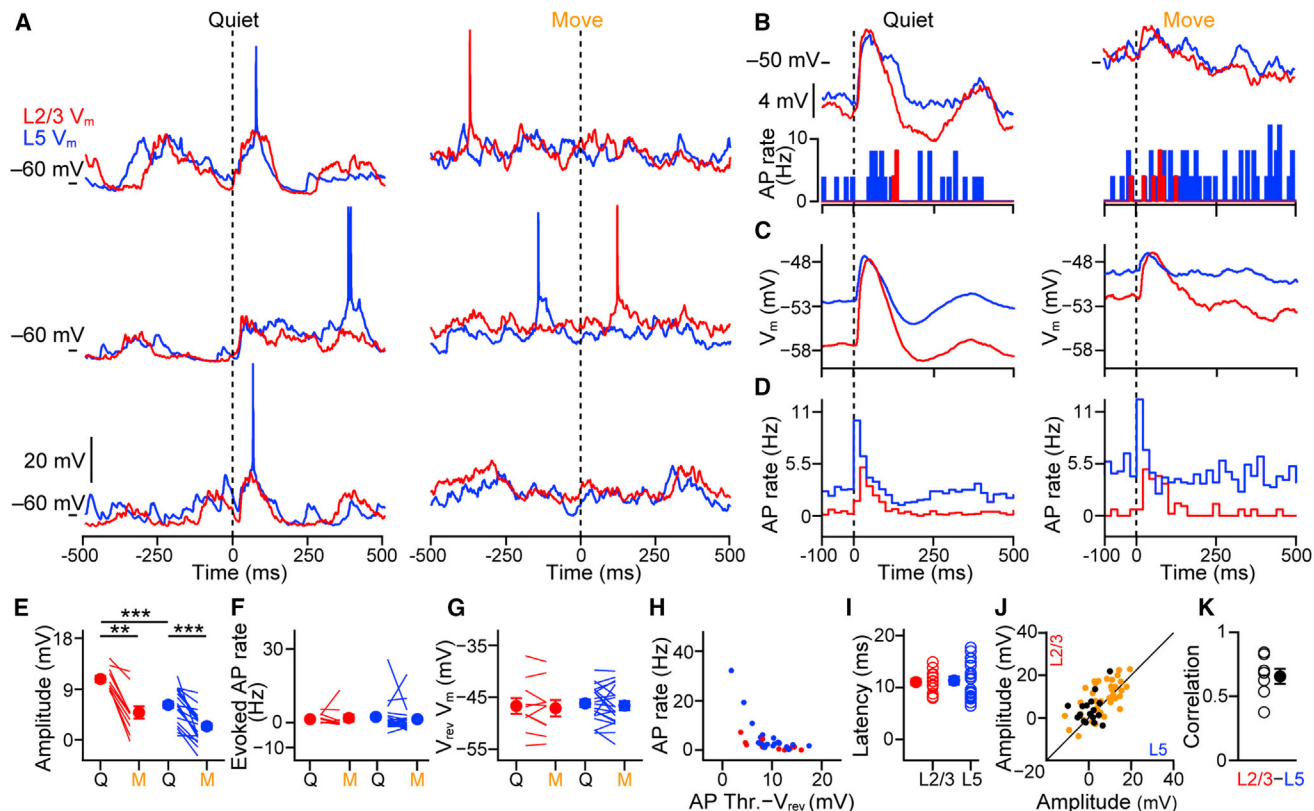


Figure 6. Tactile-Evoked Subthreshold Responses Are Highly Correlated across Cortical Layers and Modulated by Behavioral State

(A) Single-trial tactile-evoked responses from a dual L2/3 (red) and L5 (blue) whole-cell recording during quiet wakefulness (left) and movement (right). (B) Top: mean V_m tactile-evoked response of example neurons in (A) during quiet wakefulness and movement shows reduction in amplitude during moving periods. Bottom: corresponding PSTH of AP firing. (C) Population averaged subthreshold tactile-evoked response during quiet wakefulness and movement. (D) Population PSTH from L2/3 and L5 to tactile stimulation during quiet wakefulness and movement. (E) L2/3 and L5 neurons show a decrease in tactile-evoked subthreshold response amplitude as mice go from quiet (Q) to movement (M). Filled circles with error bars show mean \pm SEM. Lines show individual cells. (F) Population analysis of background-subtracted AP firing rates to tactile stimulation of the forepaw shows no difference between Q (left) and M (right) periods in both layers. AP rate measured as the difference between the 100 ms before and 100 ms after stimulus onset. (G) The subthreshold tactile-evoked reversal potential shows no significant difference between layers or behavioral states. (H) Absolute AP rate in the 100 ms after stimulus onset plotted as a function of the difference in V_m between AP threshold and the tactile stimulus-evoked response reversal potential. Filled circles show mean value of individual cells. (I) Subthreshold tactile-evoked latencies of L2/3 and L5 neurons are not significantly different. (J) Amplitude of L2/3 subthreshold tactile-evoked responses plotted against the amplitude of L5 subthreshold responses from the example pair in (A) shows highly correlated response amplitudes during Q (black) and M (orange) periods. (K) Population data of cross-correlation of mean subthreshold tactile-evoked responses (combining Q and M responses) between L2/3 and L5 neurons. Filled circles with error bars show mean \pm SEM, and open circles show individual cells. For all panels, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. See also Figures S6 and S7.

(Figure S6B), the amplitude of the V_m response was strongly dependent on the behavioral state (Figures 6A–6D). In Q periods, stimuli evoked a large-amplitude depolarizing response in both layers, whereas the amplitude of the response was reduced during M (Figures 6C and 6E; $L2/3_{Amp} Q = 10.80 \pm 0.65$ mV, $M = 4.84 \pm 1.24$ mV, $n = 10$ cells, $p = 0.037$; $L5_{Amp} Q = 6.19 \pm 0.69$ mV, $M = 2.41 \pm 0.60$ mV, $n = 20$ cells, $p = 0.001$; Q $L2/3_{Amp}$ versus $L5_{Amp}$ $p < 0.001$, and M $L2/3_{Amp}$ versus $L5_{Amp}$ $p = 0.005$). Despite the difference in subthreshold response amplitude, tactile stimulation evoked few extra APs over the background rate in both layers in both behavioral conditions (Fig-

ures 6D, 6F, and S6A; $L2/3_{AP} Q = 1.59 \pm 0.62$ Hz, $M = 1.95 \pm 1.36$ Hz, $n = 10$ cells, $p = 0.375$; $L5_{AP} Q = 2.44 \pm 1.40$ Hz, $M = 1.55 \pm 1.16$ Hz, $n = 20$ cells, $p = 0.370$; Q $L2/3_{AP}$ versus $L5_{AP}$ $p = 0.613$, M $L2/3_{AP}$ versus $L5_{AP}$ $p = 0.523$)

To examine why L5 neurons do not fire more APs in response to tactile stimulation despite having a more depolarized V_m , we measured the tactile responses as a function of the V_m just prior to stimulus onset. As neurons became more depolarized, the sensory response reduced in amplitude until eventually showing a hyperpolarizing response (Figure S7). The V_m level for the tactile-evoked reversal potential was similar during Q and M

periods and hyperpolarized relative to glutamate reversal potential (Figure 7G, $L2/3_{Rev} Q = -46.67 \pm 1.60$ mV, $M = -47.09 \pm 1.59$ mV, $n = 10$ cells, $p = 0.492$; $L5_{Rev} Q = -46.17 \pm 0.75$ mV, $M = -46.61 \pm 0.86$ mV, $n = 20$ cells, $p = 0.412$; $Q L2/3_{Rev}$ versus $L5_{Rev}$ $p = 0.644$, $M L2/3_{Rev}$ versus $L5_{Rev}$ $p = 0.775$). Notably, a minority of cells with higher mean firing rates showed a smaller difference between AP threshold and the sensory reversal potential (Figure 6H). These data provide an explanation for why the evoked rates are similar across layers and suggest that sensory-evoked GABA-ergic inhibition plays a critical role in defining the sensory reversal potential in both layers (Crochet et al., 2011; Moore and Nelson, 1998).

We next examined correlations between the timing and amplitude of sensory-evoked synaptic responses across layers during dual recordings. While the onset latencies of the synaptic and spiking responses to tactile stimulation in L5 neurons were more broadly distributed than in L2/3, there was no significant difference across layers (Figure 6I, $L2/3_{PSP_{Lat}} = 11.07 \pm 0.58$ ms, $n = 13$ versus $L5_{PSP_{Lat}} = 11.34 \pm 0.76$ ms, $n = 21$, $p = 0.972$; Figure S6B, $L2/3_{1stAP_{Lat}}$, $Q = 33.78 \pm 4.73$ ms, $n = 8$ cells, $M = 44.67 \pm 8.30$ ms, $n = 3$ cells; $L5_{1stAP_{Lat}}$, $Q = 39.52 \pm 4.72$ ms, $n = 19$ cells, $M = 31.10 \pm 5.90$ ms, $n = 9$ cells). Moreover, trial-by-trial analysis of the tactile-evoked postsynaptic potential (PSP) showed that subthreshold response amplitudes were highly correlated between L2/3 and L5 neurons (Figures 6J and 6K). Together, these data indicate that, in contrast to SDEs and spontaneous APs, tactile stimulation, like movement onset, triggers synchronized subthreshold input across layers.

L5 Neurons Signal Tactile-Triggered Movements

Following 75.2% of stimuli delivered in Q periods, the mouse's forepaw remained stationary (quiet-quiet [QQ] trials). However, 24.8% of stimuli in Q periods evoked short-latency (<100 ms) forepaw digit movements (Figure 7), termed quiet-move (QM) trials. We next compared sub- and supra-threshold responses in QQ with QM trials (Figures 7A–7C). Tactile stimulation evoked a short-latency, large-amplitude subthreshold response in QQ and QM trials in both layers (Figures 7A and 7B). Notably, the amplitude of the subthreshold response was larger in QM than QQ trials in both layers (Figure 7D; $L2/3$ QQ = 10.80 ± 0.65 mV versus QM = 11.83 ± 0.79 mV, $n = 10$ cells, $p = 0.037$; $L5$ QQ = 6.19 ± 0.69 mV versus QM = 8.16 ± 0.78 mV, $n = 20$ cells, $p = 0.002$; and QQ $L2/3$ versus $L5$ $p < 0.001$, QM $L2/3$ versus $L5$ $p = 0.005$). Moreover, we observed that the V_m prior to stimulus onset was significantly more hyperpolarized in QM trials than QQ trials in L5, but not in L2/3 neurons (Figures 7E; $L2/3$ QQ = -57.37 ± 1.36 mV, QM = -58.19 ± 1.58 mV, $n = 10$ cells, $p = 0.232$; $L5$ QQ = -52.53 ± 0.69 mV, QM = -54.24 ± 0.61 mV, $n = 20$ cells, $p = 0.009$; and QQ $L2/3$ versus $L5$ $p = 0.006$, QM $L2/3$ versus $L5$ $p = 0.033$). In QQ trials, the peak response was then followed by a brief hyperpolarization and subsequent depolarization peaking at ~ 350 ms (Figure 7B). This secondary depolarization was significantly larger in both layers during QM than QQ trials (Figure 7F) and evoked APs in L5, but not L2/3, neurons (Figures 7C and 7G; $L2/3$ QQ = -0.18 ± 0.13 Hz, QM = -0.12 ± 0.34 Hz, $n = 10$ cells, $p = 0.910$; $L5$ QQ = -0.01 ± 0.24 Hz, QM = 2.66 ± 0.81 Hz, $n = 20$ cells, $p = 0.003$; QQ $L2/3$ versus $L5$ $p = 0.613$, QM $L2/3$ versus $L5$ $p = 0.011$). Thus, movements

evoked by a tactile input are linked to a more hyperpolarized pre-stimulus V_m , a larger subthreshold early response, and a higher late increase in mean firing rate of L5 neurons.

DISCUSSION

The coordinated activity of six layers of primary sensory cortical neurons underlies sensory perception. Using whole-cell recordings in awake mice, we investigated synaptic mechanisms of translaminar synchronous activity. We show that laminar-specific differences in membrane properties drive distinct firing rates, that translaminar synchrony is dependent both on the behavioral state and the source of synaptic input (spontaneous, sensory, and movement evoked), and that L5 neurons signal spontaneous and tactile-triggered movement.

V_m Determinants of Spontaneous AP Firing Rates

Prior work has observed low firing rates of L2/3 excitatory neurons during synchronized states in resting and anesthetized animals (Barth and Poulet, 2012; Jouhannau et al., 2014) and no change (Crochet and Petersen, 2006; Polack et al., 2013; Poulet and Petersen, 2008; Zhou et al., 2014) or a reduction (Bennett et al., 2013; Sakata and Harris, 2012) of firing rates during activated cortical states. We observed that L5 neurons have a higher mean spontaneous rate of APs (2.86 ± 0.60 Hz) than L2/3 neurons (0.32 ± 0.09 Hz) in resting mice, supporting previous reports in anesthetized and awake animals (Brecht et al., 2003; Constantinople and Bruno, 2011; de Kock et al., 2007; de Kock and Sakmann, 2009; Manns et al., 2004; O'Connor et al., 2010; Sakata and Harris, 2009), and, in contrast to L2/3, a significant increase in mean firing rates during movement (Figure 2). Previous studies of deeper layers have observed both increases and decreases in firing rates of somatosensory (Curtis and Kleinfeld, 2009; de Kock and Sakmann, 2009) and motor (Carvell et al., 1996; Schieman et al., 2015; Zagha et al., 2015) cortical neurons during movement.

What synaptic and cellular mechanisms could explain the higher firing rates in L5 neurons? Our whole-cell recordings indicate that higher L5 rates were not the result of an intrinsic difference in AP threshold. Moreover, AP triggered averaging showed that the depolarizing synaptic inputs driving APs had similar amplitudes and dynamics in L2/3 and L5 neurons (Figure 3). Instead, higher mean firing rates in L5 neurons appeared to result from a larger input resistance and a more depolarized V_m , as observed in subsets of L5 pyramidal neurons in vitro (Lefort et al., 2009; Mason and Larkman, 1990), which likely combine to push excitatory input over AP threshold more often than in L2/3 neurons. Because most excitatory connections to pyramidal neurons are small in amplitude (Jouhannau et al., 2015; Lefort et al., 2009) and the rise time of the depolarization prior to an AP is negatively correlated with AP threshold (Figure S3) (Azouz and Gray, 2000; Poulet and Petersen, 2008), it is likely that coincident synaptic input is required to drive spontaneous AP firing in both layers.

Sensory-Evoked Firing Rates Are Determined by a Hyperpolarized Reversal Potential

In resting mice, the absolute tactile-evoked firing rate was higher in L5 (5.03 ± 1.74 Hz) than L2/3 (1.96 ± 0.75 Hz) neurons, but this

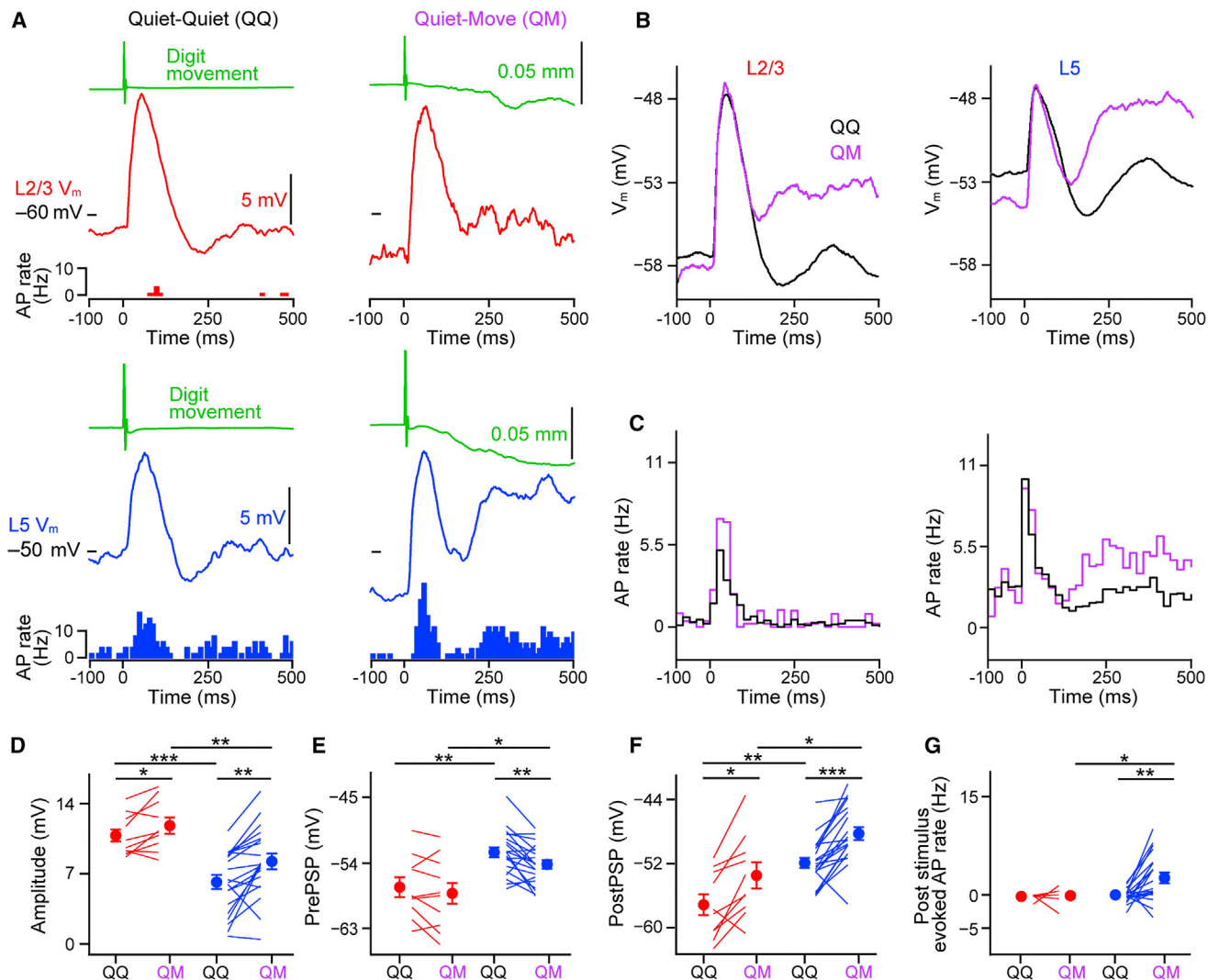


Figure 7. Tactile-Evoked Forepaw Movements Are Signaled by L5 Neurons

(A) Mean V_m tactile-evoked responses with corresponding digit movement (green) and PSTHs from an L2/3 (red, top) and an L5 (blue, bottom) neuron in resting, quiet mice that showed no behavioral response (left, quiet-quiet [QQ]) or a short-latency digit movement following the stimulus (right, quiet-movement [QM]).

(B) Grand average tactile-evoked responses from all L2/3 and L5 neurons during QQ and QM trials.

(C) Population PSTHs of firing rates in L2/3 and L5 neurons following tactile stimulation in QQ and QM trials. Note that only L5 neurons show an evoked spiking response in the later phase (300–400 ms after stimulus onset).

(D) The amplitude of the V_m response to tactile stimulation is significantly larger for QM trials than in QQ trials in both L2/3 and L5 neurons. Filled circles with error bars show mean \pm SEM. Lines show individual cells.

(E) The mean V_m in the 100 ms before stimulus onset is more hyperpolarized in QM trials than QQ trials in L5, but not in L2/3 neurons.

(F) The mean V_m in the late phase (300–400 ms following tactile stimulation) is significantly more depolarized after a QM trial than a QQ trial in both layers.

(G) Significant increase (background subtracted) in AP firing rates in QM trials as compared to QQ trials in the late phase (300–400 ms following tactile stimulation) in L5 neurons, but not in L2/3 neurons.

For all panels, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

was superimposed on different background firing rates. In fact, a brief tactile stimulus added similarly few additional APs to the background rate in both layers (evoked rate: L2/3 1.59 ± 0.62 Hz and L5 2.44 ± 1.40 Hz). Our observation of low evoked rates across layers is similar to prior studies of S1 in anesthetized animals (Barth and Poulet, 2012; Brecht et al., 2003; de Kock et al., 2007; Manns et al., 2004). Higher evoked rates have

been observed in “thick tufted” L5 pyramidal neurons (de Kock et al., 2007; Sakata and Harris, 2009). We did not differentiate between subtypes of cortical excitatory L5 neurons, but we did observe a minority (3/20) of L5 neurons with sensory-evoked rates >10 Hz (Figure 6F). Future work targeting whole-cell recordings to within-layer excitatory neuron subtypes in sensory cortex will be of great importance.

Cortical sensory responses are modulated by behavioral state in different sensory systems (Bennett et al., 2013; Crochet and Petersen, 2006; Niell and Stryker, 2010; Otazu et al., 2009; Schneider et al., 2014; Zhou et al., 2014). We report a reduction in subthreshold response amplitude in L2/3 neurons to brief tactile stimuli during movement. We go on to show that L5 neurons also have a reduced subthreshold response during movement (Figure 6). Despite this, the numbers of evoked APs remained the same in both layers during quiet and moving periods. In both layers, subthreshold responses were reduced in amplitude as the baseline V_m values became more depolarized and exhibited a reversal potential more hyperpolarized than AP threshold. Interestingly, in the cells with higher sensory-evoked firing rates, the sensory reversal potential was closer to threshold. Together, this suggests that strong, local GABAergic inhibition plays a significant role in clamping the subthreshold sensory response below AP threshold and regulating AP firing during behavior in both L2/3 (Crochet et al., 2011) and L5 neurons.

Correlated Neural Activity across Cortical Layers

Synchronous activity in cortical networks is thought to be fundamental to sensory processing and perception. Prior work has shown that spontaneous V_m activity in L2/3 neurons during resting states is more correlated than in activated states in behaving or attentive animals (Okun et al., 2010; Poulet and Petersen, 2008). We observed a similar pattern across L2/3 and L5. Neurons showed large-amplitude, highly correlated fluctuations of the V_m during resting periods and an active state with low SD, as well as a reduction in slow fluctuation amplitude and translaminar V_m synchrony (Figures 2 and 3). However, close inspection of the fine timing of subthreshold inputs evoked at different time points revealed differences in the timing of synaptic input across layers. Spontaneous APs were driven by cell-specific, depolarizing inputs (Figure 3). In contrast, movement- (Figure 5) and sensory-driven (Figure 6) synaptic input had a similar timing across layers. Interestingly, slow subthreshold fluctuations in resting mice had an earlier onset in L5, reminiscent of the earlier timing of “UPstate” onsets in deeper layers observed in anesthetized and sleeping animals (Chauvette et al., 2010; Sakata and Harris, 2009), cortical slices (Sanchez-Vives and McCormick, 2000), and extracellular recordings in awake rats (Sakata and Harris, 2009). Thus, the type of input (spontaneous, sensory, or movement evoked) determines the timing of synaptic input across layers.

What mechanisms could explain synaptic timing differences? One possibility is that they result from differences in the wiring supporting spontaneous, sensory, and motor events. In support of this suggestion, a recent anatomical study showed laminar differences in local and long-range cortico-cortical inputs to S1 excitatory neurons (DeNardo et al., 2015). Long-range inputs show laminar matching. L2/3 neurons receive proportionally more input from distant L2/3 neurons than L5 neurons, which receive a greater proportion from distant L5 neurons. Moreover, locally, L2/3 neurons receive a greater proportion of inhibitory inputs than L5 neurons. Upstates in anesthetized animals (Han et al., 2008; Luczak et al., 2007) and slow fluctuations in awake resting animals (Ferezou et al., 2007) are thought to propagate as waves of activity across cortex supported by long-range cor-

tico-cortical connections. Thus, a later onset in L2/3 neurons could result from a combination of lower firing rates, increased inhibitory input, and laminar-specific cortico-cortical wiring. In contrast, because sensory thalamic neurons are driven both by sensory stimulation and movement (Poulet et al., 2012), we suggest that punctate thalamic input drives the synchronized synaptic input following tactile stimuli or movement onset.

L5 Neurons Report Spontaneous and Tactile-Evoked Movement

Subthreshold sensory responses are correlated not only with the behavioral and cortical state but also with the behavioral outcome. Both L2/3 and L5 neurons showed a larger-amplitude subthreshold sensory response in trials that lead to short-latency forepaw movements (QM trials) compared to trials with no movement before or after the stimulus (QQ trials). In L5 neurons, a difference was also observed in the pre-stimulus V_m , with QM trials having a more hyperpolarized value than QQ trials. This suggests that the link between subthreshold response amplitude and behavioral output is due to the prestimulus cortical state rather than delivery of different amplitude stimuli. Our findings resemble recent V_m recordings in auditory cortex during an auditory discrimination go/no-go task, where neurons from L4 and L5 showed more hyperpolarized V_m value in hit trials than in false-positive trials in mice performing an auditory perception task (McGinley et al., 2015). Future experiments could address whether this is the result of a higher signal to noise ratio in a phase of low network activity resulting in an enhanced probability of signal transmission to downstream motor centers.

During evoked movement trials, neurons in both layers showed a prominent late depolarization (Figure 7), with L5 neurons also showing an increase in late spiking. Because forepaw movements necessarily occurred soon after tactile stimulation in QM trials, it was difficult to assess whether the late activity is causally related to the perception of the stimulus (Sachidhanandam et al., 2013), an intrinsic part of the transformation of sensory input to motor output and/or the start of the active cortical state associated with attention or arousal. Examining mice trained to delay sensory-triggered movements will help link late activity to perception and movement.

Functional Consequences

We observed higher background firing rates in L5 neurons but no difference in the numbers of additional, sensory-evoked spikes across layers, suggesting that L2/3 and L5 have distinct sensory coding strategies (Sakata and Harris, 2009). The fine timing differences of synaptic input may be important in the processing of dynamic sensory stimuli and for changes of synaptic strength under spike-time-dependent plasticity rules. Fast, laminar-specific manipulations of synchronized activity in trained mice are now required to define the causal role of translaminar synchrony in perception.

EXPERIMENTAL PROCEDURES

All experiments were approved by the Berlin animal ethics committee and carried out in accordance with European animal welfare law. Head-restrained 6- to 9-week-old C57bl6J mice were paw-tethered, and digit movements were

monitored by a force-feedback sensing and stimulating arm. The sensing arm ending was a 4.7-mm-diameter flat disk with one edge pressed up against the glabrous skin of digit 3. Then, 2-ms, 10-mN tactile stimuli were delivered at pseudo-randomized intervals. Blind whole-cell patch-clamp recordings were made from primary somatosensory forepaw cortex located by intrinsic optical imaging. Neurons were processed for biocytin staining using standard histological techniques. Data were analyzed with custom-written scripts in MATLAB and IgorPro. All data were statistically analyzed using non-parametric tests, paired data with a Wilcoxon signed rank test and unpaired data with a Wilcoxon rank sum test. Data are presented as the mean \pm SEM unless otherwise stated. See [Supplemental Experimental Procedures](#) for further details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.05.026>.

AUTHOR CONTRIBUTIONS

W.-J.Z. and J.F.A.P. designed the project. W.-J.Z. performed all experiments. J.K. and W.-J.Z. analyzed the data. J.F.A.P. wrote the manuscript, with comments from all coauthors.

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