Linear integration of spine Ca^{2+} signals in layer 4 cortical neurons in vivo

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Sensory information reaches the cortex through synchronously active thalamic axons, which provide a strong drive to layer 4 (L4) cortical neurons. Because of technical limitations, the dendritic signaling processes underlying the rapid and efficient activation of L4 neurons in vivo remained unknown. Here we introduce an approach that allows the direct monitoring of single dendritic spine Ca^{2+} signals in L4 spiny stellate cells of the vibrissal mouse cortex in vivo. Our results demonstrate that activation of N-methyl-D-aspartate (NMDA) receptors is required for sensory-evoked action potential (AP) generation in these neurons. By analyzing NMDA receptor-mediated Ca^{2+} signaling, we identify whisker stimulation-evoked large responses in a subset of dendritic spines. These sensory-stimulation-activated spines, representing predominantly thalamo-cortical input sites, were denser at proximal dendritic regions. The amplitude of sensory-evoked spine Ca^{2+} signals was independent of the activity of neighboring spines, without evidence for cooperativity. Furthermore, we found that spine Ca^{2+} signals evoked by back-propagating APs sum linearly with sensory-evoked synaptic Ca^{2+} signals. Thus, our results identify in sensory information-receiving L4 cortical neurons a linear mode of dendritic integration that underlies the rapid and reliable transfer of peripheral signals to the cortical network.

barrel cortex | layer 4 stellate cells | thalamo-cortical transmission | two-photon imaging

In the mammalian brain, sensory information is transmitted to the neocortex primarily through thalamo-cortical projections. Thus, in the vibrissae-related somatosensory cortex of rodents, tactile information arrives through axons of neurons that are located in the ventro-posterior-medial thalamic nucleus (VPM). It is well established that these axons provide direct input to layer 4 (L4), where they contact the dendrites of three types of excitatory neurons, namely the spiny stellate cells, the pyramids, and the star pyramids (1). In addition, there is recent evidence that axons originating in the VPM provide a direct input also to layer 5 (L5) neurons (2). A particularly interesting cytoarchitectonical feature of L4 of the rodent vibrissal cortex is the organization in distinct structures called “barrels” (3). The dendritic arborization of each spiny stellate cell is confined within the border of a barrel and is typically asymmetric, with cell bodies located at the barrel’s periphery and the dendrites facing the center (4, 5). Functionally, most spiny stellate cells and also the other L4 neurons in each barrel respond primarily to a single whisker, referred to as the principal whisker, whereas adjacent, or surround, whiskers provide a much weaker input (6). The fraction of thalamo-cortical inputs to each L4 neuron is remarkably small (about 10–20%) compared with that of intracortical inputs (7). Nevertheless, thalamocortical inputs are very efficient because of their synchronous activation by sensory stimuli (8, 9).

The dendritic processes that are associated with the synaptic activation of L4 neurons were investigated initially in vitro in acute brain slices. For example, a study involving the use of whole-cell recordings in combination with two-photon imaging characterized synaptically evoked N-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+} influx in dendritic spines (10). The analysis of

the spike timing dependence of the Ca^{2+} transient amplitudes showed a supralinearity when the synaptic stimulus preceded the back-propagating action potential (bAP) and a sublinearity when the order was reversed. Recently, a study combining whole-cell recordings in vivo and two-photon imaging in vitro investigated the cellular mechanisms of angular tuning in L4 neurons in the mouse barrel cortex (11). The results indicated that angular tuning of somatic voltage responses involves a complex nonlinear dendritic interplay of thalamo-cortical and cortico-cortical inputs. However, the dendritic Ca^{2+} signals in vivo that are associated with the rapid initial activation through whisker stimulation remained unclear.

Advances in two-photon Ca^{2+} imaging techniques have enabled direct observation of single-spine activity in neurons in the upper cortical layers in vivo (12–15). Thus, a study focusing on layer 2/3 cortical neurons in mouse barrel cortex in vivo (15) has shown that, in addition to specific inputs that are primarily activated by distinct single whiskers, neurons in the upper cortical layers also receive “shared” single-spine inputs that can be activated by multiple whiskers. These shared inputs are activated by other cortical “feeder” neurons that receive inputs from multiple whiskers. The precise identity of the feeder neurons remained unclear. Up to now, two-photon Ca^{2+} imaging of spines and dendrites was largely restricted in cortical layers 2/3 at depths of up to about 200–250 μm (12, 16, 17). The general feasibility of recordings in deeper cortical layers was recently indicated by a report that analyzed dendritic Ca^{2+} signaling underlying spontaneous activity in the mouse motor cortex (18). In the present study, we implemented an optimized method of two-photon imaging in vivo (*SI Text*), which allowed the recordings of

Significance

In the mammalian brain, sensory information reaches the cortex through thalamic axons, which provide a strong drive to layer 4 (L4). The thalamic inputs are characterized by their synchronous mode of activation and their high efficacy. Here, by using two-photon imaging in vivo, we analyze sensory-stimulation-evoked calcium transients on the level of individual excitatory synapses located on dendritic spines in L4 spiny stellate cells. We demonstrate that the rapid transfer of peripheral signals to the cortical network is associated with the activation of synapses that are widely dispersed throughout the entire dendritic tree. The dendritic integration of the synaptic responses is linear and does not involve cooperativity between individual synapses.

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Fig. 3. Trial-by-trial activation pattern of whisker-stimulation-evoked responses in single spines. (A) Top view of a reconstructed neuron. Green box indicates the location of dendrite shown in B. (B) A spiny dendritic segment from the dendrite shown in A. Red arrows mark spines that were included in the analysis. (C) The schematic representation of the dendritic segment shown in B. Red and blue circles indicate responsive and nonresponsive spines, respectively. Black rectangles mark the dendritic shafts analyzed in D. (D) Ca^{2+} transients calculated from the region of interests shown in C for consecutively selected stimulation trials under the condition of the cell body's subthreshold response. (E) Response probability distribution of responsive spines under subthreshold response conditions ($n = 112$ spines from nine neurons). (F) (Upper) The superposition of the trials with responses ($n = 12$) of spine 3 (s3) from D. Thick line shows the average. (Lower) The corresponding traces from the neighboring dendritic shaft (d3). Thick line shows the average. (G) The relative average amplitudes ($\pm$ SEM) of spine and shaft Ca^{2+} signals ($n = 37$ spines from nine neurons). (H) Histogram of the distance between nearest-neighbor active spines ($n = 61$ spine pairs from nine neurons).

proximal half of the dendritic field within a radius of about 70 μm around the cells' somata (Fig. 2 F–H). This result is entirely consistent with recently reported anatomical data (24).

Linear Dendritic Integration of Whisker Stimulation-Evoked Single-Spine Inputs. To study the dendritic processes occurring during the rapid initial activation of L4 neurons, we combined Ca^{2+} imaging with cell-attached recordings. We did not use the more invasive whole-cell configuration to avoid perturbation in membrane potential, mediated, for example, by leak currents. Fig. 3A shows the reconstruction of a representative L4 neuron and the dendritic segment used for Ca^{2+} imaging that was located at a depth of 415 μm below the pial surface (Fig. 3 B and C). Fig. 3D

illustrates the subthreshold Ca^{2+} responses that were obtained during repeated trials of sensory stimulation. The median probability of whisker stimulation-evoked spine Ca^{2+} signals was 0.24 ($n = 112$ spines from nine neurons; Fig. 3E). In this particular experiment, we observed robust and reliable Ca^{2+} transients in 5/13 spines (s1, s2, s3, s8, and s10). By contrast, the Ca^{2+} transients in the immediately adjacent dendritic shaft were small or absent (on average more than 80% smaller; $n = 37$ spines from nine neurons, paired t test, $P < 0.0001$) (Fig. 3 F and G). As in other types of neurons, the residual Ca^{2+} transients in the shafts may result from Ca^{2+} diffusion from the active spines (25).

In the next step of our analysis, we determined the distance between neighboring spines responding to PW stimulation. For the example illustrated in Fig. 3 B and C, we found that the nearest distance ranged from 1 μm (between s2 and s3) to 14 μm (between s8 and s10). Overall, the median distance between nearest-neighboring active spines was 3.2 μm (Fig. 3H, $n = 61$ spine pairs from nine neurons). The observation of this short distance between spines responding to sensory stimulation raised the question of whether the activity in a given spine has an impact on the amplitude of the Ca^{2+} transient of its coactive neighboring spines. This issue is important because a cooperativity may facilitate the generation of local dendritic spikes involving the nonlinear properties of NMDA receptors (26). To test this possibility, we compared the amplitude of Ca^{2+} transients that were recorded when a spine was activated alone with Ca^{2+} transients that were recorded when multiple neighboring spines on the same dendrite were active. In contrast to recent evidence indicating cooperativity in hippocampal neurons (27), Fig. 4 A–C ($n = 33$ spines from nine neurons) demonstrates that under both conditions the amplitudes of the Ca^{2+} transients were not different (paired t test, nonsignificant, $P = 0.86$), without indications for cooperativity.

We found that the amplitudes of synaptically evoked spine Ca^{2+} transients through sensory stimulation ("Syn," Fig. 4D) recorded under subthreshold conditions were higher than those Ca^{2+} transients produced by a spontaneous bAP in absence of sensory stimulation ("bAP," Fig. 4D and Fig. S2; paired t test, $n = 73$, $P < 0.001$). Remarkably, it turned out that the amplitudes of the Ca^{2+} transients recorded during suprathreshold sensory stimulation ("Syn + bAP") were similar to those obtained for the transients generated by the arithmetic sum of the subthreshold stimulation- and bAP-evoked transients ("Calculated," Fig. 4D and E; $n = 73$ spines from nine neurons). The linear summation is similar to what has been previously observed with *in vitro* recordings in spiny stellate cells, provided that the synaptic activation was temporally coincident (within 1–2 ms) with bAPs (10).

Next, we investigated whether the linearity is maintained in the highly active neurons thought to be involved in whisker-object-touching tasks in awake animals (28). We found that, under our recording conditions, fast passive whisker deflection activated L4 neurons in a way that resembled neuronal activation in object-touching tasks (28). Indeed, a similarly small fraction of neurons responded with intense spiking (4/32) and at a similarly high frequency (>10 Hz, Fig. 4 F–H). Fig. 4I shows the averaged images of a dendritic segment for "f0" conditions (baseline fluorescence) as well as the stimulation-evoked increase in fluorescence images, " Δf ," when either one, two, or three spikes were fired, respectively. In the dendritic shafts, the bAP-associated Ca^{2+} signals increased linearly with spike number (Fig. 4 J and K, Pearson's correlation, $r = 0.98$, $P < 0.001$) without evidence for regenerative dendritic processes. Moreover, in the active spines, whisker-evoked Ca^{2+} signals also increased linearly with spike number, except with an offset that corresponds to the major contribution of synaptic component (Fig. 4 J and K, Pearson's correlation, $r = 0.93$, $P < 0.001$). Finally, we took advantage of the fact that spine Ca^{2+} transients associated with a single bAP have much smaller amplitudes than sensory-stimulation-evoked ones (Fig. 4D and

Fig. 4. Dendritic integration of noncooperative single-spine sensory inputs. (A) Schematic representation of a spiny dendritic segment. Red arrows indicate the responsive spines. (B) Average Ca^{2+} transients from s1 marked in A, when the spine was active alone (Left, average of $n = 5$ trials) or simultaneously with other spines (Right, average of $n = 5$ trials). Dotted lines indicate start of stimulation. (C) Normalized average amplitudes ($\pm$ SEM) of spine Ca^{2+} transients when the spines were active alone ("single") or simultaneously with other spines ("multiple"); $n = 33$ spines from nine neurons; paired t test, $P = 0.86$. (D) Examples of average (average of $n = 5$ trials) spine Ca^{2+} transients under different conditions. bAP: single bAP in spontaneous conditions; Syn: evoked subthreshold synaptic event; Syn+bAP: evoked synaptic event coinciding with a single bAP; calculated: the arithmetic sum of bAP and Syn conditions. Arrow indicates the time of AP firing. Dotted lines indicate the start of stimulation. (E) The relative average amplitudes ($\pm$ SEM) of Ca^{2+} transients of the calculated versus the measured sum of synaptic and backpropagating AP events; $n = 73$ spines from nine neurons; paired t test, $P = 0.005$. (F) Examples of stimulus-evoked prolonged firing response of L4 neurons; dashed line indicates stimulation onset. The two cells were recorded separately. (G) The overall firing rate of each recorded L4 neuron with respect to the depth from the cortical surface. (H) Cumulative distribution curve of the overall firing rate for $n = 32$ neurons. (I) "f0" image of a dendritic segment at 415- μm depth (average image in 400 ms before stimulation onset), and the " Δf " images (average image in 400 ms after stimulation onset) corresponding to responses with no AP, 1 AP, 2 APs, or 3 APs. Red arrows indicated the active input spines identified under subthreshold conditions. (J) Ca^{2+} transients of an active spine (denoted by s1 in I) and its neighboring dendritic shaft corresponding to responses with no AP, 1 AP, 2 APs, or 3 APs. Ca^{2+} transients were averaged for all stimulation trials in which the given numbers of APs were evoked. Vertical dotted lines indicate whisker stimulation onset time. (K) $\Delta f/f$ values in dendritic shaft (Upper) as well as in active spines (Lower) versus the number of APs in response to whisker stimulation for 14 dendritic segments (in four neurons). Error bars: $\pm$ SD.

Fig. S2). Thus, spine activation by afferent inputs can be reliably resolved even in firing neurons. Fig. S3 A1 and A2 shows that the same set of spines is activated under both subthreshold and suprathreshold conditions. However, the probability of activation of such an input is more than twice as high under suprathreshold (Fig. S3B) as under subthreshold conditions (Fig. 3E). This changed probability leads to a significant increase in the fraction of active inputs per dendrite under suprathreshold compared with subthreshold conditions (Fig. S3C), suggesting that sensory-evoked spiking in L4 spiny stellate cells is controlled by the activity of the up-stream afferent neurons located mostly in the thalamus.

Discussion

In conclusion, our results reveal direct insights into the dendritic organization of synaptic inputs in cortical L4 neurons underlying short-latency sensory activation. Several lines of evidence suggest that the majority of these spines are activated by thalamo-cortical axons with a probably minor contribution from cortico-cortical inputs (1). Sensory stimulation activated a set of spines that remained stable throughout the recordings. The fraction of sensory-activated short-latency spines in vivo was $18 \pm 7\%$ ($n = 9$ neurons $\pm$ SD), which is similar to the fraction of thalamo-cortical inputs that was determined in previous anatomical studies (e.g., projection from thalamic ventral posterolateral nucleus occupies about 15% of spines of L4 spiny stellate cells) (2, 29). In line with the dendritic distribution of active spines obtained from our imaging recordings (Fig. 2), a recent electron and light microscopy study revealed an increased number of thalamo-cortical inputs in proximal dendritic segments compared with the distal sites (3, 24). Our analysis was restricted to short-latency EPSPs of 8–12 ms following whisker deflection (Fig. 1H). The latency of the corresponding spine Ca^{2+} transients was 10 ms (Fig. 2B). These latencies correspond to those known for thalamo-cortical inputs (6). Nevertheless, cortico-cortical inputs cannot be excluded as L4 spiny stellate cells are strongly interconnected and able to produce short-latency responses in other L4 neurons within the same barrel (30). The sparse activation of L4 neurons under our recording conditions (21/39 neurons did not spike in response to whisker stimulation at all; median response probability was $<10\%$) (Fig. S4), the single peaked distribution of the early sensory-evoked EPSPs, and the well-known powerful feed-forward inhibition narrowing the temporal window of activation to 1 ms (31) suggest that the contribution from cortico-cortical inputs is small.

It is remarkable that despite the synchronous activation of spines that are located at relative short distances from one another (median distance = 3.2 μm , Fig. 3H; SI Text), we saw little cooperativity. Moreover, despite the fact that multiple nearby spines can be simultaneously activated in a single stimulation trial (2–8 spines per dendritic segment with an overall average of 3.3 spines per 20- μm dendrite length), the dendritic shaft does not show large, regenerative supralinear Ca^{2+} signals (SI Text). This is in contrast to observations that were made in, for example, hippocampal pyramidal neurons, in which, at least in vitro, there was evidence for a strong cooperativity that is mediated by NMDA receptor activation (27). Furthermore, in hippocampal pyramidal neurons, but not in L4 spiny stellate cells (Fig. 4), there is clear evidence for supralinear summation between synaptically and bAP-evoked spine Ca^{2+} transients (32, 33). By contrast,

or 3 APs. Red arrows indicated the active input spines identified under subthreshold conditions. (J) Ca^{2+} transients of an active spine (denoted by s1 in I) and its neighboring dendritic shaft corresponding to responses with no AP, 1 AP, 2 APs, or 3 APs. Ca^{2+} transients were averaged for all stimulation trials in which the given numbers of APs were evoked. Vertical dotted lines indicate whisker stimulation onset time. (K) $\Delta f/f$ values in dendritic shaft (Upper) as well as in active spines (Lower) versus the number of APs in response to whisker stimulation for 14 dendritic segments (in four neurons). Error bars: $\pm$ SD.

in vitro recordings from L4 spiny stellate cells revealed supra-linearity only if the time interval between EPSPs and bAPs was more than 10 ms (10). For intervals of less than 5 ms, as found during the initial sensory-stimulation-dependent activation in vivo (Fig. 1 *H* and *I*), no supralinear summation is expected.

Together, our in vivo imaging experiments in L4 spines and dendrites provide direct evidence that the dendritic integration of the first wave of sensory information, preceding complex processes like angular tuning computation (11), is linear. Linear integration of synaptic potentials, generated in widely distributed spines throughout the entire dendritic field (34), may underlie the rapid thalamo-cortical transfer of sensory information in L4 neurons not only in the vibrissa but also in other cortical regions. The pronounced NMDA receptor-dependent component ensures large amplitude EPSPs, even under conditions in which a relatively small fraction of spines is activated, thereby contributing to the reliability of signal transfer in L4 neurons.

Materials and Methods

All experimental procedures were performed in strict accordance with institutional guidelines and were approved by the animal welfare committee of the state government of Bavaria, Germany.

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Animal Preparation. Experiments were performed in isoflurane-anesthetized C57BL/6 mice (P27–38, *n* = 10), and animal preparation procedures were largely similar to previous descriptions (15, 17). The position of the cortical barrel columns was determined with intrinsic signal optical imaging. Craniotomy (~2 × 1.5 mm) was made above the identified C2 barrel.

Electrophysiological Recordings. Whole-cell recordings of L4 neurons in the C2 barrel were performed by using the “shadow patch” method (35). Patch pipettes had resistances of 7–8 MΩ and contained a pipette solution consisting of 112 mM K-gluconate, 10 mM Hepes, 8 mM KCl, 10 mM Na₂-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na₂-GTP, and 25 μM Alexa Fluor 594 hydrazide sodium salt. Cell-attached recordings were performed in L4 neurons that were filled with the Ca²⁺-sensitive fluorescent indicator OGB-1 through electroporation.

Imaging. Ca²⁺ imaging of spines and dendrites of L4 neurons was performed by using the LOTOS variant of two-photon imaging (13) adapted for resonant galvo scanner-based imaging (15).

For a detailed description of materials and methods, see *SI Text*.

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