Pattern and not magnitude of neural activity determines dendritic spine stability in awake mice

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The stability of dendritic spines in the neocortex is profoundly influenced by sensory experience, which determines the magnitude and pattern of neural firing. By optically manipulating the temporal structure of neural activity in vivo using channelrhodopsin-2 and repeatedly imaging dendritic spines along these stimulated neurons over a period of weeks, we show that the specific pattern, rather than the total amount of activity, determines spine stability in awake mice.

Synaptic connections in mammalian cortex are strongly modified by experience, injury and learning1. Changes in neural connectivity in the cortex, culminating in the growth and retraction of synapses, contribute to changes in neural function. Because of the profound effects that experience has on synaptic connectivity, the role of neural activity in regulating synaptic stability has been intensively studied. This matter was recently addressed in primary sensory cortices in which in vivo vital imaging techniques were used to show that sensory deprivation is able to regulate the fates of dendritic spines2–4, the postsynaptic components of excitatory synapses. Whether changes in dendritic spine stability are influenced by changes in total neural activity or by changes in the pattern of this activity is not known.

To investigate the relative influence of total firing versus firing pattern on spine stability in the neocortex, we chronically stimulated layer 5 (L5) pyramidal neurons in awake, behaving mice for 8 h per day for a period of 7 d. We then repeatedly imaged dendritic spines from a subset of these cells using chronic in vivo two-photon imaging techniques. To achieve this, we bred mice expressing yellow fluorescent protein (YFP)-tagged channelrhodopsin-2 (ChR2) in neocortical L5 pyramidal neurons with mice expressing a cytosolic enhanced green fluorescent protein (EGFP) that labels a subset of L5 pyramidal neurons in their entirety. We confirmed that neurons expressing GFP also expressed ChR2 using laser-capture real-time qPCR (quantitative PCR) (Supplementary Fig. 1).

To select a chronic stimulation protocol, we wanted to use physiologically relevant frequencies that approximated the average firing patterns of somatosensory cortical L5 cells in behaving mice. To determine the resting firing rates of these cells, we performed cell-attached patch recordings from awake mice trained to maneuver on a freely moving Styrofoam ball (Supplementary Fig. 2a and Supplementary Video 1). We excluded traces obtained during any periods of running by the mouse in order to better approximate firing rates during periods of quiescence or normal grooming. Our recordings indicate that L5 excitatory neurons spontaneously fire at 2.4 ± 0.5 Hz under such conditions (Supplementary Fig. 2b). Recent work examining the firing rates of these cells in mice performing behavioral tasks reported average firing rates of about 10 Hz during periods of active whisking5. We therefore used 2-Hz and 10-Hz light trains to approximate firing rates seen during passive activity and active object localization, respectively. One group of mice received 2-Hz light trains applied for 1 s every 2 s (Supplementary Fig. 3 and Supplementary Video 2), whereas another group received 10-Hz light trains applied for 1 s every 10 s (Supplementary Fig. 3 and Supplementary Video 3), thus keeping the total number of light pulses between these groups the same (1 Hz average) while only varying the pattern in which we presented the stimuli. To control for the effects of overall activity, a third group of mice received no stimulation. Each light pulse was 10 ms in duration. We delivered these by implanting a cranial window over the somatosensory cortex and affixing a removable, head-mounted light-emitting diode (LED) directly over the cranial window (Supplementary Fig. 2). We tethered mice to a stimulator by means of a flexible headphone wire, and the mice were free to explore their cages and had unlimited access to food and water (Supplementary Videos 2 and 3). We delivered light bursts for 8 h each day for a 7-d period, totaling 201,600 light flashes. Notably, 10-Hz light trains did not increase surface brain temperature (data not shown). We also affixed control mice with the head-mounted LED and tethered them to the stimulator, but they received no light pulses.

To determine how these two patterns of light pulses affected network activity, we performed cell-attached patch recordings from L5 and L2/3 excitatory neurons and from L2/3 fast-spiking interneurons in awake, head-restrained mice. L2/3 excitatory neurons, which do not express ChR2 in these mice, did not respond to light stimulation (n = 8 cells) at either frequency (Fig. 1a). Notably, spiking of L2/3 fast-spiking interneurons, which also do not express ChR2 in these mice, precisely matched the frequency of light pulses at both 2 Hz and 10 Hz (n = 5 cells; Fig. 1a). This was likely to have been caused by the light-induced activity of L5 axons expressing ChR2, which innervate L2/3 fast-spiking interneurons6,7.

Recordings from L5 excitatory neurons showed unexpected, though serendipitously useful, results. First, we found that L5 neurons were sparsely responsive: out of 11 neurons recorded, two responded to light stimulation, indicating that spiking in one L5 neuron does not drive responses in neighboring neurons. Second, in the two responsive neurons there was clear trial-to-trial variability in light-evoked response probability. During 2-Hz light trains, light pulses evoked spiking approximately half the time—some light trains elicited two spikes, others one and others zero (Fig. 1a,b). During 10-Hz light trains, the probability of evoked response increased to 80%; the lowest

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Received 26 March; accepted 14 May; published online 17 June 2012; doi:10.1038/nn.3134
number of spikes evoked during 10-Hz trains was five and the highest ten (Fig. 1a,b). Notably, light trains at the two frequencies had different effects on network activity. During 10-Hz trains, spontaneous activity of L5 neurons dropped by a factor of 5.5 compared with periods of no stimulation (Fig. 1c, P < 0.001), an effect that is likely to be due to the recruitment of fast-spiking interneurons, as shown above, which would suppress extraneous spiking. In contrast, spontaneous activity during 2-Hz bursts was not significantly different than during periods of no stimulation (Fig. 1c, P = 0.75). When we counted the total number of spikes that occurred during 10-s epochs (inclusive of evoked and spontaneous activity) when cells were either stimulated with 2-Hz bursts every 2 s (n = 29 epochs) or 10-Hz bursts every 10 s (n = 29 epochs), we found no significant difference in the distributions between the two groups (Fig. 1d, P = 0.18). Thus, the total amount of activity was held constant between the two groups, whereas the evoked firing frequency differed by a factor of 8 in awake, behaving mice.

To examine how these different firing patterns affect spine stability, we used two-photon excitation to image the fine structure of the apical dendrites of EGFP-expressing L5 pyramidal neurons in the somatosensory cortex in vivo. We first imaged dendrites and dendritic spines on postnatal day 28 (P28), 1 d before the onset of optical stimulation. We imaged them again at P36, 1 d after the end of the weeklong stimulation trial (Fig. 2a), and a third time at P43, 1 week after the termination of optical stimulation. We imaged the same dendritic domains on all three time points and tracked the fates of dendritic spines (control: n = 3 mice, 6 cells, 19 dendritic branches, 737 spines; 2 Hz: n = 4 mice, 7 cells, 21 branches, 696 spines; 10 Hz: n = 6 mice, 8 cells, 24 branches, 749 spines). Repeated optical stimulation with 10-Hz trains for 7 d resulted in an approximately 25% reduction in the percentage of eliminated spines compared with unstimulated or 2-Hz-stimulated mice (Fig. 2b). In contrast, 2-Hz trains did not significantly affect rates of spine elimination compared with controls (Fig. 2b; unstimulated control: 24.5 ± 1.8% loss, 10-Hz stimulated mice: 18.0 ± 1.1%, 2-Hz stimulated mice: 22.5 ± 1.6%; analysis of variance (ANOVA): P < 0.01; post hoc test: 10 Hz versus control, P = 0.005; 2 Hz versus control, P = 0.66). Measures of the relative size of eliminated spines—a measure of synaptic strength—showed no differences across groups (Fig. 2c), indicating that our stimulation paradigm was not causing the loss of a specific subgroup of spines. In addition

![Figure 1](https://example.com/image1.png)

**Figure 1** Chr2-mediated and spontaneous activity in awake mice. (a) Laminar and cell-type responses to 2-Hz (left) and 10-Hz (right) blue light trains. Example traces from an L2/3 excitatory neuron (Ex) and a L2/3 fast-spiking inhibitory neuron (FS) are shown. Filled circles indicate a failure to respond to a blue light pulse. Open circles identify successes. Note that Ex neurons do not respond at either frequency, whereas FS neurons respond to every light pulse. The lower three traces are from the same L5 excitatory neuron and show the variation in the evoked firing rate in this layer at the different stimulation frequencies. Probability p of response is indicated for each trace. (b) Plot of evoked firing rate during 1-s trains of 2-Hz or 10-Hz light trains. (c) Plot of spontaneous activity during 1-s periods of normal wakefulness, and during 1-s trains of 2-Hz or 10-Hz light trains. (d) Scatter plots of total spiking measured over 10-s intervals in L5 neurons stimulated with the 2-Hz and 10-Hz light trains. 0, normal background activity; 2, 2-Hz stimulation; 10, 10-Hz stimulation. ***P < 0.001. Error bars are s.e.m.; n.s., not significant.

![Figure 2](https://example.com/image2.png)

**Figure 2** Spine stability is determined by the pattern of activity. (a) Left, low-magnification image of the dendritic tree of a single L5 pyramidal neuron. The region highlighted in the yellow box is shown to the right. Scale bar, 100 μm. Right, high-magnification images of a dendritic region showing dendritic spines. We acquired images at P28, P36 and P43. This example is from a mouse stimulated at 2 Hz from P28–P35 and unstimulated thereafter. Arrows show examples of gained (green) or lost (red) spines. Scale bar, 10 μm. (b) Plot of percentage of spines gained or eliminated in each experimental group over a period of 1 week. *P < 0.05; **P < 0.01. (c) Measure of relative spine size for eliminated spines in control (black line) and 10-Hz-stimulated mice (dashed line). (d) Plot of change in spine density over 1 week in control (solid line), 2-Hz (dashed line), and 10-Hz (dotted line) groups. (e) Plot of percentage of spines gained or eliminated in each experimental group in the week after the cessation of optical stimulation. Error bars are s.e.m.; n.s., not significant.
to reduced spine elimination, we also found an approximately 30% reduction in the percentage of new spines formed in the 10-Hz-stimulated mice compared with unstimulated controls (Fig. 2b). Stimulation with 2 Hz did not appreciably alter fractional spine gain (Fig. 2b; unstimulated control: 15.3 ± 1.5% gain, 10-Hz-stimulated mice: 10.5 ± 1.3%, 2-Hz stimulated mice: 14.2 ± 1.3%; ANOVA: P < 0.05; post hoc test: 10 Hz versus control, P = 0.03; 2 Hz versus control, P = 0.81).

Spine density in unstimulated mice decreased by 11% between P28 and P36 (0.45 ± 0.03 spines per µm at P28, 0.40 ± 0.02 spines per µm at P36; P < 0.01). We observed similar reductions in spine densities in both the 10-Hz-stimulated (~8% decrease; 0.51 ± 0.02 spines per µm at P28, 0.47 ± 0.02 spines per µm at P36; P < 0.01) and 2-Hz-stimulated groups (~9% decrease; 0.48 ± 0.02 spines per µm at P28, 0.44 ± 0.02 spines per µm at P36; P < 0.001). Although the reduction in spine density was greater in the control mice than in either stimulated group, this difference was not statistically significant (Fig. 2d; ANOVA: P = 0.54).

To examine whether the observed effects on synaptic stability were permanent or whether they depended on continual stimulation, we stopped delivering optical stimuli after the first week (P36) and then reimaged the same dendrites 1 week later at P43 (Fig. 2a). We found no significant differences in fractional spine gain or loss between any of the groups over this week (Fig. 2e; elimination, ANOVA P = 0.56; gain, ANOVA P = 0.34). Accordingly, we found no significant differences in overall spine density (unstimulated, 0.39 ± 0.02 spines per µm; 10 Hz, 0.45 ± 0.02 spines per µm; 2 Hz, 0.42 ± 0.02 spines per µm; ANOVA, P = 0.11).

Sensory experience alters the pattern of neural activity more than it alters the magnitude of this activity9,10, and learning enhances temporal correlations in neural ensembles in the cortex11. Our results show that the pattern of neural activity, and not the absolute number of spikes fired, determines the stability of dendritic spines and, by extension, of excitatory synapses in the cortex. Comparable experiments in the developing neuromuscular junction yielded similar conclusions12. Taken together, these results support a view in which firing pattern is the major conveyor of information in the nervous system, a view that has gained recent support by investigations of axon segregation in the developing colliculus13. Our findings leave open a number of explanations for the source of increased spine stability. One possibility, which we favor, is that the firing pattern of the parent neuron, rather than the pattern of global network activity, drives spine stability. Alternatively, spiking of L5 neurons may recruit local GABAergic circuitry in L1, the layer in which the dendrites we imaged reside. Local dendritic inhibition may influence spine stability more strongly than the intrinsic activity of the L5 neuron. Fast-spiking interneurons, which preferentially innervate excitatory cell somas, are strongly recruited by optical stimulation of L5 neurons (Fig. 1a). Less clear is whether somatostatin-expressing interneurons, which innervate the apical dendrites imaged here and would therefore be the likely source of dendritic inhibition14, are also recruited by L5 activity. In either scenario it is the pattern rather than the magnitude of activity that matters most. The importance of firing pattern on synaptic stability is perhaps most elegantly observed in songbirds, in which juveniles learn to sing by matching their own song to a memorized tutor song. During the period of exposure to a specific pattern of instructive experience (the tutor song), dendritic spines are rapidly stabilized15—an effect that was mirrored in our experiments by 10-Hz bursts.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We wish to thank S.T. Carmichael for his help with quantitative real-time PCR. A. Silva for generously providing access to his two-photon microscope for some of these experiments, S. Kuhlman for her help with spike waveform analysis, E. Ruthazer for critical comments on earlier versions of this manuscript and G. Feng (Massachusetts Institute of Technology) for generously supplying the Thy1-Chr2-YFP mice. This work was funded by grants from the US National Eye Institute (EY016852) and from the US National Institute for Mental Health (MH077972).

AUTHOR CONTRIBUTIONS

R.M.W. conducted the longitudinal dendritic spine imaging experiments, analyzed the data and wrote the manuscript. E.T. conducted the awake, behaving cell-attached patch recordings. J.T.T. designed and supervised the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nn.3134. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
ONLINE METHODS

Subjects. Mice expressing the Chr2 transgene driven by the thy1 promoter\textsuperscript{16} (line 18) were purchased from Jackson Laboratories (stock no. 007612) and also generously given to us by G. Feng. These mice were bred with Thy1-GFP mice\textsuperscript{17} (M line). Offspring from these crossings were used for our experiments. GFP expression was confirmed visually by examining ear clippings under a fluorescence microscope. Chr2 expression was confirmed by PCR using the following primers: 5′-TCT GAG TGG CAA AGG ACC TTA GG-3′ (forward); 5′-GAA GAT GAC CTT GAC GTA TCC G (3′-reverse). All experimental procedures were approved by the University of California Los Angeles Office of Animal Research Oversight and the Chancellor’s Animal Research Committee.

Laser capture microdissection (LCM). LCM was performed on brains from four double-transgenic mice (Thy1-Chr2-YFP × Thy1-GFP) and two wild-type mice. Briefly, brains were dissected, frozen on dry ice and sectioned at 10 µm. Sections taken from the somatosensory cortex were placed on uncoated glass slides, followed by dehydration in 80% (1 min), 90% (30 s), 2× 100% ethanol (30 s) followed by two rinses in xylene. Slides were air-dried and immediately submitted to LCM. EGFP-positive cells from L5 were laser captured (Veritas System, Molecular Devices) with the following parameters: spot size 11 µm, pulse duration 1.5 ns, power 55–60 mW. Fifteen to twenty neurons were captured from L5 of both cortical hemispheres in each section through the somatosensory cortex, for a total of 300 cells from each group. To avoid RNA degradation, cells were captured within 60 min and transferred to the RNA lysis buffer. Total RNA was extracted from isolated cells with the mini RNA isolation kit (Zymo Research) and captured within 60 min and transferred to the RNA lysis buffer. Total RNA was normalized to GAPDH. Values are expressed as the concentration ratio of gene expression normalized to GAPDH.

Recordings of spontaneous activity in awake mice on a running ball. We followed the protocol outlined in ref. 18. To habituate mice to head restraint, mice were handled on the first day of training by repeatedly picking them up. On the second day, a metal bar later used to restrain the animal was fixed to the mouse’s skull. On the third day, mice were placed on a floating Styrofoam ball, head fixed and allowed to run for two 10-min sessions with a 10-min break in between. This was repeated daily for 3 d. On the day of recording a small burr hole was made directly over somatosensory cortex. Loose cell-attached patch recordings were made from 18 L5 neurons. Laminar position was based on depth. Contact of the pipette tip with the cell was judged by the concurrence of a sudden increase in resistance and proximity of the pipette tip to the soma. Pressure was released to 0 mbar. Spike waveforms were used to distinguish between regular-spiking and fast-spiking cells\textsuperscript{19}. Electrode resistances were between 3 and 5 MΩ. Seals of 0.03–1 GΩ resistance were found to be sufficient to detect and isolate the spikes of single neurons. Five-minute recordings were made from each cell. Electrophysiological signal was acquired with a Multiclamp amplifier in current-clamp mode, a National Instruments digitizer and WinEDR software (J. Dempster, Strathclyde University). Data were analyzed in MATLAB and Clampfit. Signal was sampled at 10 kHz and filtered at 6 kHz. Pipette capacitance was compensated. Solutions were as follows, internal (in mM): 105 K-glucosone, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-mg, 0.3 GTP. Cortex buffer (in mM): 125 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 CaCl\textsubscript{2}, 2 MgSO\textsubscript{4}.

Recordings of light-evoked activity in awake mice on a running ball. Mice were habituated as above. Instead of a burr hole, a 2-mm craniotomy was made and a coverslip was placed over the craniotomy, leaving a 1-mm gap for recording access. Furthermore, pipettes were filled with cortex buffer. For each recorded cell, 5-min runs were recorded with 10-Hz light trains, and, subsequently, 5-min runs were recorded with 2-Hz light trains. Recordings were made blind. Fast-spiking interneurons were identified using waveform analysis as detailed in ref. 19. Spikes were classified as light evoked if they were time locked with either the onset of the LED stimulus (fast-spiking interneurons) or the offset (L5 excitatory neurons fired as the local field potential was decaying). All spikes that were not time locked to the stimulus during the 1-s light stimulation period were classified as spontaneous.

Cranial window surgery. Cranial windows were prepared as described in ref. 20. To optically stimulate through the cranial window, a blue high-powered LED (Nichia Corporation; NF5B036BT-E) was fixed to a removable head cap that could be secured directly over the cranial window. The pattern of light stimulation was controlled using a Master-8 stimulator (AMPI). Each pulse to the LED was 3.8-V and 10-ms in duration. Highly flexible earphone wire was used to connect the stimulator to the head-fixed diode on the mouse. Mice were stimulated for 8 h each day for 7 d in the light, were able to freely move around their cages, and had free access to food and water. At the end of each 8-h stimulation period, the LED head caps were removed, the mice were placed back in their home cage and were returned to the vivarium.

Cortical temperature. Temperature was measured during a 1-h exposure to 10-Hz blue light trains with the LED head cap secured directly over the cortex as during awake stimulation experiments. We used a HYPO-33-1-T-G-60-MP-M thermocouple from Omega (http://www.omega.com/) with a needle diameter of 0.2 mm. Temperature measures were made every 10 s. Brain temperature fluctuated over a 0.3-degree temperature range over the course of the hour.

Longitudinal in vivo 2-photon imaging. GFP-labeled neurons in the somatosensory cortex were imaged in vivo using a custom-built two-photon laser scanning microscope and ScanImage acquisition software written in MATLAB\textsuperscript{21}. GFP was excited at 910 nm. Emitted photons were filtered with a Semrock FF01-514/30 band-pass filter and a Semrock FF01-730/SP laser-blocking emission filter. Filtered photons were detected with a Hamamatsu R3896 photomultiplier tube. Analysis of spines was performed using ScanImage software according to the guidelines established in ref. 20. The percentage of gained or eliminated spines was calculated as the number of spines added or lost between two time points, respectively, divided by the total number of pre-existing spines. Relative spine size, a measure that includes both the number of pixels and the pixel intensity of each spine, was calculated as in ref. 22. Briefly, the background-subtracted summed pixel value of a spine was divided by the average background-subtracted pixel intensity of a nearby segment of dendritic shaft to correct for inhomogeneities in fluorescence excitation. In this measure, the resultant units are in pixels and reflect the normalized size of the spine. Spine turnover and density data is presented as means ± s.e.m.; P values across the three experimental groups were obtained by means of a one-way ANOVA. Tukey-Kramer multiple comparison post hoc tests were performed to compare means between individual groups.

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