An excitatory basis for divisive normalization in visual cortex

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Neurons in visual cortex are connected not only locally, but also through networks of distal connectivity. These distal networks recruit both excitatory and inhibitory synapses and result in divisive normalization. Normalization is traditionally thought to result from increases in synaptic inhibition. By combining optogenetic stimulation and intracellular recordings in mouse visual cortex, we found that, on the contrary, normalization is a result of a decrease in synaptic excitation.

Distant neurons in primary visual cortex (V1) influence each other through polysynaptic networks of distal intracortical connectivity. These networks involve horizontal connectivity in V1 and feedback from higher areas1. Their effect depends on the activity of the target region, increasing its firing when it is at rest, but suppressing its firing when it responds to visual input2. Arithmetically, these effects are well described by the normalization equation2,3: distal network activation causes mostly summation at low contrast and mostly division at high contrast.

Divisive normalization is widespread across neural systems and species4 and is often assumed to rely on the level of synaptic inhibition. This assumption has been shown to be correct in some circuits, such as the olfactory system of Drosophila5 and zebrafish6. In visual cortex, however, the evidence is mixed. For instance, there is disagreement as to whether the level of inhibition does7,8 or does not9 underlie the preference of V1 neurons for smaller stimuli, which are not10–12 underlie the preference of V1 neurons for smaller stimuli, which are not10–12, others rest on alternative explanations4,9,13–15.

To establish the synaptic basis of normalization mediated by distal network connectivity in mouse V1, we activated source neurons in the binocular zone (BZ) and we recorded from target neurons in the monocular zone (MZ), ~60 degrees away in the retinotopic map (~0.8 mm away in cortex). We first used in utero electroporation to express ChR2-venus in layer 2/3 pyramidal neurons of V1 in the left hemisphere. We then recorded from layer 2/3 neurons in the left MZ, under isoflurane anesthesia, while activating the left BZ through antidromic optogenetic stimulation2 of its contralateral callosal projections (Fig. 1a).

We first measured MZ firing rates with extracellular recordings and confirmed that the effects of distal network activation depended markedly on visual stimulation2 (Fig. 1b–d). If the MZ was not visually stimulated (0% contrast), BZ activation drove MZ spiking 50–150 ms afterwards (Fig. 1b). If, instead, the MZ was stimulated with higher contrast (Fig. 1c,d), the drive turned into suppression, particularly at later times (150–300 ms).

These effects are well summarized by the normalization equation, where MZ responses depend on local contrast c and on the time t after distal network activation:

\[ R(t) = \frac{c^q + p(t)}{c^q_0 + c^n + q(t)} \]

Here, \( c_0 \) and \( n \) determine responses to visual contrast, and \( p \) and \( q \) determine distal contributions2,4. These rose after distal network activation, with the additive term \( p \) preceding the divisive term \( q \) (Fig. 1e). This equation provided good fits to the population firing rate, explaining >98% of its variance (Fig. 1f). At low contrast \( (c < < c_0) \) and at short latencies (0–150 ms), distal network activation increased firing rate (because \( p > 0 \) and \( q \approx 0 \)). At high contrast \( (c >> c_0) \) and longer latencies (150–300 ms), it suppresses firing rate (because \( p < q \)).

To study the cellular basis of these effects, we recorded membrane potential \( (V_m) \) of MZ neurons using whole-cell somatic patch-clamp recordings (Fig. 2a–f). We studied two conditions of visual stimulation, 0% and 100% contrast, as these show, respectively, the largest additive and divisive effects.

In the absence of visual stimulation, distal network activation caused depolarization (Fig. 2a,b). In the 450 ms after optogenetic activation of BZ, MZ cells depolarized by 1.9 ± 0.4 mV (Wilcoxon signed rank test, \( P = 0.0006, n = 14 \)). Depolarization often involved two phases (Fig. 2b), starting with a transient that rose rapidly and reliably within 150 ms (Supplementary Fig. 1a–c). In contrast with the effects of local network activation, depolarization was rarely followed by hyperpolarization16 (Fig. 2a), and depended little on the prior17 level of \( V_m \) (Supplementary Fig. 1d–f).

In the presence of 100% contrast visual stimulation, however, distal network activation caused hyperpolarization (Fig. 2c,d). Between 150 and 300 ms after optogenetic activation of BZ, MZ cells hyperpolarized by 1.1 ± 0.4 mV (\( P = 0.005, n = 14 \)).

How can the same distal network activation have opposite effects on \( V_m \) depending on visual contrast? One possibility is that the effects depend simply on baseline \( V_m \), which is ~10 mV more depolarized at 100% contrast than at rest. However, when we depolarized MZ cells by ~10 mV by positive current injection at 0% contrast, BZ activation depolarized them further (1.4 ± 0.4 mV for 0–450-ms period, \( P = 0.004, n = 14 \); Fig. 2e and Supplementary Fig. 2a,c). Likewise, when we hyperpolarized them with negative current during visual stimulation, BZ activation hyperpolarized them further (−1.4 ± 0.4 mV for 150–300-ms period, \( P = 0.0004, n = 14 \); Fig. 2f and Supplementary Fig. 2b,d,f). Thus, the baseline \( V_m \) of MZ cells cannot explain why the effects of BZ activation depend on visual contrast.

Moreover, the hyperpolarization caused by distal network activation at high contrast seems unlikely to result from increases in the level

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of GABA_A inhibition^{10–12}. Indeed, the hyperpolarization resulting from a GABA_A conductance would decrease in the presence of negative current, which is the opposite of what we observed (Fig. 2d,f).

In addition, when we increased internal chloride concentration, which makes GABA_A inputs depolarizing, distal network activation at 100% contrast still hyperpolarized V_m (Supplementary Fig. 3). Might the hyperpolarization caused by distal network activation instead be a result of a decrease in excitation?^{9}

To measure synaptic inhibition and excitation, we performed voltage-clamp experiments using a cesium-based internal solution. At zero contrast, distal network activation recruited both inhibitory postsynaptic currents (IPSCs, conductances of 1.15 ± 0.28 nS, 0–450 ms, P = 0.002, n = 10; Fig. 2g,h) and excitatory postsynaptic currents (EPSCs, 0.28 ± 0.08 nS, P = 0.002, n = 10; Fig. 2k,l). Consistent with measurements of V_m, recruitment of EPSCs consisted of two phases and did not depend on the prior spontaneous activity level (Supplementary Fig. 4).

Visual stimulation completely changed these effects: instead of increasing inhibitory and excitatory currents, distal network activation decreased them both (Fig. 2i,j,m,n and Supplementary Fig. 5). At 100% contrast, distal network activation decreased both IPSCs (−0.91 ± 0.25 nS, 150–300 ms, P = 0.006, n = 10; Fig. 2l) and EPSCs (−0.34 ± 0.08 nS, P = 0.002, n = 10; Fig. 2m,n). Overall, excitation and inhibition remained roughly proportional: following distal network activation, they decreased and recovered together (Fig. 3a,b).

This result indicates that the hyperpolarization caused by distal network activation at high visual contrast is a result of a decrease in excitation, and not of an increase in inhibition. Indeed, in control experiments in which we recorded in both current clamp and voltage clamp in the same neurons, the hyperpolarization and the decrease in excitation had similar time courses (Supplementary Fig. 6). A simple calculation confirmed that the decrease in the level of excitation explains the suppressive effects seen in membrane potential (Fig. 3c). Inhibition contributes the opposite effect: by decreasing following distal network activation, it depolarizes the target cells. It thus counteracts,
Figure 3 Roles of excitation and inhibition in divisive suppression. (a) Average excitatory and inhibitory conductances following distal network activation at 100% contrast. Conductances are normalized by their values in the 100 ms before distal network activation. Shaded areas indicate mean ± s.e.m. (n = 10 neurons). (b) Relationship between inhibition and excitation. Thick gray and black lines are the trajectories in the 100 ms before distal network activation at 0% contrast (gray) and 100% contrast (black). When distal network activation arrives in the presence of 100% contrast, it decreases both inputs proportionally (thin black, 0–700 ms). The cross shows mean ± s.e.m. (n = 10). (c) Predictions of V<sub>n</sub> based on synaptic conductances, all measured at 100% contrast. The measured V<sub>n</sub> averaged over 14 neurons following distal network activation (dashed) is poorly predicted by inhibitory conductance alone (cyan), as inhibition would predict a depolarization. It is better predicted by synaptic excitation (pink) especially in combination with inhibition (gray). Shaded areas indicate mean ± s.e.m. (n = 10 neurons).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.K.S., B.H., M.H. and M.C. designed the study. T.K.S. and B.H. performed the experiments. T.K.S., B.H. and M.C. analyzed the data. T.K.S., B.H., M.H. and M.C. wrote the paper.

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METHODS

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In vivo electroporation. We expressed ChR2-Venus in layer 2/3 pyramidal neurons over visual cortex via in vivo electroporation onto C57Bl6 × CD1 mice at embryonic day 15.5. We used the offspring of a cross between CD1 females and C57BL/6 males (Charles River), taking advantage of the fertility and fostering capability of CD1 females. Crossed mice had brown or black coats as described previously8 and showed normal features in the pigmented epithelium of eye, confirmed with fundus images and sectioned images (data not shown). E15.5 timed-pregnant CD-1 mice were anesthetized with 2% isoflurane in oxygen. Up to 1 µl of DNA solution with Fast Green (Sigma) was pressure-injected into left lateral ventricle of embryos. The solution2,8,21 contained pCAGGS-ChR2-Venus (Addgene 15753, 1.5 µg µl−1) and pCAG-mCherry (0.5 µg µl−1). Electroporation was achieved with five square pulses (50 V, 50 ms, 1 Hz, CUY21, NepaGene). mCherry fluorescence was used to screen for positive animals at P0 under a fluorescent stereoscopic microscope (MVX10, Olympus). Images showing ChR2-Venus expression in a whole brain in vivo and in sectioned slices are available in our previous study (Fig. 1d,e in ref. 2).

Animals were maintained with a light-dark cycle of 12:12 h, and up to four mice were kept in one cage after weaning.

Initial surgery. At postnatal day 21–28 the electroporated mice were implanted with a cranial window over V1 contralateral to the electroporated hemisphere. Electroporated mice (n = 48, both sexes) were implanted with a head post and a cranial window (3 h). Anesthesia was obtained with 2% isoflurane and temperature was maintained at 37 °C using a feedback-controlled heating pad (TR-200, FST). Carprofen (10 mg per kg of body weight), atropine (0.3 mg per kg) and dexamethasone (2 mg per kg) were applied to prevent pain, secretions and brain edema. Eyes were covered with ointment (chloramphenicol, Martindale Pharmaceuticals). A head-plate was implanted to the skull with black dental cement (Ortho-Jet powder, Lang Dental, USA). A cranial window was embedded at the callosal stimulation side (Fig. 1a). Through the window, callosal axon terminals were clearly seen as a Venus-labeled band under a fluorescent stereoscopic microscope (MVX10, Olympus). Images showing ChR2-Venus expression in a whole brain in vivo and in sectioned slices are available in our previous study (Fig. 1d,e in ref. 2).

The space beneath the window glass was filled with aCSF-agarose (0.25%) rather than using layered glass. Because we needed to align the laser spot onto the callosal band, only mice showing a clear band (48 of 96) was used. No experimenter blinding was done. No experimenter blinding was done.

Pre-recording surgery. A pre-recording surgery was performed (2 h), 3–7 d after implantation of the cranial window. An implanted animal was anesthetized with isoflurane (2%), and was given Carprofen, atropine and dexamethasone as described above. The animal was held with a head-plate holder, and its temperature was maintained at 37 °C. The eye for visual stimulation was covered with a contact lens (Pmma 003, Veterinary Specialty Products, UK), and the other eye with a black piece of aluminum foil. The bone over left visual cortex was thinned to 2.5 mm lateral and 0.5 mm rostral to lambda (a square of 1 × 1 mm). Then a vessel-free area was identified for a craniotomy (<300 µm) and the border between two solutions. This junction potential compounds the voltage drop across the barrier of two solutions. In most experiments, we used an internal solution based on potassium gluconate (potassium gluconate 135 mM, KC1 6 mM, HEPES 10 mM, MgATP 4 mM, NaGTP 0.3 mM, EGTA 0.1 mM, phosphocreatine 4 mM, pH 7.3 adjusted with KOH). In some recordings (Supplementary Fig. 3), we used an internal solution based on potassium chloride (substituting K gluconate with KCl) to make GABA A input depolarizing. In some cases, Vm was linearly detrended for slow DC drift26.

For measurements of postsynaptic currents, series resistance (39.1 ± 3.4 MΩ) and membrane capacitance were corrected and compensated by 50–60%. The recording was aborted if series resistance was >50 MΩ. To isolate EPSCs or IPSCs, we selected a holding potential of −60 mV or +20 mV. With our cesium-based solution, these values approximate, respectively, the reversal potentials for GABA A input and for glutamatergic input. We first measured EPSCs, then IPSCs. In most experiments, we used a solution based on cesium together with internal blockers (cesium methansulfonate 140 mM, MgATP 4 mM, NaGTP 0.3 mM, EGTA 0.3 mM, phosphocreatine 4 mM, TEA-Cl 5 mM, QX314-Cl 4 mM, pH 7.3 adjusted with CsOH) to facilitate measurements of synaptic conductances. In a few experiments (Supplementary Fig. 6), we used a solution based on potassium gluconate to achieve both voltage- and current-clamp recordings in the same neurons.

Signals from the amplifier were low-pass filtered at 10 kHz (Multiclamp 200B) and then acquired at 30 kHz with a DAQ board (National Instruments).

Liquid junction potential. In our readings of membrane potential we did not correct for the liquid junction potential, the electrochemical potential generated at the border between two solutions. This junction potential compounds the voltage readings during experiments27. We estimated the junction potential (Clampex, Molecular Devices) to be 12 mV, 13 mV, and 1 mV for the solutions based on potassium gluconate, cesium methansulfonate, and potassium chloride.

Optogenetic stimulation. A blue laser light (SDL-473-2007, DreamLasers) was directed into an optical fiber (50-µm diameter), and diverging light from the fiber end was collimated and refocused to a 500-µm diameter spot using convex lenses. Laser power density at the focused spot was adjusted to 250 mW mm−2 (ref. 2) with a rotatable neutral density filter. The laser spot was aligned onto the callosal band. Laser illumination lasted 2 s and was controlled with a high-speed shutter (LS372, Uniblitz). A small fraction of laser output was monitored with a photodiode (PDA100A, ThorLabs). The illumination commenced 1 s after each condition started. The interstimulus interval for laser was >2.5 s.

Visual stimulation. Visual stimuli were presented on two LCD monitors (E2273HD5, Iiyama, mean luminance 50 cd m−2, refresh rate 60 Hz, gamma corrected), covering an angle of 100° horizontal and 65° vertical in the right visual hemifield contralateral to the recording site. We presented dynamic white
noise (bright and dark 6° sized-squares, 10.7 frames per s, 1.3 s) stimulating only the far monocular visual field (55–95° azimuth)². The random noise was different across blocks but the same within a block. There were at least 20 blocks for current-clamp measurements and 15 blocks for voltage-clamp measurements. Each block involved 4–16 conditions in a random order, the combination of contrast (0 or 100%), laser stimulation (absence or presence) and, if in the current-clamp experiments, current injection (3–4 different currents including zero). Interstimulus interval for visual stimulation was >1 s.

Data analysis. Data were analyzed in MATLAB (MathWorks) and were shown as mean ± s.e.m., unless otherwise stated. For statistical pairwise tests, the two-sided Wilcoxon signed-rank test was used, unless otherwise stated. The alpha level (0.05) was appropriate for the sample size.

Analysis of current-clamp data. To analyze subthreshold membrane potential (V_m), action potentials were detected as an upstroke in the 1st derivative of V_m and were replaced with an interpolated straight line for 1 ms before and 9 ms after the upstroke. We then smoothed the V_m signal with a 10 ms Gaussian window (s.d. 2 ms). Mean, s.d. and s.e.m. were calculated among trials for each condition. To evaluate the effect of distal network activation on V_m, we calculated the difference in V_m with and without the activation, and took the mean and s.e.m. across trials (Fig. 2a–c). This helped remove variability among trials inevitably introduced by dynamic white noise, which was different across trials.

Analysis of synaptic currents. We smoothed the current signal as described and took the mean and s.e.m. across trials (for ten neurons based on their conductance measurements, and then averaged the values based on current-clamp experiments, current injection (3–4 different currents including zero). We then derived G_i and G_e underlying the EPSC and the IPSC, we first corrected the potential drop at the uncompensated series resistance²⁸

\[ V(t) = V_{\text{hold}} - I(t) \times R_{\text{series}} \]

where \( V(t) \) is the holding membrane potential after correction, \( V_{\text{hold}} \) is either 20 mV or −60 mV, \( I(t) \) is measured current, and \( R_{\text{series}} \) is the portion of series resistance that was not compensated during experiments (40–50%). We then derived \( G_i \) and \( G_e \) from the following equation²⁸

\[ I(t) = G_{\text{rest}} \times (V(t) - E_{\text{rest}}) + G_i(t) \times (V(t) - E_i) + G_e(t) \times (V(t) - E_e) \]

Here, \( G_{\text{rest}} \) and \( E_{\text{rest}} \) are the resting leak conductance and membrane potential, and \( E_i \) (13 mV) and \( E_e \) (−63.2 mV, Supplementary Fig. 9f) are the reversal potentials.

For robustness, we chose the values \( V_{\text{hold}} \) so that after correction for junction potentials they would be close to the reversal potentials for inhibitory and excitatory inputs. In fact, in the case of the cesium-methansulfonate solution, the junction potential of 13 mV means that commanding \( V_{\text{hold}} = −60 \) mV resulted in a corrected \( V_{\text{hold}} = −73 \) mV, and commanding \( V_{\text{hold}} = 20 \) mV means that the corrected \( V_{\text{hold}} = 7 \) mV. Nonetheless, using the equation above does not require that the holding values correspond precisely to the actual reversal potentials.

\( V_m \) predictions based on conductance measurements. To predict \( V_m \) based on derived synaptic conductance (Fig. 3c and Supplementary Fig. 7b), we used the equation above setting \( I = 0 \) and \( G_i \) and \( G_e \) to the measured conductances (Fig. 2n). \( E_i \) and \( G_i \) were set to −61.9 mV and 4.3, mS respectively. These values were based on current clamp experiments (Fig. 2b,c), not on voltage clamp experiments where many intrinsic conductances were blocked. We set \( E_i \) and \( E_e \) at 12 mV and −71.4 mV taking into consideration the liquid junction potential for a potassium gluconate solution (Supplementary Fig. 9c). \( V_m \) traces were predicted for ten neurons based on their conductance measurements, and then averaged (Fig. 3c and Supplementary Fig. 7b). \( V_m \) prediction was also made based on excitatory conductance alone (\( G_i = 0 \), pink) and on inhibitory alone (\( G_e = 0 \), cyan).

To test for the robustness of our conclusions, we repeated analysis above with different values for \( E_i \) resulting from the potassium gluconate solution (−65 or −75 mV), and for \( E_e \) in the equation above (−60 or −70 mV). In all of those cases, the conclusions remained the same (data not shown): the prediction based only on inhibition goes in the wrong direction, while the prediction based only on excitation is too hyperpolarizing. The prediction based on the combination of excitation and inhibition best captures the data.

Confirming the estimates of reversal potential. To compare the results obtained when measuring conductance and those obtained when measuring membrane potential (Fig. 3c), we need to estimate reversal potentials for the two experimental conditions: voltage clamp (a cesium methansulfonate solution) and current clamp (a potassium gluconate solution). We established these reversal potentials by estimating the liquid junction potential and the chloride ion concentration outside and inside the cell. There might be errors in these estimates. For instance, the chloride concentration outside the cell is affected both by natural CSF and by our ACSF, and we don’t know which one predominates.

To test our estimates quantitatively, we measured the reversal potentials for directly activated GABA_A inputs in voltage clamp and in current clamp (Supplementary Fig. 9). We recorded from Pvalb-IRES-Cre;Ai32 mice, which express ChR2 in PV cells, and evoked GABA_A input optogenetically²⁹,³⁰, while we recorded intracellularly from excitatory neurons. We found that GABA_A input reverses at −63.2 ± 1.3 mV in voltage clamp and −71.4 ± 1.5 mV in current clamp (Supplementary Fig. 9f), consistent with predicted values for chloride concentrations in our two internal solutions with external ACSF (−62 and −74 mV).

Scaling of different measurements in the same neurons. To facilitate comparison between different measurements in the same neurons (for example, the EPSC versus the IPSC in Fig. 3b and Supplementary Fig. 7a), \( V_m \) versus EPSCs (Supplementary Fig. 6i), we also subtracted the values measured at rest (0% contrast) so that the normalized average values before distal network activation were 0 in the absence of visual stimulation, and 100% in the presence of visual stimulation.

Trial variability in the absence of visual stimulation. To characterize transient and slow \( V_m \) depolarizations in the absence of visual stimulation, we measured the height of the \( V_m \) response from the lower boundary in \( V_m \) (V_bottom, see below), and evaluated its reliability as mean divided by s.d. (the reciprocal of the coefficient of variation across trials, 1/C.V., Supplementary Fig. 1b). If a transient event within 150 ms had mean/s.d. > 2, the response was counted as a significant depolarization. Similarly, if the time-averaged \( V_m \) response between 300 and 450 ms had mean/s.d. > 2 across trials, the response was regarded as a slow \( V_m \) depolarization. The same evaluation was adopted for EPSCs and IPSCs (Supplementary Fig. 4).

To evaluate the effects of prior \( V_m \) onto \( V_m \) depolarizations in the absence of visual stimulation (Supplementary Fig. 1d), we separated trials into two groups: a more quiescent (hyperpolarized) group and a less quiescent (depolarized) group. We based this separation on averages of \( V_m \) taken 0–100 ms before the distal network activation. If this average was below a criterion voltage, we classified the trial as more quiescent, and otherwise as less quiescent. To determine the criterion voltage, we proceeded as follows. First, we determined the upper and the lower boundaries in spontaneous \( V_m \) as average of the most depolarized \( V_m \) or the most hyperpolarized \( V_m \) among trials (\( V_{\text{top}} \) and \( V_{\text{bottom}} \)). Then we set the criterion voltage at 20% distance from \( V_{\text{bottom}} \) to \( V_{\text{top}} \). To compare the size of early and slow depolarizations between the two groups (Supplementary Fig. 1f), we measured the \( V_m \) response size relative to \( V_{\text{bottom}} \). We adopted the same grouping for EPSCs and IPSCs (Supplementary Fig. 4).

Fits of the normalization model. We analyzed the multiunit activity in the superficial layers in the far MZ which was collected in our previous study³ (n = 14). We first fit the firing rate of the control condition (no activation) with a hyperbolic ratio function²⁹

\[ r(c) = r_0 + \frac{r_{\text{max}}}{c_0 + c^\nu} \]

where \( r_0 \) is the baseline firing rate, \( r_{\text{max}} \) is the maximum rate, \( c_0 \) is the saturation contrast, and \( \nu \) is a constant determining the slope of the function. The values of \( r_0 \) and \( r_{\text{max}} \) were then used to normalize each unit’s response to
values $R$ ranging from 0 to 1. We then fitted the control responses together with the responses measured with the activation using the full normalization equation\(^4\) (given in main text). We imposed the same $c_{50}$ and $n$ across conditions, and obtained parameters $p$ and $q$. Model parameters were obtained by weighted least-squares fit. To investigate temporal dynamics in additive and divisive contributions (Fig. 1e), we used a sliding window of 100 ms to measure response and obtain parameters $p$ and $q$.

To assess fit quality\(^2\) we measured the percentage of variance in the responses $R$ explained by the model predictions $m$

$$\nu = 1 - \frac{\sum_{i=1}^{n} (R_i - m_i)^2}{\sum_{i=1}^{n} (R_i - \bar{R})^2}$$

where the indices $i$ indicate one of visual contrast and $\bar{R}$ is the mean of the responses.

A Supplementary Methods Checklist is available.

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