Recruitment of Local Inhibitory Networks by Horizontal Connections in Layer 2/3 of Ferret Visual Cortex

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INTRODUCTION

In primary visual cortex, neurons with similar receptive field properties are grouped into functional modules, and within each module, inhibitory and pyramidal cells are densely interconnected in a local neuronal circuit. In addition to local connections, the axons of pyramidal cells in layer 2/3 extend up to several millimeters and form clusters interconnecting iso-orientation domains (Bosking et al. 1997; Gilbert 1992; Gilbert and Wiesel 1989), providing the potential for both local and long-range neuronal interactions, but how these interactions may affect the spatial and temporal distribution of neuronal activity in layer 2/3 is uncertain.

Long-range horizontal connections contact both inhibitory and pyramidal cells (Hirsch and Gilbert 1991; McGuire et al. 1991; Weliky et al. 1995), evoking a balance of excitation and inhibition that is poorly understood. Thus it is unknown whether the excitation delivered by multiple horizontal connections increases pyramidal cell excitability or recruits local inhibitory circuits and suppresses pyramidal cells. Following electrical stimulation in brain slices, horizontal connections generate subthreshold excitatory postsynaptic potentials in pyramidal cells (Yoshimura et al. 2000), but the convergence of a sufficient number of horizontal inputs may enable pyramidal cells to generate action potentials. Excitatory postsynaptic potentials (EPSPs) delivered by horizontal connections may be augmented by temporal summation (Thomson and Deuchars 1994), persistent sodium currents (Stafstrom et al. 1985), and N-methyl-d-aspartate (NMDA) receptors (Artola and Singer 1990; Sutor and Hablitz 1989), supporting the idea that convergent horizontal pathways may increase pyramidal cell excitability. However, an alternative possibility is that convergent horizontal connections may reduce pyramidal cell activation by recruiting local inhibition. While horizontal connections evoke purely excitatory events after weak electrical stimulation, they generate compound EPSP/inhibitory postsynaptic potentials (IPSPs) following strong stimulation. Thus convergent horizontal connections, which generate purely excitatory events when stimulated individually, may evoke inhibition when stimulated in combination. This idea is supported by evidence that excitation and inhibition are inseparable (Douglas and Martin 1991; Somers et al. 1995, 1998).

To determine how local neuronal populations and pyramidal cells in layer 2/3 integrate convergent horizontal connections, we stimulated two sets of horizontal connections in tangential slices of layer 2/3 of ferret visual cortex, and assayed the spatiotemporal distribution of neuronal activity with intracellular recording and voltage-sensitive dye imaging. We found that responses generated by horizontal connections were integrated nonlinearly due to the emergence of strong inhibitory synaptic potentials.

METHODS

Slice preparation and optical recording

Adult ferrets (P40—P60, Marshall Farms, North Rose, NY) were killed under pentobarbital sodium anesthesia (100 mg/kg, ip), and tangential brain slices of layer 2/3 were cut to 350 m using a vibratome. Slices were incubated in an interface chamber with voltage-sensitive dye (0.1 mg/ml, RH461, Grinvald et al. 1987, Molecular Probes, Eugene, OR) in normal artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 1.3 MgSO4, 2.8 CaCl2, 4 KCl, 1 KH2PO4, 10 dextrose, and 26 NaHCO3) for approximately 90 min at 28°C, and subsequently transferred to a submersion chamber perfused with warmed, oxygenated ACSF at 30°C on a Zeiss Axiovert 100 TV microscope for recording. Electrical stimuli were delivered with concentric bipolar electrodes (FHC, Bowdoinham, ME) driven with isolated current sources (A360, WPI, Sarasota, FL) and triggered by a pulse generator (Master-8, AMPI, Jerusalem, Israel). A 250-W lamp driven by a stable power supply (ATM75–15M, Kepco, Flushing, NY).

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NY) delivered light through a 546 ± 20-nm filter (Chroma Technology, Brattleboro, VT), the epifluorescence port, and a 10× objective lens (Zeiss Fluar, 0.5NA) to the slice. Emitted light was filtered at 590 nm longpass, and directed to the bottom port to a photodiode array with 256 elements collecting images at 2 kHz (16 × 16 square array, CS897, Hamamatsu, Bridgewater, NJ). In each trial, slices were exposed for 325 ms with intertrial intervals of 10 s, using an electro-mechanical shutter (VS35, Vincent Associates, Rochester, NY). Images were averaged over 25–50 trials to improve the signal/noise ratio, and then processed digitally by tripling the number of pixels in each dimension (yielding images 48 × 48 pixels), temporally filtered at 500 Hz and spatially filtered with a Gaussian kernel (5 × 5). For displaying the images as three-dimensional movies in pseudocolor, additional processing was performed using IP-Lab (Scanalytics, Fairfax, NJ), and IDL (Research Systems, Boulder, CO).

**Electrophysiology**

Sharp electrodes were fabricated from borosilicate glass tubing (WPI, Sarasota, FL) to have resistances of 90–130 MΩ and filled with 3M KAc. Intracellular recordings were obtained in current-clamp mode (AxoClamp-2B, Axon Instruments), low-pass filtered at 3 kHz (Model 410, Brownlee Precision, San Jose, CA), and digitized at 20 kHz (AT-MIO-16E, National Instruments, Austin, TX). Optical and electrical recordings were simultaneously acquired on PC computer running custom software written in C++. Following a recording, cells were filled with neurobiotin (Vector Labs, Burlingame, CA) by current injection (+0.5 nA, 500-ms pulses at 1 Hz), and the slices were fixed for subsequent processing. Neurons included in the analysis had pyramidal cell morphologies and resting potentials more negative than −65 mV with overshooting action potentials. Using a programmable micromanipulator (SM1, Luigs and Neumann, Ratingen, Germany), intracellular recordings were targeted to selected neuronal populations during image acquisition, enabling simultaneous acquisition of optical and electrical recordings from the same cortical locus. Following an experiment, the exact locations of the stimulating and recording electrodes were determined by acquiring a brightfield image with a CCD camera (C2400, Hamamatsu) and aligning it with images acquired from the photodiode array.

**RESULTS**

In previous experiments, we imaged tangential slices of visual cortex stained with voltage-sensitive dyes to observe
patterns of optical activity evoked by horizontal connections, delivering electrical stimuli at single cortical sites. We found that these optical patterns included two features: a diffuse zone of activation surrounding the stimulation site, and numerous ovoid domains of activity, or optical clusters, corresponding to axonal clusters of horizontal connections (see companion paper). Here, we use a similar approach to study interactions between patterns of activity driven by horizontal connections, delivering electrical stimuli at two cortical sites. In the first part, we investigate how integration of horizontal inputs is represented in the spatiotemporal distribution of population-based neuronal activity, imaging with voltage-sensitive dyes. In the second part, we use intracellular recording to investigate how horizontal inputs are integrated by individual pyramidal cells.

Spatial distribution of emergent inhibition

To investigate the integration of horizontal inputs by neuronal populations, we positioned two stimulating electrodes up to several hundred microns apart in tangential slices of ferret visual cortex layer 2/3, enabling the stimulation of two predominantly distinct sets of horizontal connections. As the resulting patterns extended over 1.2 mm in radius, there was substantial overlap in the two patterns of activity, enabling interaction between the neuronal populations driven by each set of connections. The experiments were conducted by first stimulating each electrode separately to determine the pattern of responses evoked by each individual set of connections (Fig. 1, A and B), and then stimulating both electrodes simultaneously to observe interactions between the two patterns. As the simplest outcome would have been linear summation, we added the two patterns evoked by separate stimulations to obtain an “expected response” (Fig. 1C) for comparison with the “actual response” of stimulating both electrodes together (Fig. 1D).

The actual spatiotemporal distribution of responses was substantially different from expected, indicating that the interaction could not be described by simple addition. To quantify these differences, we subtracted the expected from actual response and obtained a “difference image” (Fig. 1E) that facilitated the identification of regions that deviated most strongly from the expected. These difference images revealed large ovoid regions of negative-going responses approximately 500 μm in diameter (full-width at half-maximum [FWHM]), indicating that the interaction between the two patterns of connections yielded domains of strong suppression.

As described in the companion paper, optical responses evoked by electrical stimulation of horizontal connections consist of a diffuse zone and optical clusters. We considered three different types of interactions, including those between 1) two optical clusters, 2) an optical cluster and a diffuse zone, and 3) two diffuse zones. We found that suppression domains with similar characteristics were evoked by all types of interaction, suggesting that they depended primarily on the overlap in the activity patterns, rather than the specificity of horizontal connections formed by optical clusters or diffuse zones.

In general, suppression domains were centered on optical clusters when two discrete sets of horizontal connections evoked patterns having overlapping optical clusters (Fig. 1). Next, there were cases in which an optical cluster evoked by one horizontal pathway overlapped the diffuse zone generated by a different pathway. In this situation, suppression domains were centered on optical clusters residing within the diffuse.

FIG. 1. Spatiotemporal patterns of neuronal activity driven by 2 discrete sets of horizontal connections reveal domains of suppression. Left column: in these 3-dimensional (3D) plots of optical activity, the x and y axes represent cortical distance parallel to the cortical surface (1.76 mm/side); and the z axis indicates the intensity of the optical response. Intensity is also represented in pseudocolor, using the rainbow scale for A–D and the red-white-blue scale for E. The arrow marks the location of the intracellular recording electrode, while the asterisks mark the locations of the stimulating electrodes. Center column: contour plot showing the topography of the 3D surface. Right column: at the cortical site marked by the arrow, the time courses are shown for both the electrical response of an intracellularly recorded pyramidal cell (ΔV) and the optical response from a single photodiode (ΔF), corresponding to the same site. For clarity, the traces have been both labeled and color-coded. Black and red traces are responses evoked by separate stimulations of S1 and S2, respectively. Blue and green traces are the expected and actual responses, respectively. The difference traces are shown in gray, indicating emergent inhibition. Note that the optical and electrical responses in C and D have been superimposed to facilitate the comparison of the actual and expected responses. A and B: patterns of optical activity evoked by stimulating electrode S1 (A) or S2 (B). Left column: the stimulation sites (asterisks) in A and B are several hundred microns apart, ensuring that each site activated a distinct set of horizontal connections. Ovoid domains of activity (“optical clusters”) revealed the locations of axonal clusters formed by the horizontal network. Note that these stimulation sites generated optical clusters in the same locations (e.g., arrow), indicating that the horizontal connections projecting from each stimulation site formed axonal clusters in the same neuronal populations. Right column: S1 generated a series of inhibitory postsynaptic potentials (IPSPs; ΔV, S1; black trace) while S2 generated a series of excitatory postsynaptic potentials (EPSPs; ΔV, S2; red trace), shown with the corresponding optical responses (ΔF, S1 and ΔF, S2). For this figure only, the stimulation protocol was a train of 4 pulses delivered at 100 Hz (shown top right corner). Single pulse stimuli and 4 pulse stimuli both generated emergent inhibition, but the magnitude of the emergent inhibition was commonly augmented by multiple pulses, as in this example. C: the expected response for simultaneous stimulation. Left column: this image is the arithmetic sum of the images in A and B. Right column: the blue traces show the sum of the optical responses (ΔF, expected = ΔF, S1 + ΔF, S2) and the electrical responses (ΔV, expected = ΔV, S1 + ΔV, S2). D: the actual response of simultaneous stimulation. Left column: the amplitude of the optical cluster marked by the arrow was smaller in D than C. Right column: in the optical traces, the actual response (ΔF, actual; green trace) was smaller than expected (ΔF, expected; blue trace), and in the intracellular records, the actual response (ΔV, actual) was more hyperpolarizing than expected (ΔV, expected). E: neuronal populations activated by both stimulation sites reveal domains of suppression. Left column: this image was calculated by subtracting image C from D. Note the large ovoid region of suppression marked by the arrow, centered on the same location as the optical cluster. A 2nd region of suppression was located in the upper right corner of image where another optical cluster was activated by both stimulation sites. Right column: the optical traces revealed the emergence of suppression, determined by subtracting the expected from actual response (ΔF, difference = ΔF, actual − ΔF, expected). The intracellular recording revealed emergent IPSPs, calculated by subtracting the expected from actual response (ΔV, difference = ΔV, actual − ΔV, expected). Pseudocolor ranges for images (min, max): 0, 2.8 × 10−4 ΔF/F for A and B; 0, 4.4 × 10−4 ΔF/F for C and D; −2.8 × 10−4, 0 ΔF/F for E. For all traces, vertical scale is 5 mV and 6.5 × 10−4 ΔF/F; horizontal scale is 25 ms. The time point of these images was 11 ms after the 4th stimulus.
zone, and optical clusters outside of this perimeter were unaffected (Fig. 2). Finally, when the stimulation sites were separated by several hundred microns or less, there was substantial overlap between two diffuse zones, and the resulting interaction yielded suppression domains coincident with the overlapping area (Fig. 3).

To quantify these responses, we identified the center of suppression domains and used this location to compare the time courses of the actual and expected responses. At this site, the duration of the actual response was significantly shorter than expected, having a peak width (FWHM) of approximately 13 versus 25 ms. The amplitude of the actual response was also substantially smaller than expected throughout its entire time course, but the strongest suppression occurred during the decay phase, 4.9 ± 2.2 ms after the peak. At this point the amplitude of the actual response was typically about 70% of the expected response (Table 1).

The suppression domain’s combined effect on amplitude and time course bears the hallmarks of synaptic inhibition. To investigate the cellular mechanism of suppression, we obtained targeted intracellular recordings from neurons within suppression domains, monitoring both optical and electrical responses simultaneously. Following two-site stimulation, the intracellular recordings invariably revealed the emergence of large inhibitory postsynaptic potentials, events that were absent following single-site stimulation. Given the strong correlation between optical and electrical recordings, and the evidence that suppression domains rely solely on the convergence of horizontal connections, we focused on the mechanism of this “emergent inhibition” using intracellular recording.

**Intracellular recordings of emergent inhibition**

In visual cortex, horizontal connections elicit spikes in inhibitory, but not pyramidal cells (Hirsch and Gilbert 1991). Therefore EPSPs recorded in pyramidal cells following horizontal activation are strictly monosynaptic, whereas IPSPs are mediated by disynaptic connections through local inhibitory interneurons. Due to the absence of polysynaptic excitation, intracellular recordings from pyramidal cells provide a direct

**FIG. 2.** Domains of suppression are generated in neuronal populations receiving horizontal connections from an axonal cluster and a diffuse zone. Same convention as in Fig. 1. A and B: patterns of optical activity evoked by stimulating S1 (A) or S2 (B), separately. Left column: (A) stimulation of S1 (asterisk) evoked a series of optical clusters along the upper left edge of the image; (B) the 2nd electrode was positioned close to the optical clusters evoked by S1 to activate a diffuse zone of horizontal connections projecting from S2. The arrow marks the location of the intracellular recording electrode. Right column: time courses of optical and electrical responses at location marked by arrow. S1 evoked an EPSP (ΔV, S1; black trace), and S2 evoked a weak IPSP (ΔV, S2; red trace). C: the response expected for simultaneous stimulation of S1 and S2. Left column: the resultant image from the simple addition of images A and B; D: the actual response evoked by simultaneous stimulation of both electrodes. Left column: the activation at the arrow was significantly weaker than expected (C). Right column: the actual optical response (ΔF; actual; green trace) was smaller than expected (ΔF; expected; blue trace). In the intracellular recordings, the actual response was much more hyperpolarizing than expected (ΔV; actual vs. ΔV; expected). E: (Left column) a domain of strong suppression was evident at the location marked by the arrow in this image, obtained by subtracting image C from D; (right column) the optical record showed emergent suppression (ΔF; difference), and the electrical record revealed an emergent IPSP (ΔV; difference). Pseudocolor ranges for images (min, max): 0, 4.4 × 10⁻⁴ ΔF/F for A and B; 0, 6.8 × 10⁻⁴ ΔF/F for C and D; −3.4 × 10⁻⁴, 0 ΔF/F for E. For all traces, vertical scale bar is 5 mV and 6 × 10⁻⁴ ΔF/F; horizontal scale is 25 ms.
measurement of both excitation and inhibition driven by horizontal connections. We used this framework to determine how the interaction between two discrete sets of horizontal connections recruited the activity of local inhibitory networks.

Pyramidal cells had resting potentials near the chloride reversal potential, making chloride-mediated IPSPs small and difficult to quantify; therefore cells were moderately depolarized (10 ± 2 mV) via current injection (200 ± 50 pA) to facilitate detection and measurement of IPSPs. Using the same stimulation protocol described above, electrodes were stimulated separately to determine individual contributions to response; these were added to determine the expected response, and simultaneous stimulation of electrodes yielded the actual response. The actual responses consistently included large inhibitory PSPs, much more hyperpolarizing than responses to separate stimulations or the expected response (Fig. 4, A and B). The emergence of large IPSPs was particularly striking when separate stimulations generated a pair of depolarizing events, but simultaneous stimulation evoked a large hyperpolarization (Figs. 5, A and B, and 6, A and B).

To quantify this disparity between responses evoked by separate and simultaneous stimulations, we subtracted the expected from the actual responses, which yielded large hyperpolarizing potentials up to −10 mV in amplitude (−3.5 ± 2.2 mV, n = 46). As these inhibitory PSPs emerged during simultaneous stimulations, but were absent from the sum of separate stimulations, we termed these “emergent IPSPs.” Thus the emergent IPSP is the additional inhibition evoked by simultaneous stimulation, which was not anticipated from the responses evoked by separate stimulations. Through the remain-

### TABLE 1. Statistics of suppression zones visualized by optical imaging

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<th>(Actual/expected) %</th>
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Values are means ± SE. n, number of responses.

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**FIG. 3.** Suppression in neuronal populations receiving convergent horizontal connections from 2 diffuse zones. Same convention as in Fig. 1. A and B: pattern of optical activity generated by separately stimulating electrode S1 (A) and S2 (B). Left column: each stimulation site evoked a mound of activity corresponding the diffuse zone of horizontal connections. The center of the image (arrow) was activated by both stimulation sites, indicating a convergence of horizontal connections onto this neuronal population. Right column: time course of optical and electrical responses, showing that S1 evoked a compound EPSP/IPSP and S2 evoked an EPSP. C: theoretical response produced by the arithmetic sum of images in A and B. D: the actual response generated by simultaneous stimulation of both electrodes. Left column: this image shows a depression located midway between the 2 stimulation sites, while the expected response C does not. Right column: comparing the actual and expected responses, the actual response was smaller in the optical recording and more hyperpolarizing in the intracellular recording. E: (left column) a large suppression was seen in the center of the image between the 2 electrodes. This image is the subtraction of image C from D; (right column) the emergent IPSP and the suppression of the optical response had very similar time courses. Pseudo-color ranges for images (min, max): 0, 4.6 × 10⁻⁴ ΔF/F for A–D; −4.6 × 10⁻⁴ ΔF/F for E. For all traces, vertical scale is 5 mV and 6.3 × 10⁻⁴ ΔF/F; horizontal scale is 25 ms.

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had an onset latency consistent with disynaptic activation, occurring 3.2 ± 0.8 ms after the onset of the EPSP. Because EPSPs are delivered directly to pyramidal cells, but IPSPs are delivered through intervening inhibitory cells, the onset of IPSPs is delayed relative to EPSPs. Second, the time course of the hyperpolarization is consistent with IPSPs mediated by GABA<sub>\lambda</sub> receptors. For emergent IPSPs, the rise time (7.4 ± 4.2 ms), the FWHM (30.7 ± 7.9 ms), and the decay time constant (36.0 ± 18 ms), were all in the range of values reported for GABA<sub>\lambda</sub> receptor-mediated IPSPs (Buhl et al. 1994; Thomson and Deuchars 1997; Thomson et al. 1996; Tamas et al. 1997, 1998).

Because hyperpolarizing responses may be driven by intrinsic conductances as well as GABAergic inhibitory postsynaptic

FIG. 4. Integration of synaptic activation by horizontal connections produces large inhibitory potentials. A and B: simultaneous electrical stimulation at 2 distant cortical sites yields responses that deviate strongly from linear summation. A: responses evoked by stimulating each electrode separately. Superimposed traces showing postsynaptic potentials evoked by brief electrical stimulation (100 μs, 32 μA) of electrode S1 (top) or S2 (bottom). B: response evoked by stimulating both electrodes simultaneously (actual, bottom), overlaid with the predicted response (expected, top). The expected response was obtained by simply adding the 2 responses from A. The actual response was much more hyperpolarizing than expected. Inset: the position of the intracellularly recorded pyramidal cell relative to the stimulation electrodes (asterisks), distances indicated in microns. C and D: in the same cell, responses evoked by weak stimuli are predicted by linear summation. C: responses evoked by delivering stimuli to each electrode separately after reducing the stimulation strength on both electrodes (100 μs, 28 μA). Postsynaptic potentials generated by S1 (top) and S2 (bottom) are superimposed. Note that this small reduction in stimulation strength caused a negligible change in the postsynaptic potentials evoked by S1 or S2, comparing these responses with those in A. D: response evoked by simultaneous stimulation of both electrodes. The superimposed traces show that actual response and expected response were identical, indicating that the responses had summed linearly. The large hyperpolarizing event evoked by the stronger stimulus (B) was abolished by a slight reduction in stimulation strength. E: isolation of emergent inhibitory postsynaptic potentials. Emergent IPSPs were calculated by subtracting the expected response from the actual response. These differences traces were calculated for the responses in both (B, bottom) and (D, top). The large hyperpolarizing potential evoked by 32-μA stimulation was abolished by reducing the stimulation strength to 28 μA. F: histogram of emergent IPSPs. From 60 recordings, emergent IPSPs were obtained from difference traces as in E, and their amplitudes measured from baseline to the negative peak. In 46 recordings, these events ranged in amplitude from 1 to 10 mV.

FIG. 5. Emergent inhibition requires GABA<sub>\lambda</sub> receptor-mediated synaptic transmission. A: superimposed traces showing EPSPs generated by stimulating in separate trials either electrode S1 (top) or S2 (bottom). B: response evoked by simultaneous stimulation of electrodes S1 and S2. The actual response is purely depolarizing, revealing that linear summation failed to predict emergent inhibition. Inset: electrode positions with distances in microns. C and D: bicuculline abolishes emergent inhibition. C: superimposed responses evoked by separately stimulating S1 (top) and S2 (bottom) after perfusion of BMI (3 μM, same cell as A and B). The response evoked by S2 was unchanged by the treatment, indicating that the PSP was purely excitatory. The response generated by S1 was larger than that in A, indicating that this response had a weak inhibitory component that was abolished by BMI. D: actual and expected responses from simultaneous stimulation of S1 and S2, superimposed. While the actual response in the control was hyperpolarizing (B), after treatment with BMI, the response was purely depolarizing. E: emergent IPSPs are abolished by BMI. The superimposed traces show emergent IPSPs before (bottom) and after (top) application of BMI. Emergent IPSPs were calculated as in Fig. 4. F: summary of the amplitudes of emergent inhibition before and after application of BMI showing that emergent IPSPs were abolished by BMI (n = 3).
potentials, we tested directly the identity of the hyperpolarizing response by partially blocking GABA_A receptors with low concentrations of bicuculline (3 μM, Fig. 5). This eliminated all hyperpolarizing events evoked by both separate and simultaneous stimulations, abolished the emergent IPSP (−4.1 ± 2.0 vs. −0.02 ± 0.49 mV, n = 3), and made actual and expected responses nearly identical.

The behavior of the emergent IPSP was investigated over a broad range of stimulation strengths. Using strong stimuli, large IPSPs were evoked in the recorded pyramidal cell when either of the two sites was stimulated separately, and the sum of these two responses yielded potentials that were more negative than the chloride reversal potential, a physiologically unrealistic prediction that confounded assessment of the emergent IPSP. Weak stimuli produced purely excitatory PSPs that summed linearly, indicating the absence of emergent IPSPs (Fig. 3, C and D). Thus emergent IPSPs were evoked using a range of moderate stimulation strengths.

**Temporal properties of the emergent IPSP**

As the emergent IPSP relied on integration of synaptic potentials generated from both stimulation sites, we next investigated the temporal properties of this integration by varying the interval between the two stimuli (Fig. 6). The emergent IPSP was largest when stimuli were delivered simultaneously, and decayed progressively as the interval was increased, becoming small or absent when stimuli were delivered 10 ms apart. Plotting the amplitude of the emergent IPSP against the time interval between stimuli showed that the emergent IPSP arises from a process of integration having a time constant of 6.2 ± 0.8 ms (n = 7). This time constant is very similar to that of inhibitory cells (Tamas et al. 1997, 1998), supporting the idea that emergent IPSPs arise from a local inhibitory network which integrates the excitatory PSPs delivered by convergent horizontal inputs.

While the previous experiments indicated that emergent IPSPs arise from two sets of horizontal connections driving a local neuronal population, they did not reveal whether these connections drive the local population equally. To test the possibility that horizontal connections make different contributions to the emergent IPSP, we introduced a temporal delay between the two stimuli and alternated the order of stimulus delivery (Fig. 7). By stimulating one electrode 5 ms both before and after the other electrode, we found in 86% of cases that the emergent IPSP was larger using one protocol rather than the other, having an average difference of −1.3 ± 0.4 mV (n = 7), indicating that the order of stimulation was significant.

This asymmetry is unlikely to arise as an artifact of preparing tangential slices. First, the ability of both pathways to generate EPSPs and IPSPs indicated that the local neuronal circuit of inhibitory cells and pyramidal cells was intact, and that long-range horizontal connections were uninjured by slicing. Second, emergent IPSPs required inhibitory cells to receive connections from both pathways. Thus even if the slicing process selectively damaged only the synapses made by one set of horizontal connections onto local inhibitory cells, a highly unlikely event, then none of the affected inhibitory cells could...
connections. To test this idea, we constructed two model neuronal circuits to probe the subthreshold and superthreshold activation of inhibitory cells and to determine how synaptic potentials arising from horizontal activation were integrated by pyramidal cells and their local inhibitory network.

Both models A and B contain two pyramidal cells (P1 and P3, “projection cells”) whose long-range horizontal connections make excitatory synapses with a local population of inhibitory cells (I1 and I2) and a pyramidal cell (P2, “recipient cells”). The difference between the two models is that each inhibitory neuron in model A is driven by only one projection cell (P1 or P3), whereas each is driven by both cells (P1 and P3) in model B (Fig. 8, A and B). The difference in circuit behavior between the two models is illustrated in Fig. 8, C and D. Stimulating projection cells separately evoked purely subthreshold potentials in the recipient cells of both models. In contrast, simultaneous stimulation yielded subthreshold activation of inhibitory cells for model A and superthreshold activation of inhibitory cells in model B, evoking an emergent IPSP in the recipient pyramidal cell (Fig. 8, C and D, bottom row). Thus model B is capable of generating emergent IPSPs, but model A is not.

Models A and B were compared with a third model, linear summation, by varying stimulation strengths and measuring response amplitudes evoked by simultaneous stimulation at a single time point near the peak amplitude of IPSPs (Fig. 8E, inset, vertical line). For each model, as stimulus strength was increased, responses initially grew more depolarizing as the amplitude of EPSPs increased, but then they became hyperpolarizing as inhibition intensified. However, due to the neuronal circuitry, each model generated different amounts of inhibition. In model A, simultaneous stimulation generated responses which were largely identical to those of linear summation, but responses more negative than about $-1$ mV were less hyperpolarizing due to inhibitory shunting. In model B, responses diverged dramatically from linear summation as small increases in stimulus strength evoked large, stepwise increases in hyperpolarization due to generation of emergent IPSPs. The steep, linear progression in IPSP amplitude was enabled by the recruitment of increasingly larger numbers of inhibitory cells, which offset the effect of shunting.

Next, we made a direct comparison of models A and B and linear summation by plotting the actual against expected responses (Fig. 8F). On this graph, linear summation yields a straight line having unity slope. Model A responses diverged from the linear model at negative potentials due to the effect of inhibitory shunting. In contrast, model B responses were disparate from those of linear summation, because the actual responses were much more hyperpolarizing than expected due to emergent IPSPs.

Using model B, we were able to assess the number of inhibitory cells contributing to the emergent IPSPs in our experimental results (Fig. 8G). Plotting our experimental data in the same format, actual versus expected responses, and generating a series of curves from the model to indicate how many inhibitory cells must be recruited by simultaneous stimulation to obtain various levels of hyperpolarization, we estimated that $\approx 20$ inhibitory cells contribute to the intracellularly recorded emergent IPSPs.

Model of emergent inhibition

In principle, emergent IPSPs may be generated by inhibitory cells that integrate synaptic inputs from multiple horizontal connections. To test this idea, we constructed two model neuronal circuits to probe the subthreshold and superthreshold activation of inhibitory cells and to determine how synaptic potentials arising from horizontal activation were integrated by pyramidal cells and their local inhibitory network.

Both models A and B contain two pyramidal cells (P1 and P3, “projection cells”) whose long-range horizontal connections make excitatory synapses with a local population of inhibitory cells (I1 and I2) and a pyramidal cell (P2, “recipient cells”). The difference between the two models is that each inhibitory neuron in model A is driven by only one projection cell (P1 or P3), whereas each is driven by both cells (P1 and P3) in model B (Fig. 8, A and B). The difference in circuit behavior between the two models is illustrated in Fig. 8, C and D. Stimulating projection cells separately evoked purely subthreshold potentials in the recipient cells of both models. In contrast, simultaneous stimulation yielded subthreshold activation of inhibitory cells for model A and superthreshold activation of inhibitory cells in model B, evoking an emergent IPSP in the recipient pyramidal cell (Fig. 8, C and D, bottom row). Thus model B is capable of generating emergent IPSPs, but model A is not.

Models A and B were compared with a third model, linear summation, by varying stimulation strengths and measuring response amplitudes evoked by simultaneous stimulation at a single time point near the peak amplitude of IPSPs (Fig. 8E, inset, vertical line). For each model, as stimulus strength was increased, responses initially grew more depolarizing as the amplitude of EPSPs increased, but then they became hyperpolarizing as inhibition intensified. However, due to the neuronal circuitry, each model generated different amounts of inhibition. In model A, simultaneous stimulation generated responses which were largely identical to those of linear summation, but responses more negative than about $-1$ mV were less hyperpolarizing due to inhibitory shunting. In model B, responses diverged dramatically from linear summation as small increases in stimulus strength evoked large, stepwise increases in hyperpolarization due to generation of emergent IPSPs. The steep, linear progression in IPSP amplitude was enabled by the recruitment of increasingly larger numbers of inhibitory cells, which offset the effect of shunting.

Next, we made a direct comparison of models A and B and linear summation by plotting the actual against expected responses (Fig. 8F). On this graph, linear summation yields a straight line having unity slope. Model A responses diverged from the linear model at negative potentials due to the effect of inhibitory shunting. In contrast, model B responses were disparate from those of linear summation, because the actual responses were much more hyperpolarizing than expected due to emergent IPSPs.

Using model B, we were able to assess the number of inhibitory cells contributing to the emergent IPSPs in our experimental results (Fig. 8G). Plotting our experimental data in the same format, actual versus expected responses, and generating a series of curves from the model to indicate how many inhibitory cells must be recruited by simultaneous stimulation to obtain various levels of hyperpolarization, we estimated that $\approx 20$ inhibitory cells contribute to the intracellularly recorded emergent IPSPs.
D I S C U S S I O N

By combining intracellular recording and voltage-sensitive dye imaging in tangential slices of ferret visual cortex layer 2/3, we investigated properties of integration in local cortical circuits driven by different sets of horizontal connections. At cortical sites where horizontal connections converged onto the same neuronal populations, neuronal responses deviated from linear integration due to the emergence of large inhibitory postsynaptic potentials, or emergent inhibition. These inhibitory PSPs reduced the amplitude and time course of membrane potential fluctuations in pyramidal cell recordings, and suppressed population-based neuronal activation in optical recordings. The results indicate that horizontal connections, by converging onto neuronal populations, recruit strong inhibition from local inhibitory networks. As the recruitment of inhibition was not specific to the different types of connections formed by horizontal collaterals, axonal clusters or diffuse zones, emergent inhibition reflects a general property of local inhibitory networks. Furthermore, these results indicate that the balance of excitation and inhibition driven by horizontal connections is strongly biased toward inhibition.

Physiological properties of emergent inhibition

The large amplitude of emergent IPSPs in pyramidal cells indicates that the convergence of horizontal connections strongly activates local inhibitory networks. The present experiments indicate that the largest emergent IPSPs would require the concerted activation of ≈20 inhibitory cells. Previous experiments have shown that single inhibitory cells elicit rather small changes in conductance and weak IPSPs (<1 mV in pyramidal cells depolarized by ≈20 mV; Gupta et al. 2000; Thomson and Deuchars 1997). In contrast, in vivo intracellular recordings reveal much larger changes in inhibitory conductance during visual processing (Anderson et al. 2000; Borg-Graham et al. 1998; Hirsch et al. 1998). Taken together, these data indicate that inhibitory cells recruited by horizontal connections do not perform in isolation during cortical processing, but as part of coordinated inhibitory networks.

There are two factors contributing to the ability of inhibitory neurons to perform as a local network. First, the synapses formed by axonal clusters are distributed over many hundreds of neurons in a cortical area of a few hundred microns in diameter. By delivering EPSPs to numerous inhibitory cells in a local population, horizontal connections evoke coordinated depolarization of inhibitory cells, priming these cells for co-activation. Second, cortical inhibitory cells are coupled by gap junctions, facilitating synchronous activation (Galarreta and Hestrin 1999; Gibson et al. 1999). In combination, these factors may coordinate the activity of local inhibitory networks, yielding large emergent IPSPs during convergent activation by multiple sets of horizontal connections.

Role of emergent inhibition in visual processing

The potency of this effect made it possible to visualize the spatiotemporal dynamics of emergent inhibition by imaging with voltage-sensitive dyes. The images revealed large domains of suppression extending up to several hundred microns, sculpting the distribution of population-based neuronal activity. The behavior of suppression domains and emergent inhibition suggests a number of ways that they may contribute to visual processing. First, neuronal responses in vivo have a complicated dependence on stimulus contrast which might be explained in terms of emergent inhibition (Levitt and Lund 1997; Sceniak et al. 1999). For example, simply by varying contrast it is possible for combinations of visual stimuli to evoke either facilitation or suppression (Polat et al. 1998), suggesting that discrete populations of neurons in cortex may deliver either EPSPs or IPSPs. Although this seems paradoxical, such opposing effects can be explained by local circuit integration yielding emergent inhibition. Our experimental results suggest that high contrast stimuli may evoke emergent inhibition yielding suppression, while low contrast stimuli may fail to recruit inhibitory networks and yield facilitation, consistent with a previous model (Somers et al. 1998).

Second, delivering temporally offset electrical stimulation to spatially distant horizontal connections yielded emergent IPSPs with different amplitudes, suggesting that emergent inhibition may participate in direction selectivity. When a visual stimulus is moved between two points in visual space, the locus of neuronal activity is shifted between two areas of cortex. By analogy to this situation, we delivered stimuli at two separate cortical sites in layer 2/3 at slightly different times, simulating the movement of visually-evoked neuronal activity in cortex. Delivering these temporally offset stimuli revealed that the amplitude of the emergent IPSP depended on the order of stimulation, indicating that different sets of horizontal connections do not drive inhibitory networks equally. By analogy to the visual stimulation paradigm, reversing the order of electrical stimulation corresponds to reversing the direction of motion of a visual stimulus, implying that visual stimuli drifting in opposite directions would generate different amounts of inhibition in the local cortical circuit, yielding direction selective responses. Such asymmetries in the spatial or temporal distribution of inhibition are a component of many models of direction selectivity in cortical neurons (Maex and Orban 1996; Mineiro and Zipser 1998; Suarez et al. 1995).

The emergence of large IPSPs during coincident activation of a neuronal population by horizontal connections implicates local inhibitory networks in detecting the coincidence of neuronal signals. Because emergent IPSPs were generated only when horizontal connections were stimulated within about 10 ms of each other, the local inhibitory network acted as a coincidence detector of horizontal activation. The result of this coincidence detection was a significant reduction in the time course of neuronal activity, because emergent IPSPs transformed long duration depolarizations into brief, transient events. As transient depolarizations generate temporal precision in neuronal action potentials (Mainen and Sejnowski 1995), and inhibitory cells generate synchronous firing of pyramidal cells (Cobb et al. 1995), coincident activation by horizontal connections may lead to synchronous activation of local pyramidal cells. Because emergent IPSPs are generated by horizontal inputs having temporal disparities ≲10 ms, neurons receiving slightly temporally offset horizontal inputs would be expected to generate more temporally synchronized output.
APPENDIX: CIRCUIT MODELS

Circuit models were constructed with a modified version of Neuron software (Hines 1989; Moore and Stuart 2000) using idealized integrate-and-fire neurons. The local inhibitory neuronal circuit consisted of 20 inhibitory cells, each making a single connection on pyramidal cell P2 with a synaptic conductance of 10 nS and reversal potential of $-75$ mV ($E_{inh}$), enabling unitary IPSPs of 1 mV during depolarizing current injection (Fig. 8). In model A, inhibitory cells were contacted by either of two pyramidal cells, P1 or P3, establishing two distinct inhibitory cell populations, I1 and I2, having 10 cells each. In model B, each inhibitory cell received two synaptic connections, one from each pyramidal cell, P1 and P3. P2 received excitatory synaptic connections from P1 and P3.

For simplicity, the conductance of excitatory synapses made by P1 and P3 were assigned as a function of synaptic strength. Synapses made by P1 and P3 with P2 had conductance calculated by $G_{ep} = \frac{S_p}{100}$, where $G_{ep}$ is the conductance of excitatory synapses on pyramidal cells in nanosiemens (nS), and $S_p$ is the stimulation strength for...
pyramidal cells that varied from 0 to 35 units, enabling P2’s EPSPs to range from 0 to 6 mV with reversal potential of 0 mV ($E_{\text{rev}}$). Synapses made by P1 and P3 with inhibitory cells had conductance calculated by the formula $G_{\text{inh}}(S_i) = S_i N$, where $G_{\text{inh}}$ is the conductance of excitatory synapses on inhibitory cells, $S_i$ is the ordinal number of the inhibitory cell, and $N$ is a formula for varying synaptic strengths across a distributed population of inhibitory cells in the network. The inhibitory cell-adjusted synaptic strength, $S_i$, was calculated by $S_i = S_j - 20$. Thus inhibitory cells had a higher threshold for activation than pyramidal cells, but above this threshold, the inhibitory drive increased twice as fast as excitation delivered to P2. The network factor for distributing synaptic weights across the population of inhibitory cells was calculated by $N = \exp(-J/10)$, where $J$ is the ordinal number of inhibitory cell, enabling synaptic strength to decrease systematically across the population. For model A, ordinal numbers ranged from 1 to 10 for each population, and for model B, P1 and P3 contacted all 20 inhibitory cells, and ordinal numbers ranged from 1 to 20 for assigning P1 synapses; for assigning P3 synapses, cells were numbered in reverse order (from 20 to 1) so that an inhibitory cell receiving a strong synapse from one population received a weak synapse from the other. Thus this factor accomplished two aims: it made the number of recruited inhibitory cells dependent on stimulation strength, and by assigning ordinal numbers in a crisscrossing pattern, it yielded a heterogeneous distribution of inhibitory cells, including those driven primarily by one population and those driven relatively equally by both. To study the behavior of these models, P1 and P3 were driven to spike by 200-μs electrical stimuli, while P2 was depolarized by 12 mV via current injection of 500 pA.

Idealized neurons were constructed from cylindrical compartments. Pyramidal cells had two dendritic compartments ($5 \times 500 \mu m$, diam-by-length), an axon ($0.5 \mu m \times 2 \mathrm{~mm}$), and a soma ($30 \times 30 \mu m$). Inhibitory cells had two dendrites ($5 \times 500 \mu m$), an axon ($0.5 \times 100 \mu m$), and a soma ($20 \times 20 \mu m$). Inhibitory synapses were located on the soma, and excitatory synapses were placed on the dendrites, and their conductances were modeled by an alpha function having a 5-ms time constant for excitatory synapses and 6-ms time constant for inhibitory synapses. Axons contained Hodgkin-Huxley style sodium and potassium channels with $G_{\text{Na}} = 0.5 S/cm^2$ and $G_{\text{K}} = 0.1 S/cm^2$. Additional parameters were $E_{\text{Na}} = -75 mV$, $E_{\text{K}} = 50 mV$, $E_{\text{K}} = -77 mV$, $C_m = 1 \mu F/cm^2$, $\tau = 2 ms$, $R = 25 \mathrm{M\Omega}$, $R_e = 200 \Omega$, $C_{\text{leak}} = 300 \mu S/cm^2$, and $E_{\text{leak}} = -75 mV$.

Using model B, we estimated the number of inhibitory cells that were responsible for generating emergent IPSPs in the experimental data (Fig. 8G). Across the full range of stimulation strengths, we controlled the number of inhibitory cells recruited by simultaneous stimulation of S1 and S2 and generated curves for actual and expected responses (each inhibitory cell activating a synaptic conductance of 10 nS). Assigning the recruitment by simultaneous stimulation to zero inhibited inhibitory cells yielded responses identical to model A. The majority of experimental data points were located among curves obtained by varying the recruitment of inhibitory cells in model B from 2 to 20, allowing an estimate of the number of inhibitory cells contributing to the emergent IPSP of each pyramidal cell response.

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FIG. 8. Neuronal circuit models for investigating the mechanism of emergence. A and B: hypothetical neuronal circuits of layer 2/3 neurons. The behavior of these model circuits was probed by monitoring the activity of pyramidal cell P2, shown with an intracellular recording electrode. P1 and P3 are pyramidal cells driven by stimulating electrodes S1 and S2, respectively. The axons of these pyramidal cells deliver long-range horizontal connections to P2, and a local network of inhibitory cells, I1 and I2. In model A, inhibitory cells are contacted by 1 pyramidal cell, whereas in model B, each inhibitory cell receives input from both P1 and P3. C and D: neuronal responses from model A (C) and model B (D). In this array of neuronal responses, 4 neuronal populations yield the same response across the stimulation protocol (stimulation protocol and parameters are shown at left. Top and middle row: In model A, a stimulus delivered to S1 evoked a spike in P1 that delivered subthreshold EPSPs to I1 and P2, while a stimulus delivered to S2 evoked a spike in P2, delivering subthreshold EPSPs to I2 and P1. In model B, stimulating either S1 or S2 delivered subthreshold responses in I1, I2, and P2. Bottom row: stimuli delivered simultaneously to S1 and S2 generated responses in both P1 and P2. In model A, P1 and P2 integrated synaptic potentials from P1 and P3, revealing a large EPSP, while inhibitory cells remained subthreshold. In model B, I1 and I2 neurons integrated synaptic input from P1 and P3, surpassing threshold and generating an IPSP in P3. E: amplitude of postsynaptic potentials generated in P2 by varying stimulation strength. Responses are shown for model A (●) and model B (▲) following simultaneous stimulation of S1 and S2. In addition, responses are shown for linear summation (□) from adding responses obtained by stimulating S1 and S2 separately. Inset: responses generated by models A and B and linear summation for stimulation strengths 20, 25, 26.5, and 28.5 units. For graphing, responses were measured at a single time point near the peak of the IPSP (dotted line). Scale bar is 2 mV and 10 ms. Responses were purely excitatory for stimulation strengths <25 units and are identical for each model. For stronger stimuli, responses diverged due to different behaviors of inhibitory networks. Model A generated responses which were less hyperpolarizing than linear summation due to shunting inhibition. Model B generated stronger hyperpolarizations because simultaneous stimulation recruited more inhibitory cells, yielding emergent inhibition. Notably, for each model, there were many discontinuities along the line through hyperpolarizing potentials due to the quantal nature of IPSPs. For example, small increases in stimulation strength yielded small increases in the excitatory response unless this was true strong enough to recruit an additional inhibitory cell that hyperpolarized the PSP by about −1 mV, creating the appearance of numerous steps along this region of the curve. F: nonlinearity of responses generated by simultaneous stimulation in models A and B. The expected response, the addition of responses generated by stimulating S1 and S2 separately (linear summation) is plotted on the abscissa, while the actual responses, obtained by stimulating S1 and S2 simultaneously are plotted for models A (●) and B (▲) on the ordinate. For reference, the points obtained by linear summation (□) are shown with a unity slope line. Responses from model A diverged from linear summation at negative potentials due to inhibitory shunting. Responses from model B were displaced from the other curves, because they had more hyperpolarized potentials due to the recruitment of inhibitory cells yielding emergent inhibition. To determine from each point to the unity line indicates the amplitude of the emergent IPSP. These points were poorly fit by a straight line due to the following 2 factors. First, the steep slope in the recruitment of inhibition (Fig. 8E) caused large changes in actual response to occur with small changes in expected response, making points appear to cluster vertically. For example, when the stimulation strength was increased from 26.75 to 27 units, the expected response showed a negligible increase from −1.24 to −1.19 mV, but the actual response jumped from −5 to −6 mV, due to the recruitment of an additional inhibitory cell, giving the appearance of points aligned vertically. Second, discontinuities in expected responses caused gaps between the columns of points. G: comparison of experimental responses with model results. Experimental data from intracellular recordings were overlaid with curves obtained from model B, showing the number of inhibitory cells required to generate the emergent IPSP.