Spatiotemporal Patterns of Excitation and Inhibition Evoked by the Horizontal Network in Layer 2/3 of Ferret Visual Cortex

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INTRODUCTION

Horizontal connections are thought to play an important role in psychophysical and physiological phenomenon (Field et al. 1993; Gilbert and Wiesel 1990; Maffei and Fiorentini 1976; Ramachandran and Gregory 1991; Toyama et al. 1981; Ts’o et al. 1986), but the mechanisms of these effects are uncertain because the physiological properties of lateral connections remain poorly understood. In intracellular recordings, horizontal connections generate weak, subthreshold excitation in pyramidal cells, but can drive strong local inhibition (Bringuier et al. 1999; Hirsch and Gilbert 1991; McGuire et al. 1991; Weliky et al. 1995; Yoshimura et al. 2000). How excitation and inhibition interact to shape spatiotemporal patterns of activity evoked by horizontal connections remains unclear, however.

Lateral connections in layer 2/3 are formed by pyramidal cell axons that extend up to several millimeters, revealing two distinct zones of connectivity. Within several hundred microns of the soma, axons branch to form diffuse connections (Bosking et al. 1997; Malach et al. 1993), but beyond this zone, axons target neurons with similar orientation preferences, making clustered connections in iso-orientation domains (Gilbert and Wiesel 1989). While the data suggest that the two zones may have distinct functional properties, how these zones are represented in the spatiotemporal distribution of synaptic activation is uncertain.

Due to a variety of synaptic mechanisms and circuit-based phenomena, neuronal responses are highly sensitive to temporal patterns of activation (Abbott et al. 1997; Thomson and Deuchars 1997; Tsodyks and Markram 1997). During stimulus trains, the synaptic efficacy of excitation decreases and inhibition increases, contributing to suppression of neuronal responses. In contrast, pyramidal cell responses are augmented by temporal summation and by a variety of voltage-gated conductances which enhance depolarization, contributing to facilitation. Taken together, these mechanisms yield combinations of facilitation and suppression, making it difficult to predict how repetitive stimulation may affect dynamics of activity in spatiotemporal patterns evoked by horizontal connections.

Horizontal connections have been implicated in synchronizing neuronal activity (Amzica and Steriade 1995), but the mechanism is unresolved. As inhibitory networks synchronize populations of pyramidal cells (Cobb et al. 1995; Lytton and Sejnowski 1991; Tamas et al. 2000; Van Vreeswijk et al. 1994; Whittington et al. 1995), one possibility is that horizontal connections synchronize neuronal activity by driving inhibition. This idea is bolstered by a number of models (Bush and Sejnowski 1996; Traub et al. 1996; Wilson and Bower 1991), but physiological support is lacking due to the absence of information about spatiotemporal properties of horizontal networks.

Previous imaging studies using voltage-sensitive dyes have elucidated spatiotemporal patterns of activity in coronal slices of visual cortex (Contreras and Llinas 2001; Nelson and Katz 1995; Tanifuji et al. 1994; Yuste et al. 1997), but these slices greatly disrupt horizontal networks. Thus to determine the spatiotemporal patterns of activity generated by the horizontal network, we combined intracellular recording with voltage-sensitive dye recording in tangential slices of layer 2/3 from ferret visual cortex. The resulting patterns revealed that the synaptic potentials driven by horizontal connections are highly dynamic in both space and time. Pharmacological experiments and targeted intracellular recordings suggested that the effects may rely on inhibition.

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METHODS

Slice preparation and optical recording

Methods for isolating tangential slices from ferret visual cortex were similar to those described previously (Nelson and Katz 1995). Young adult ferrets (P47–P60, Marshall Farms, North Rose, NY) were decapitated under pentobarbital sodium anesthesia (100 mg/kg, ip). Using a vibratome and slicing parallel to the pial surface, the initial 200 μm containing layer 1 was discarded, and then a 350-μm-thick tangential slice of layer 2/3 was cut. Slices were transferred to an interface chamber and incubated with voltage-sensitive dye (0.1 mg/ml RH461, Molecular Probes, Eugene, OR) (Grinvald et al. 1987) in normal saline (in mM: 125 NaCl, 1.3 MgSO₄, 2.8 CaCl₂, 4 KCl, 1 KH₂PO₄, 10 dextrose, and 26 NaHCO₃) at 28°C for approximately 90 min. For imaging, slices were transferred to a submersion chamber on a Zeiss Axiovert 100 TV microscope and perfused at 4 ml/min with a peristaltic pump delivering oxygenated saline warmed to 29 ± 2°C. Light was supplied from a 250-watt lamp in a custom-built enclosure driven by a current-regulated power supply (ATM75–15M, Kepco, Flushing, NY). Incident light was delivered through the epifluorescence port, filtered at 546/40 nm (Chroma Technology, Brattleboro, VT) and reflected by a 550-nm dichroic mirror through a 10× objective (Zeiss Fluar, 0.5NA) to the slice. Emitted light was long pass filtered at 590 nm and directed through the bottom port to a 256 element photodiode array (16 × 16, C5897, Hamamatsu, Bridgewater, NJ), acquiring frames at 2 kHz. Trials were acquired every 10 s, and the duration of illumination was limited by an electromechanical shutter (VS35, Vincent Associates, Rochester, NY) to 325 ms per trial; the last 125 ms included the stimulus and response. Stimuli were delivered on alternate trials (every 20 s) enabling the acquisition of interleaved, unstimulated background images that were subtracted from the stimulated trials. A pulse generator (Master-8, AMPI, Jerusalem, Israel) was used as a trigger for stimulus delivery, shutter opening and acquisition of the electrical and optical responses. Light intensity was adjusted to nearly saturate the 16-bit A/D converters by increasing the intensity to nearly saturate the 16-bit A/D converters by nearly saturating the 16-bit A/D converters by opening and acquisition of the electrical and optical responses. Light was supplied from a 250-watt lamp in a custom-built enclosure driven by a current-regulated power supply (ATM75–15M, Kepco, Flushing, NY). Incident light was delivered through the epifluorescence port, filtered at 546/40 nm (Chroma Technology, Brattleboro, VT) and reflected by a 550-nm dichroic mirror through a 10× objective (Zeiss Fluar, 0.5NA) to the slice. Emitted light was long pass filtered at 590 nm and directed through the bottom port to a 256 element photodiode array (16 × 16, C5897, Hamamatsu, Bridgewater, NJ), acquiring frames at 2 kHz. Trials were acquired every 10 s, and the duration of illumination was limited by an electromechanical shutter (VS35, Vincent Associates, Rochester, NY) to 325 ms per trial; the last 125 ms included the stimulus and response. Stimuli were delivered on alternate trials (every 20 s) enabling the acquisition of interleaved, unstimulated background images that were subtracted from the stimulated trials. A pulse generator (Master-8, AMPI, Jerusalem, Israel) was used as a trigger for stimulus delivery, shutter opening and acquisition of the electrical and optical responses. Light intensity was adjusted to nearly saturate the 16-bit A/D converters by varying current. Typical power settings were between 170 and 200 W.

Electrophysiology

Intracellular recordings were obtained in current-clamp mode (AxoClamp-2B, Axon Instruments) using sharp electrodes (90–130 MΩ) filled with 3 M KAc and supported with a programmable motorized manipulator (SM1, Luigs and Neumann, Ratingen, Germany). Signals were filtered at 3 kHz (Model 410, Brownlee Precision, San Jose, CA) and digitized at 20 kHz (AT-MIO-16E, National Instruments, Austin, TX), Following a recording, cells were filled with neurobiotin (Molecular Probes) 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 were regular-spiking cells (McCormick et al. 1985) with resting potentials of −70.5 ± 5.8 mV, overshooting action potentials and pyramidal cell morphologies.

Image processing

Image processing was performed with custom programs written in Visual C++ on a Pentium computer, and with IP-Lab (Scanalytics Inc, Fairfax, NJ) and IDL (Research Systems, Boulder, CO) on a Macintosh computer. All signals were inverted so that excitation appears as an increase in intensity. To improve the signal/noise ratio, optical traces were temporally filtered at 500 Hz. Spatial filtering was accomplished by tripling the number of pixels on each axis, and smoothing the resultant image (48 × 48 pixels) with a 5 × 5 Gaussian kernel.

Electrical stimulation

Electrical stimuli (100 μs) were delivered from a current isolator (A360, WPI, Sarasota, FL) to a concentric bipolar electrode (FHC, Bowdoinham, ME). In all cases, electrical stimuli were extremely weak (10–28 μA), yielding optical signals far below the level of dye saturation; the amplitude of optical signals could be increased more than 10-fold by increasing stimulation strength, indicating that optical responses are well within a linear operating range of the dye. Within this range of weak stimuli, optical signals related to fibers of passage and intrinsic (slow) signals were undetectable.

Analysis of temporal coordination

The analysis of temporal coordination required measurements that were relative, in which the time courses of optical clusters were compared after each of four stimuli in a train (Fig. 9, G and H). To accurately quantify this effect, it was necessary to exclude slices from analysis in the following two situations. First, a slice was exempted from analysis if the time courses of optical clusters were “saturated.” Following sufficiently strong stimuli and large enough inhibitory responses, time courses did not exhibit relative temporal shifts because responses could not become any faster; their rate was saturated. In this situation, there was no change in variance, because response rates were at their physical limit. Second, a slice was excluded if it yielded “synchrony by default”. When optical clusters were located at the same distances from the stimulation site, they commonly had identical time courses. In this situation, the responses had no initial variance, and temporal coordination was an artifact of geometrical properties in the slice. Thus we included in data analysis slices which were capable of temporal changes (unsaturated responses) and revealed an initial variance (heterogeneous optical clusters).

Even so, the variance in timing following the first stimulus covered a broad range for different slices (2–42 ms2, Fig. 9G). The reason for this can be attributed to the two factors described above. Namely, due to the interrelationship between stimulation strength, response acceleration, and variance in timing, the initial variance depended on the strength of the stimulus and the amount of inhibition recruited by it. The largest initial variances were observed for weak stimuli, for which optical clusters typically exhibited a greater distribution in their latencies to peak response following the first stimulus. In addition, due to the relationship between spatial distribution, axonal propagation delays, and temporal properties of optical clusters, the largest initial variances were observed for slices having widely dispersed optical clusters.

Histology

Extracellular injections of biocytin were used to determine the pattern of anatomical clusters. Electrodes were pulled from capillary glass and broken to diameters of 5–10 μm and filled with 2% biocytin in 0.9% sodium chloride. After an imaging experiment, the stimulating electrode was retracted and the biocytin-filled electrode was advanced into the same site. Pulses of positive current (0.1 mA) were delivered from a current source (Stoelting, Wood Dale, IL) for approximately 20 min. After the injection, alignment markings were made by advancing an optical fiber into the slice at several peripheral sites, creating perforations in the slice (100 μm diam) that were maintained through subsequent processing. At each of these sites, the position of the fiber was recorded by capturing images of the emitted light on both the CCD camera and the photodiode array. Finally, the slice was returned to the interface chamber for 3–6 h before fixation (2–4 days in 4% paraformaldehyde in PBS at 4°C). After processing, sections were mounted on slides, and their images were acquired in
brightfield with a CCD camera (Princeton Instruments, Trenton, NJ). Aided by redundant sets of alignment markings, images of histological sections and voltage-sensitive dye records were overlaid for analysis. To determine the correspondence between optical and anatomical clusters, optical clusters were identified and demarcated by 320-μm-diam circles, equivalent to their mean full-width at half-maximum, and these circles were transferred to identical regions in the anatomical image. Optical clusters were quantified by normalizing their intensities to their brightest pixel, and summing all of the pixel values within the encircled area. Anatomical clusters were quantified by converting the image to binary format and calculating the percentage of labeled pixels within the circle. To determine whether these circled regions, or “assigned clusters,” were significant, we first excluded the diffuse zone and then randomly selected 3,000 circular regions with 320 μm diam, centered on pixels throughout the image. For these randomly selected regions, the measured values were significantly smaller than assigned clusters, both optical and anatomical.

RESULTS

The paper is presented in four sections. In the first section, we use voltage-sensitive dyes and electrical stimulation to characterize the spatiotemporal patterns of activation evoked by horizontal connections. Investigating how these patterns evolved during repetitive stimulation led to two novel observations, discussed in the second and third sections: 1) stimulus trains generated propagating waves of suppression and 2) stimulus trains reduced the variance in timing of optical responses despite large variations in distance and latency. In the fourth section, we investigated the mechanism of these phenomena by combining voltage-sensitive dye imaging with intracellular recording so that optical and electrical responses could be obtained simultaneously from the same cortical locus. Analyzing these responses and the effects GABAergic receptor blockers suggested that inhibition contributed to both the suppression wave and variance reduction.

These experiments describe activity patterns evoked by stimulation at single cortical sites, forming the foundation for studying cortical interactions mediated by horizontal connections evoked by stimulating two cortical sites, the topic of the companion paper (Tucker and Katz 2003).

Characterization of activity patterns generated by horizontal connections in tangential slices

We first determined the patterns of activity generated in space and time by focal electrical stimulation in tangential slices of layer 2/3 from ferret visual cortex. As connections from lower cortical layers were absent in these slices, this enabled us to visualize the pattern of subthreshold activity mediated primarily by horizontal connections. To simulate the trains of action potentials generated by visual stimuli, we delivered trains of electrical stimuli (4 pulses, 100 Hz), and the resulting optical responses were recorded in regions 1.76 × 1.76 mm with 0.5-ms resolution, using a 16 × 16 photodiode array.

Brief trains of weak electrical stimuli produced complex patterns of optical activity (Fig. 1). The initial response was a large increase in activity in a restricted region (approximately 200 μm diam) immediately surrounding the stimulating electrode. During the next several milliseconds, the activation propagated outwardly, and a number of distinct, ovoid domains of activity appeared 400–1,200 μm from the stimulation site. After each subsequent stimulus, this pattern of activation was regenerated, as activity arose at the stimulation site, expanded, and intensified the “patchy” appearance in localized regions. Thus the basic layout of the spatial pattern was largely the same after each stimulus, although the amplitude and timing of signals changed, as discussed in subsequent sections. After the final stimulus, the optical signals slowly decayed over the ensuing tens of milliseconds.

The patterns consisted of two components: a large uniform response around the stimulation site (“diffuse zone”), and an assortment of localized regions of activity that we termed “optical clusters.” These patterns were consistent with the anatomy of horizontal connections, which are iso-tropically distributed within a zone extending several hundred microns and form axonal clusters beyond this zone (Bosking et al. 1997; Malach et al. 1993). In the optical patterns, the diffuse zone extended 596 ± 177 μm; optical clusters were well-fit by a Gaussian function with a full-width at half-maximum (FWHM) of 319 ± 102 μm (n = 100 optical clusters), and there were an average number of 3.2 ± 1.6 such clusters per field of view (3 mm²). Optical activity propagated away from the stimulation site at a velocity of 0.24 ± 0.2 m/s (at 28–30°C), comparable to that observed for horizontal connections in vivo (Bringuier et al. 1999) and in vitro (Nelson and Katz 1995). Taken together, these data strongly suggest that horizontal connections are the anatomical basis of the optical patterns.

Direct evidence that the axonal clusters of pyramidal cells are the anatomical substrate of the optical clusters was obtained by targeting the stimulation site with extracellular injections of biocytin (Fig. 2). Consistent with other reports (Bosking et al. 1997; Malach et al. 1993), the resulting pattern included a diffuse zone of anterograde label with a radius of several hundred microns, and clusters of connections residing outside of this perimeter (n = 3). Overlying anatomical sections with the corresponding optical recordings demonstrated alignment between the anatomical diffuse zone and optical responses proximal to the stimulation site, and colocalization of optical and anatomical clusters. Quantification of the density of labeling and the intensity of fluorescence indicated that the anatomical and optical clusters, compared with other regions in the image, were highly significant (Fig. 2, C and D).

To further investigate whether optical clusters were generated by horizontal connections, we delivered electrical stimuli at several different locations within the slice. Because axonal clusters target iso-orientation domains, and orientation preferences are mapped systematically in visual cortex (Blasdel and Salama 1986; Grinvald et al. 1986), layer 2/3 contains numerous distinct networks of horizontal connections, each corresponding to a specific orientation preference. Therefore stimulating different locations in a tangential slice should reveal multiple, distinct patterns of optical clusters. In every slice tested, we observed nonoverlapping patterns of optical clusters, indicating the presence of multiple networks of horizontal connections, and also overlapping patterns of optical clusters, revealing sites interconnected by the same horizontal network (n = 18 slices, Fig. 3, A–E). Overlying all of the patterns observed within an individual slice produced composite images
resembling orientation preference maps visualized with intrinsic signal imaging in vivo (Fig. 3F). This suggests that optical clusters represent activation of clustered horizontal connections.

Spatial and temporal characteristics of the suppression zone

The optical patterns elicited by each stimulus in the train were similar, but also showed consistent and systematic
changes in spatiotemporal characteristics. After each stimulus, responses within a roughly circular region surrounding the stimulation site were strongly suppressed. This region grew larger after each stimulus, extending up to several hundred microns, indicating a progressive expansion of suppression. We quantified responses within this “suppression zone” in terms of amplitude, time course, and bicuculline-sensitivity.

Response amplitudes in the suppression zone progressively declined in the course of a stimulus train. In some cases, this effect was so strong that responses either decreased to zero or became negative-going (Fig. 4, A and B), which is indicative of hyperpolarizing inhibition. Analyzing the slices which exhibited negative-going responses, we visualized patterns of hyperpolarization by plotting the absolute value of negative-valued pixels and applying a distinct pseudocolor scheme to distinguish this processed image from normal images (Fig. 4C). The resulting images showed that negative-going responses formed either a full or partial ring around the stimulation site. The size of this ring expanded progressively during the stimulus train from 379 ± 129 to 806 ± 105 μm (n = 5 slices). This result suggests that during repetitive stimulation, the horizontal connections augment inhibition at increasingly greater distances from the stimulation site.

Responses not only became smaller, but faster as well. After four pulses, decay rates were over five times faster inside the suppression zone compared with outside (r = −6.7 ± 2.1%/ms, d < 400 μm vs. −1.3 ± 0.7%/ms, d > 800 μm). This rapid decay led to the appearance of a flattened ring encircling the stimulation site, outside of which responses were largely unaffected (Fig. 5, A and B). To visualize these patterns of rapidly decaying responses, we calculated the first derivative with respect to time of the entire image series (Fig. 5C), obtaining spatiotemporal patterns of the optical signal’s rate of change. In these processed images, the largest amplitudes indicate the most rapid decay rates. Comparing images after each stimulus in the train revealed that the radius of rapidly decaying optical responses expanded progressively from 302 ± 147 to 631 ± 164 μm (Fig. 5, D–G; n = 18 slices).

To determine whether the suppression of optical responses was due to inhibition, we attenuated inhibitory currents by applying low concentrations of the GABAa receptor blocker, bicuculline (BMI, 3 μm). This increased the peak amplitudes of optical responses within the suppression zone by 78 ± 12% and decreased the decay rates fourfold (from −6.7 ± 2.1 to −1.6 ± 0.9%/ms). Thus BMI caused the responses within the suppression zone to grow larger and slower, generating a diffuse mound of activity centered on the stimulation site which often merged with optical clusters on the perimeter (Fig. 6, A and B). By subtracting images obtained before and after bicuculline application, we obtained optical patterns related to the magnitude of inhibition. Measuring the spatial extent of the bicuculline-sensitive region after each stimulus revealed a progressive expansion from 291 ± 140 to 631 ± 181 μm (Fig. 6, C–F; n = 13 slices). Thus the suppression zone results from inhibition driven by horizontal connections.

Nearby optical clusters were often included within the periphery of the suppression zone. Consistent with responses within the rest of suppression zone, during the stimulus train, the amplitude of optical clusters decreased and the decay rate increased, but in addition, their precise location appeared to drift (Fig. 7). To quantify this phenomenon, the location of the optical cluster’s peak amplitude was determined after each stimulus. Between the first and fourth stimulus, optical clusters whose centers were initially located 400 ± 50 μm from stim-
ulation site progressively shifted further away by 239 ± 143 μm (Fig. 7G, n = 30 optical clusters). These spatial shifts may result from an interaction between the suppression wave and an optical cluster. Because the magnitude of suppression declined steadily over distance from the stimulation site, an optical cluster would be more inhibited on the side closer to the stimulating electrode. This asymmetric distribution of suppression would shift the location of the peak amplitude, and during repetitive stimulation, as the suppression grows stronger and travels further, the peak would shift further toward the optical cluster’s outer perimeter.

Taken together, these data indicate that repetitive stimulation of horizontal connections generates an expanding zone of suppression, which approaches a maximal extent of several hundred microns (Fig. 7H). By propagating further after each stimulus, the suppression zone envelopes responses over progressively larger distances, sculpting the spatial and temporal distribution of neuronal activity.

Temporal properties of individual optical clusters

Outside the suppression zone, the responses of optical clusters grew progressively faster after each stimulus in a train. To quantify this effect, we measured the interval of time from stimulus delivery to peak response (time-to-peak) within optical clusters after each stimulus. From the first to fourth stimulus, this interval decreased by 30% (from 9.5 ± 1.4 to 6.7 ± 0.9 ms, n = 36 optical clusters, P < 0.001, Fig. 8), an effect we termed “response acceleration.”

Because the balance of excitation and inhibition driven by horizontal connections depends on stimulation strength (Hirsch and Gilbert 1991), we varied the strength of stimulus trains to determine the effect on response acceleration. Reducing the
FIG. 7. Spatial shifts of optical clusters evoked by the suppression zone. A–D: time series showing the spatial shift of an optical cluster, during the time that the suppression zone was expanding. Note the progressive outward drift of the optical cluster (arrows) away from stimulation site (asterisk) toward the black circle in A. While the stimulation site and optical cluster had adjacent peaks in the 1st image, they were well separated in the 4th. Comparing the bottom right quadrant of each image, negative-going responses emerged between stimulation site and the point marked by the black square in A, forming a partial ring around stimulation site. Comparing the top right corner of each image, an optical cluster outside the zone of suppression was relatively unaffected. E and F: spatial profiles showing the concurrent emergence of suppression and the translocation of an optical cluster. Corresponding to images shown in A–D, after each stimulus (1–4) the spatial profile of response amplitude is plotted along a straight line from the stimulation site (asterisk) to the black circle marked in A and displayed in E. In addition, the responses are plotted along a straight line from the stimulation site to the black square marked in A and displayed in F. E: corresponding to the orientation of images A–D, the abscissa has the stimulation site (asterisk) on the right and the black circle on the left. Note that the peak of the optical cluster migrated further to the left (leftward arrow) as, midway between the optical cluster and the stimulation site, a trough of suppression (upward arrow) emerged. F: the abscissa has the stimulation site (asterisk) on the left and the black square on the right. Note that the trough of suppression (upward arrow) became negative-going as it deepened and expanded progressively outward. G: incremental spatial shift of optical clusters. Optical clusters with initial positions within the diffuse zone (approximately 400 microns from the stimulation site, squares) migrated incrementally after each stimulus, while those outside of this zone (>800 microns from the stimulation site) did not change position (circles). H: incremental expansion of suppression wave assessed by maximal spatial extent, following each stimulus pulse, of hyperpolarization (circles), negative derivative (triangles), and bicuculline-sensitivity (squares). In each case, the suppression zone expanded to a maximum of 600–800 μm.

FIG. 8. Acceleration of responses in optical clusters during repetitive stimulation. A: time course of optical response in 2 regions from Fig. 1. These responses were acquired on 2 different photodiodes that viewed the stimulation site (gray line) and an optical cluster (black line), marked by the asterisk and the circle in Fig. 1E, respectively. Four stimuli were delivered at times marked along the top. B: optical clusters accelerate during stimulus trains. Using the response from the optical cluster shown in A, the response after each stimulus pulse was translated in time (along the x axis) to align the responses temporally. In this figure, each electrical stimulus occurs at t = 0 ms, and the corresponding responses are numbered 1–4, representing the stimulus number. After each stimulus, the time-to-peak became shorter and the decay rate became faster. Comparing the responses after the 1st and 4th pulses, the peak shifted to the left by 5.5 ms (indicated by arrow). The 1st response did not form a distinct peak before the onset of the 2nd, and the time of the peak was assigned in the following way. Due to the distance of this optical cluster from the stimulus site, there was an onset latency of 4.5 ms. Because the 2nd stimulus was delivered 10 ms after the 1st, but the 1st response remained unaffected ±14.5 ms (10 + 4.5 ms) due to onset latency, this value was assigned as the most conservative estimate of the time of the peak. C: temporal acceleration is specific to optical clusters. Using the time of the peak response after the first stimulus as a reference point, the time-to-peak of responses in optical clusters exhibited a progressive reduction following subsequent stimulus pulses (squares). In contrast, regions outside of these optical clusters, located at equal distances from the stimulation site, showed no significant change (circles).
stimulation strength to a level at which an optical cluster was discernable but weak, which would evoke mostly excitatory events in pyramidal cells, the latency to peak amplitude was long after each stimulus, and response accelerations were small or absent (10.3 ± 1.3 and 8.9 ± 1.4 ms for time-to-peak after pulses 1 and 4, respectively, n = 6). Increasing stimulation strength to a level which would evoke strong inhibition in pyramidal cells, the latency to peak was far shorter. In this case, the temporal accelerations were also not observed, presumably because responses after the first stimulus could not be accelerated further (5.4 ± 0.8 and 5.2 ± 0.7 ms for time-to-peak after pulses 1 and 4, respectively, n = 6). Therefore the largest accelerations were observed by adjusting the stimulation strength to moderate levels at which accelerations between 4 and 6 ms were achieved.

Beyond the suppression zone, response acceleration was only observed in optical clusters (Fig. 8C). At sites peripheral to the suppression zone and optical clusters, responses were weak but measurable, and they showed no change in time course during repetitive stimulation (9.2 ± 1.2 and 8.5 ± 1.0 ms for time-to-peak after the first and fourth pulses, respectively, P < 0.052). Thus response acceleration was observed in regions activated by horizontal connections and not in surrounding areas.

Temporal properties in patterns of multiple optical clusters

Each stimulation site evoked a number of optical clusters, each of which showed response acceleration. By plotting the responses of multiple optical clusters on a single graph, we observed that their differences in timing declined progressively during the stimulus train (Fig. 9). Following the first stimulus, the peaks of optical clusters were widely distributed in time, but following the fourth stimulus, they were nearly coincident. To quantify this effect, we measured for each optical cluster in the pattern the time of the peak response, and using these values, we calculated the mean and the variance following each stimulus. Following the first stimulus, the peaks had a relatively large variance in timing, but following the fourth stimulus, the variance was markedly reduced by 71% (11.8 ± 2.6 to 3.4 ± 1.0 ms², P < 0.005, n = 18 slices, Fig. 9, G and H). The points representing the variance after each stimulus were well-fit by an exponential function with an asymptote at 1.5 ± 2.4 ms², suggesting that the variance in timing among optical
clusters could be reduced even further by additional stimuli. Thus during stimulus trains, optical clusters became more similar in time course, their responses converging within a narrower time window.

We analyzed peripheral regions, those areas outside of optical clusters and the suppression zone, to determine the variance in their responses. For these sites, the variance in timing was much larger than that of optical clusters (20.6 ± 3.2 vs. 3.4 ± 1.0 ms$^2$), and it was unchanged by repetitive stimulation (Fig. 9H). These results suggest that the timing of responses within a pattern of optical clusters is coordinated by repetitive stimulation of horizontal connections.

Correlation of voltage-sensitive dye responses with synaptic physiology

We determined the synaptic basis of the optical responses using intracellular recording and pharmacology. The optical signals described above originate primarily from postsynaptic neurons activated by horizontal connections. We verified this by blocking glutamatergic transmission with APV (20 μM) and CNQX (20 μM), which abolished optical clusters ($n=5$ slices), demonstrating that responses do not arise from the axonal clusters themselves but rather from their postsynaptic targets (Fig. 10). In the absence of glutamatergic transmission, optical responses were completely eliminated except for a small residual response within a 200 μm radius of the stimulation site, indicating the region of cells driven directly by the electrode (Grinvald et al. 1982). As the responses in this region were driven nonsynaptically, they were excluded from all subsequent analyses.

To directly determine the effect of inhibition on the time course of the optical response, we targeted defined optical clusters for intracellular recording, enabling simultaneous recording of electrical and optical signals ($n=36$ pyramidal cells). Knowing the position of the electrode relative to the photodiode array, the electrical response from an individual cell could be compared directly with the optical response from a single photodiode, which yields an aggregate measure of the electrical activity in a population of a few hundred cells including both pyramidal and inhibitory neurons.

In the intracellular recordings, hyperpolarizing inhibitory potentials were typically weak or absent, indicating that the resting potential was close to the chloride reversal potential of GABA$\alpha$ receptors. Therefore to facilitate our study of the correlation between inhibition and the time course of optical responses, neurons were depolarized by injecting current to enhance inhibitory potentials, a procedure that could not be applied to the population of cells that contribute to the optical response.

Under these conditions, weak electrical stimuli generated small optical responses and pure excitatory postsynaptic potentials (EPSPs) whose time courses were very similar (Fig. 11A). Stronger electrical stimuli generated compound EPSP/ inhibitory postsynaptic potentials (IPSPs) and larger optical signals whose peaks were much sharper in time than those observed following weak stimulation (Fig. 11, B and C). Plotting the time-to-peak and the FWHM of the optical signal against the maximum amplitude of hyperpolarization in the electrical recording revealed that as the strength of inhibition increased, the FWHM of narrowed (30 ± 6 to 5 ± 2 ms, optical; 24 ± 5 to 1.5 ± 0.2 ms, electrical) and the time-to-peak shortened (9.5 ± 0.8 to 4.3 ± 0.4 ms, optical; 7 ± 0.6 to 1.9 ± 0.5 ms, electrical, Fig. 11, D and E, $n=36$). These effects of inhibition on the time course of the optical response yield a signature of inhibition that can be quantified even though the optical signal itself does not undergo sign reversal.

When GABA$\alpha$ receptors were blocked with increasing concentrations of bicuculline (BMI, Fig. 11, F and G), concerted changes were seen in the optical and electrical records. As inhibition was attenuated, responses became substantially larger and broader, increasing the FWHM (7.1 ± 4.2 to 39.5 ± 28 ms, optical; and 2.7 ± 1.2 to 22.8 ± 2.6 ms, electrical) and increasing the time-to-peak (3.8 ± 0.8 to 10.7 ± 6 ms, optical; 2.3 ± 0.2 to 6.6 ± 1.5 ms, electrical, $n=8$). Traces indicated that purely excitatory responses required a relatively long time to reach their peak amplitude, but when this peak was truncated by the onset of inhibition, the peak shifted earlier in time. The magnitude of this temporal shift supports the idea that inhibition accounts for the response acceleration observed in the optical responses during repetitive stimulation described above.

DISCUSSION

Role of the horizontal network in sculpting population-based neuronal activity

Spatiotemporal dynamics of neuronal activity have been described in coronal slices of visual cortex (Contreras and Llinas 2001; Nelson and Katz 1995) and numerous other systems, revealing propagating waves (Devor and Yarom 2002; Ermentrout and Kleinfeld 2001; Leznik et al. 2002; Precht et al. 1997; Senseman 1999; Senseman and Robbins).
reaching the peak of the EPSP (indicated by arrows above traces). Resting to the left, shortening the time-to-peak, because the signal began to decay before responses. Also note that in the presence of large IPSPs, the peak amplitude shifted excitatory PSP. Note the close correspondence between the optical and electrical prolonged the time-to-peak. In 4 μM BMI, the time course was similar to a purely excitatory postsynaptic potential (EPSP) matching the shape of the optical signal.

B: a weak electrical stimulus generated a purely excitatory postsynaptic potential. Vertical scale bar is 2 mV (intracellular) or 0.01% peak shortened, the full-width at half-maximum (FWHM) narrowed, and the decay of the IPSP. The peak of optical and electrical responses occurred earlier in time when the signal was truncated by inhibition. The time-to-peak was measured as the time from signal onset to the maximal value of the depolarization phase of the response. E: the FWHM was inversely correlated with magnitude of IPSP (Data compiled from 36 recordings). F and G: blockade of inhibition prolonged time course of responses. Simultaneous recordings of optical (F) and electrical (G) signals at the same cortical site. Increasing the concentration of bicuculline stepwise caused progressive slowing of response (c, control; 1 μM, 2 μM; and 4 μM). Each addition of BMI slowed the decay rate, broadened the FWHM, and prolonged the time-to-peak. In 4 μM BMI, the time course was similar to a purely excitatory PSP. Note the close correspondence between the optical and electrical responses. Also note that in the presence of large IPSPs, the peak amplitude shifted to the left, shortening the time-to-peak, because the signal began to decay before reaching the peak of the EPSP (indicated by arrows above traces). Resting potential ~70.1 mV, injected current +200 pA.

FIG. 11. Simultaneous imaging and intracellular recording correlates time course of optical signals with inhibitory postsynaptic potentials. A–C: paired intracellular and optical recordings at 3 stimulation strengths. Current (+200 pA) was injected through the recording electrode to depolarize the membrane potential and enhance inhibitory postsynaptic potentials (IPSPs; resting potential ~74 mV). A: a weak electrical stimulus generated a purely excitatory postsynaptic potential (EPSP) matching the shape of the optical signal. B: a moderate strength stimulus generated a PSP with a weak inhibitory potential that truncated the duration of response, and the same shape is reflected in the optical signal. C: a strong stimulus evoked a compound (EPSP/IPSP) with a large hyperpolarization. This feature was absent from the optical signal because cells in the neuronal population are resting near the chloride equilibrium potential. In the electrical recording, the IPSP caused the depolarization phase of the PSP to be a brief, transient event, as the time-to-peak shortened, the full-width at half-maximum (FWHM) narrowed, and the decay rate accelerated. All of these features were also evident in the shape of the optical response. Vertical scale bar is 2 mV (intracellular) or 0.01% ΔF (optical), and horizontal scale bar is 20 ms. D and E: the time course of optical and electrical responses revealed the effect of inhibition. Responses were categorized as IPSPs if there was a measurable hyperpolarization below baseline, otherwise they were categorized as EPSPs (“c”). D: the time-to-peak was correlated with the magnitude of the IPSP. The peak of optical and electrical responses occurred earlier in time when the signal was truncated by inhibition. The time-to-peak was measured as the time from signal onset to the maximal value of the depolarization phase of the response. E: the FWHM was inversely correlated with magnitude of IPSP (Data compiled from 36 recordings). F and G: blockade of inhibition prolonged time course of responses. Simultaneous recordings of optical (F) and electrical (G) signals at the same cortical site. Increasing the concentration of bicuculline stepwise caused progressive slowing of response (c, control; 1 μM, 2 μM; and 4 μM). Each addition of BMI slowed the decay rate, broadened the FWHM, and prolonged the time-to-peak. In 4 μM BMI, the time course was similar to a purely excitatory PSP. Note the close correspondence between the optical and electrical responses. Also note that in the presence of large IPSPs, the peak amplitude shifted to the left, shortening the time-to-peak, because the signal began to decay before reaching the peak of the EPSP (indicated by arrows above traces). Resting potential ~70.1 mV, injected current +200 pA.
establish two distinct functional zones in cortical processing. Because the correspondence between axonal clusters and orientation domains is well documented (Gilbert and Wiesel 1989), optical clusters are a vestige of orientation domains, marking neuronal populations that generated iso-orientation specific responses in the intact animal. In comparison, the marking neuronal populations that generated iso-orientation domains is well documented (Gilbert and Wiesel 1989), optical clusters are a vestige of orientation domains, generating nonorientation specific responses. This dichotomy between regions having iso- and nonoriented responses suggests that horizontal connections may delimit two specialized regions, corresponding to signals narrowly and broadly tuned for orientation. In addition, the distribution of optical activity in these regions changed dramatically during repetitive stimulation, suggesting that horizontal connections may contribute to the dynamics of receptive field properties in vivo (Celebrini et al. 1993; Pei et al. 1994; Ringach et al. 1997; Shevelev et al. 1993). By evoking spatial and temporal dynamics in the distribution of excitatory and inhibitory synaptic potentials, horizontal connections provide a synaptic physiological correlate for the temporal evolution of receptive fields.

A role for horizontal connections in neuronal timing

A remarkable feature of the optical patterns evoked by horizontal connections was the reduction in timing variance during trains of stimuli. This suggests that the synaptic activity evoked by horizontal connections, despite propagation across long cortical distances, leads to coordinated fluctuations in subthreshold membrane potentials.

While the activity generated in pyramidal cells by horizontal connections is subthreshold, the spatiotemporal patterns revealed two features that would facilitate synchronous firing of action potentials. First, pyramidal cells are more likely to spike synchronously during transient depolarization (Mainen and Sejnowski 1995; Nowak et al. 1997; Reyes and Fetz 1993). While the probability of spiking is increased by any excitatory event, only brief depolarization (i.e., EPSPs truncated by IPSPs) constrains spike timing. As optical clusters became more coordinated and temporally sharpened during pulse trains, the probability of synchronous spiking would increase. Second, pyramidal cells are more likely to spike when the arrival of EPSPs are synchronous, rather than temporally distributed (Sotký and Koch 1993; Stevens and Zador 1998).

Optical clusters represent net membrane potential fluctuations integrated over hundreds of neurons resulting from large numbers of coincident EPSPs, providing further evidence that the synaptic potentials generated by horizontal connections would facilitate spike synchrony.

Because optical clusters had time courses which were bicuculline-sensitive and highly correlated with the magnitude of IPSPs, these data provide evidence that their coordination was generated by inhibition. This conclusion is consistent with other results that inhibitory cells, by delivering coincident hyperpolarizing potentials to large numbers of pyramidal cells, coordinate both subthreshold and superthreshold pyramidal cell activity (Cobb et al. 1995). As cortical inhibition has a spatial extent of a few hundred microns (DeFelipe and Jones 1985; Hess et al. 1975; Kisvarday et al. 1985; Somogyi et al. 1983), local inhibitory cells may coordinate activity within individual orientation domains, but not across multiple domains.

However, the present data support the idea that horizontal connections may synchronize neuronal activity between multiple orientation domains by interconnecting distributed inhibitory networks, as has been proposed in a number of models (Bush and Sejnowski 1996; Lytton and Sejnowski 1991; Traub et al. 1996; Wilson and Bower 1991). In the spatiotemporal patterns revealed by voltage-sensitive dye imaging, the timing of activity in multiple optical clusters during stimulus trains became both faster and more coordinated, suggesting that wide-spread correlations may result from the ability of horizontal connections to drive strong inhibition in widely distributed cortical locations (Fig. 12). In sum, local inhibitory networks may be able to synchronize pyramidal cells only locally when they are not connected to each other, but synchrony over long distances may be enabled when inhibitory networks are interconnected by horizontal connections.

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**FIG. 12.** Model cortical circuit using horizontal network for generating temporal coordination between optical clusters. A: the circle in the center of the figure (ds) depicts an optical cluster whose cells are driven by an electrical stimulus delivered in a tangential brain slice. These pyramidal cells project to 3 optical clusters (d1–d3), forming synaptic connections on both pyramidal cells and inhibitory cells. Outside of these optical clusters, connections are only sparse (d4). B: hypothetical postsynaptic potentials generated in d1–d4 pyramidal cells by horizontal connections arising from the stimulated optical cluster (ds). When the activity of ds pyramidal cells is weak, d1–d3 pyramidal cells reveal purely excitatory postsynaptic potentials, because inhibitory cells are not driven strongly enough to spike. However, when ds pyramidal cells are strongly activated, as occurs during a stimulus train, these inhibitory cells spike and deliver large IPSPs to local pyramidal cells, truncating the time course of the EPSPs. In outlying regions (d4), inhibition is much weaker due to the sparseness of synaptic connections. C: hypothetical spike trains generated by pyramidal cells in d1–d4, and hypothetical peristimulus time histogram (PSTH) for d1–d3. When the horizontal network evokes pure EPSPs in pyramidal cells, spikes are more likely to occur at any time during the ensuing tens of milliseconds, resulting in uncorrelated spikes in pyramidal cells. In contrast, when EPSPs are truncated by strong IPSPs, spike probability increases during a narrow time window lasting only a few milliseconds. This transient depolarization would facilitate synchronous firing among pyramidal cells in iso-orientation domains. Because pyramidal cells in peripheral areas (d4) lack this transient depolarization, their action potentials would remain uncorrelated with those in domains connected by the horizontal network.
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