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Hebbian and non-Hebbian Timing-Dependent Plasticity in the Hippocampal CA3 Region

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Abstract

The timing between synaptic inputs has been proposed to play a role in the induction of plastic changes that enable neural circuits to store information. In the case of spike timing dependent plasticity (STDP) this relates to the interval between a synaptic input and a post-synaptic spike, thus providing a conceptual link to the Hebb learning rule. Experiments have documented STDP in many synapses and brain regions, and computational models have tested its utility in many neural network functions. However, questions remain about whether timing plays a role in plasticity during natural activity, and whether it can function in information storage. The present study used imaging with voltage sensitive dye to investigate the effectiveness of input timing in the plasticity of responses in the CA3 region of hippocampal slices. Plasticity was induced by sequential dual-site stimulation at 10 msec intervals of either synaptic inputs and cell bodies (synaptic-somatic induction) or of two sets of synaptic inputs (synaptic-synaptic induction). Both protocols potentiated responses, with greater potentiation of responses to the first stimulation of the sequence than the second. Neither of these protocols induced depression. Synaptic-somatic stimulation was much more effective than synaptic-synaptic stimulation in evoking somatic action potentials, but both protocols potentiated responses equally well. This suggests that sequential dual-site stimulation can potentiate equally well with very different degrees of somatic action potential firing. With synaptic-somatic induction, potentiation was focused at the sites of stimulation. By contrast, with synaptic-synaptic induction the distribution of potentiation varied greatly. Changes in the spatial distribution of responses indicated that sequential dual-site stimulation functions poorly in the storage of activity patterns. These results suggest that in the hippocampal CA3 region, timed sequential activation of two inputs is less effective than theta bursts, both in the induction of LTP and in the storage of information.

Introduction

Timing differences between synaptic inputs are thought to serve as signals for plastic changes in synaptic strength. Experiments in many synapses and brain regions have demonstrated that the timing difference between a synaptic input and a postsynaptic action potential serves as a determinant of plasticity (Caporale and Dan, 2008; Markram et al., 2012). In general, when a postsynaptic spike follows a synaptic input within a narrow time interval the synapse becomes stronger. This condition for strengthening a synapse fits

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

nicely with the Hebb learning rule in which synapses are reinforced when they contribute to post-synaptic firing. This flags the synaptic input as salient and worthy of strengthening. Conversely, a synaptic input that follows a spike plays no role in driving the post-synaptic neuron to fire, and this 'tag-along' synapse is then targeted for weakening. The potential importance of input timing has spurred considerable interest in spike timing dependent plasticity (STDP), and timing has been incorporated into many computational models for information storage and remodeling in neural networks (Song et al., 2000; Morrison et al., 2008; Clopath et al., 2010; Fiete et al., 2010).

This conceptual appeal of input timing as a cue for plasticity is tempered by questions about its relevance to nervous system function, and whether information about timing differences between synaptic inputs can be exploited to reshape neural networks during learning. Of particular importance is the requirement for post-synaptic spikes. Although STDP has been induced in retinotectal synapses by synaptically-evoked spikes (Zhang et al., 1998), nearly all other experimental studies of STDP employ current injection through an intracellular electrode to evoke post-synaptic action potentials (Lisman and Spruston, 2005, 2010). Thus, further study of the relation between potentiation and post-synaptic spikes would improve our understanding of the role of input timing. The requirement of a post-synaptic spike bears on the biological relevance of STDP (Lisman and Spruston, 2005, 2010), but the issue of the role of timing between two synaptic inputs is actually distinct from the question of whether plasticity depends on spikes. It remains unclear whether timed activation of different synaptic inputs is more effective at inducing potentiation than either sequential activation of the same synaptic inputs, or simultaneous activation of different synaptic inputs. Much of the appeal of timing as a cue for plasticity hinges on its utility in extracting information encoded by the temporal relation between multiple synaptic inputs. But even if plasticity can be induced by sequential activation of distinct synaptic inputs, the effectiveness of timing in storing information in a neural network is a separate issue that requires testing.

The hippocampal CA3 region has an extensive network of recurrent collaterals capable of undergoing long-term potentiation (LTP) (Zalutsky and Nicoll, 1990; Debanne et al., 1998; Rebola et al., 2017), and LTP in these synapses has been proposed to serve in the storage of information (Rolls and Treves, 1998; Cerasti and Treves, 2013; Jackson, 2013; Rolls, 2013). STDP can also be seen in these synapses. One study found that positive and negative timing differences between synaptic inputs and spikes elicited potentiation of similar magnitude, with no depression (Mishra et al., 2016), while another study in organotypic slices reported depression of synaptic inputs when they were activated following a post-synaptic spike (Debanne et al., 1998). However, as with other STDP studies, the action potentials in these experiments were evoked by current injection. To test the efficacy of timing in the plasticity of the CA3 region without using current injection to evoke action potentials, the present study employed extracellular stimulation of two sites sequentially in hippocampal slices. Stimulation of dendritic layers activated synaptic inputs and stimulation of the cell body layer evoked somatic action potentials. Voltage imaging was then used to evaluate the magnitude and spatial distribution of plasticity. Sequential stimulation of two sites potentiated responses, supporting the idea that timing can serve as a cue for plasticity. Similar amounts of potentiation were observed with very different amounts of spiking in

the cell body layer, suggesting that somatic spiking is not required. Although sequential dual-site stimulation protocols induced plasticity, they were ineffective in the storage of spatial patterns of activity in the CA3 network.

Materials and Methods

Hippocampal slices were prepared as described previously (Jackson, 2013), using procedures approved by the Animal Care and Use Committee of the University of Wisconsin. Male rats 4–7 weeks old were killed by decapitation after rendering unconscious with CO₂. Horizontal slices 350 µm thick were cut with a Leica 1200S vibratome in cutting solution (124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 1 mM CaCl₂, 6 mM MgSO₄, 10 mM glucose, saturated with 95% O₂/5% CO₂) at 0 °C. After cutting, slices were incubated for 30 minutes at room temperature in cutting solution, and then transferred to aCSF (cutting solution, but with 1.3 mM MgSO₄ and 2.5 mM CaCl₂) at room temperature. Slices were stained in the voltage-sensitive absorbance dye RH482 (0.075 mg/ml) for 45–60 minutes and returned to aCSF for use within 6 hours.

Voltage imaging experiments followed previous methods (Jackson, 2013), with a few minor modifications. Images were formed using a fiber-optic coupled photodiode camera with 464 hexagonally-arranged photodiodes (Wu and Cohen, 1993; Jin et al., 2002). Photocurrent from each photodiode was amplified to 5V/nA, low-pass filtered at 500 Hz, and digitized at a frame rate of 8–10 kHz with a DAP 5400 data acquisition system (Microstar Laboratories) running on a PC. Slices were illuminated with a UHP-white-T-LN LED (Prizmatix, Southfield, MI) passed through 700/20 or 702/36 nm bandpass filters, to illuminate the slice with 18 mW or 43 mW (measured with a Thorlabs power meter), respectively. Light was collected with a Zeiss Plan-Apochromat 10X objective (NA 0.5) and directed with a sliding mirror either to the photodiode camera or to a CCD camera. With this objective the field of each photodiode was 67 µm. Thus, in the cell body rich stratum pyramidale with ~15 µm cell bodies each photodiode receives signals from well over a hundred somata in a 350 µm thick slice; signals will reflect responses from many more cells in process rich regions. These optical signals are therefore population responses.

Data acquisition, stimulation, and illumination were controlled by a computer program (PhotoZ) developed in this laboratory (Chang, 2006). Responses were clear in each trial, but to improve signal-to-noise, frames were acquired at 30 second intervals, and data displayed as averages of 4 trials. In general, experiments cycled through stimulation of site 1, site 2, and both in succession at the stated timing interval, with a 10 sec interval between separate stimulation trials. For response quantification optical signals were divided by resting light intensity and the maximum signal was read from within a 20 msec window after stimulation. To construct time course plots the peak amplitude was averaged over 6 photodiodes located from maps as the sites with highest potentiation. All data were acquired with slices perfused with aCSF at 30–32 °C.

Slices were stimulated at one or two sites with ~10–20 µm tipped glass micropipettes filled with aCSF positioned with a micromanipulator in the stratum radiatum or stratum pyramidale of the CA3 region (for example, see Fig. 1A). When stimulated at two sites the

electrodes were separated by 350–500 μm . Stimulation pulses of $-75 \mu\text{A}$, 200 μsec were applied with WPI A385 stimulus isolators triggered by the computer. Note that the use of a negative stimulus pulse results in a local negative optical signal in the immediate vicinity of the stimulating electrode caused by a direct action of the stimulus on nearby neurons. All positive optical signals thus represent active neuronal responses. For induction the two sites were stimulated in sequence at 10 msec intervals 100 times. Induction was achieved with 5 episodes of 20 pulses at 10 Hz, and 10 second intervals between episodes. In general, the site labeled ‘Site 1’ (always on the left) received the first stimulation during induction and the site labeled ‘Site 2’ (always on the right) received the second stimulation 10 msec later.

Experimental Design and Statistics.

Imaging data was filtered temporally and spatially with PhotoZ. Color maps of response distributions and potentiation were prepared with PhotoZ. Optical traces and peak response timecourses from selected locations were exported to Origin 2017 for display and analysis. The statistical significance of differences in potentiation was evaluated with the two-sampled *t*-test applied to the 5 time points from time course plots 10–18 minutes after induction. In other evaluations of significance, the *t*-test was used. Error bars in figures are standard errors.

Results

To induce potentiation electrical stimulation was applied to two sites sequentially with a 10 msec delay (20 msec delays were also tested but the potentiation was less robust). Fig. 1A illustrates the positions of stimulating electrodes in the stratum radiatum and stratum pyramidale (stimulating electrodes highlighted with white double arrows). Fig. 1A2 presents a schematic to highlight the neural processes targeted by stimulating the two locations. The electrode in the stratum radiatum activates collaterals of CA3 pyramidal cell axons, along with local pyramidal cell dendrites. The axon collaterals extend broadly through the CA3 region and activate synapses on pyramidal cell dendrites over a large area. The electrode positioned in the stratum pyramidale activates the surrounding cell bodies. Their axons join the CA3-CA3 collateral system, but fewer axons are activated compared with stratum radiatum stimulation so distant responses are smaller. Results presented here support the interpretation that stratum pyramidale stimulation is more effective in eliciting action potentials in the local pyramidal cell somata, and that stratum radiatum stimulation is more effective in activating synaptic inputs. Induction with one stimulation site in the stratum pyramidale and one in the stratum radiatum thus mimics the standard single-cell STDP protocol in which spikes are elicited by current injection (synaptic-somatic induction). Induction with two sites in the stratum radiatum provides a contrast by testing the role of timing between non-identical synaptic inputs (synaptic-synaptic induction).

Response properties and propagation—Electrical stimulation of the two sites indicated in Fig. 1A1 evoked changes in voltage sensitive dye absorbance to generate an optical readout of electrical response versus time over the $\sim 1.6 \text{ mm}$ field of view. This signal represents voltage changes within the cell membrane at a given location, providing an indicator of electrical activity arising from both action potentials and synaptic potentials in

a population of neurons at a given site. Dividing recorded light by resting light generates a response amplitude (I/I) that can be used to track the spread and compare locations. Maps of I/I encoded as color (red=maximal response and dark blue=no response) show responses originating close to the electrodes in the frame acquired 2.5 msec after stimulation (Figs. 1B1 and 1C1), and spreading several hundred microns in frames acquired 1 msec (Figs. 1B1–1B3) and 2 msec (1C1–1C3) later. This sequence of snapshots illustrates the temporal dynamics of responses initiating near the site of stimulation and spreading widely.

To view the spatial extent of responses, the peak of I/I within a time window 3.5 – 23.5 msec after stimulation was encoded as color (Fig. 1D1–1D3). Starting the window 3.5 msec after stimulation excluded the initial spikes that tend to dominate response maps and produce a sharp focus around the stimulating electrode. The 3.5–23.5 msec window excludes the initial synchronous spike and selects the later component representing synaptic potentials as well as asynchronous spikes. Slight increases of the 3.5 msec start time produced qualitatively similar response maps, and since the peak in I/I occurs early in the window, changing the end time of the measure window has no impact. Instead of snapshots at a particular time point, these peak response maps highlight the regions activated, and can be used to evaluate differences in overall spread. Stimulation of the stratum radiatum (Fig. 1D1) induced wider spread than stimulation of the stratum pyramidale (Fig. 1D2), due to the abundance of CA3 pyramidal cell axons in the stratum radiatum (Fig. 1A2). The electrode in the stratum pyramidale stimulated cell bodies, eliciting large action potentials locally as well as synaptic potentials in the vicinity of the stimulating electrode. The response map for sequential stimulation of both sites at 10 msec shows a summation of the single site distributions (Fig. 1D3). The response to site 2 stimulation dominates in this image because of the large spikes elicited in the stratum pyramidale. Comparison of the single-site (Figs. 1D1 and 1D2) and successive dual-site response maps (Fig. 1D3) reveals substantial overlap of response fields, indicating that plasticity which integrates the two inputs can be studied.

The signals from individual photodiodes of the hexagonal array provide a readout of a population-averaged voltage change for the cells at a particular location. These signals generally reveal two components, an initial rapid spike-like component reflecting synchronous action potentials and a slower decaying component. This slow component comprises both excitatory synaptic potentials as well as asynchronous spiking and polysynaptic responses arising from excitatory reverberation through the strongly interconnected circuitry of the CA3 region. Due to this connectivity, voltage sensitive dye signals decay more slowly in the CA3 region than in regions such as the dentate gyrus (Wright and Jackson, 2014) and CA1 region (Chang and Jackson, 2006) which are less interconnected. Pharmacological experiments with Na^+ channel blockers, Ca^{2+} reduction, and receptor antagonists have established the origins of these two components (Grinvald et al., 1982; Jackson and Scharfman, 1996; Kojima et al., 1999). Furthermore, in the present study Ca^{2+} channel blockade with cadmium eliminated the slow component and spared the fast component (Fig. 10), providing additional support that they represent synaptic potentials and action potentials, respectively. Optical traces from selected locations (marked with colored *'s in Fig. 1A) illustrate these characteristics. The traces all have a synaptic component. Near the stimulating electrode in the stratum radiatum, responses have small initial rapid spikes (purple trace, Fig. E1), while more distant sites have only the synaptic

component (green, yellow, red traces, Fig. 1E). Stratum pyramidale stimulation evokes much larger spikes near that electrode (yellow trace Fig. E2), indicating that stimulation in the cell body layer elicits action potentials very effectively. Responses to successive stimulation of site 1 and site 2 show summation to varying degrees depending on location (Fig. E3).

The sequence of snapshots illustrate that responses spread rapidly (Figs. 1B1–1B3 and 1C1–1C3). Since potentiation depends on timing differences, propagation velocity was examined in detail. Fig. 2 illustrates propagation of responses to stratum radiatum stimulation. Traces from three locations (Fig. 2A) demonstrated that in the region between the two sites of stimulation the responses propagated in opposite directions (Fig. 2B). Plots of distance versus response latency (time to half maximum) yield propagation velocity as the slope. These velocities generally averaged ~ 0.25 mm/msec, for propagation either toward or away from the dentate gyrus for both sites (Fig. 2C; proximal signifies closer to the dentate gyrus). This velocity is in the range expected for small unmyelinated axons (Waxman and Swadlow, 1977; Debanne et al., 2011; Ma et al., 2017). Furthermore, the same velocity was measured in 100 μ M cadmium to block synaptic transmission and focus on axonal conduction. This supports the hypothesis that the speed of response spread reflects axonal action potential conduction. Stimulating electrodes were separated by 463 ± 75 μ m (mean $\pm$ SD; N=22), so the timing delays will be altered by < 2 msec due to propagation times from the two sites. With the 10 msec interval between stimulation times employed here, differences in propagation time will prolong the intended delay to no more than 12 msec. Thus, conduction time will not have a significant impact on the timing delays used here to induce potentiation.

Potentiation with Synaptic-Somatic Induction—Synaptic-somatic induction employs the arrangement illustrated in Fig. 1, with stimulation of the stratum radiatum and stratum pyramidale. This protocol imitates STDP induction in single cell studies with spikes evoked by current injection. Fig. 3A presents an image of a slice with two stimulating electrodes positioned accordingly. After collecting baseline responses to alternating stimulation of the two sites, potentiation was induced with 100 pairs of stimulation of the two sites with a 10 msec delay (see Methods for details). Alternating stimulation of the two sites was then resumed. Figs. 3B1 and 3B2 display traces from selected locations to show responses to stimulation of the two sites before and after induction (colored traces correspond to marked locations in Fig. 3A). In this example the stratum radiatum received the first induction pulse (Site 1) and the stratum pyramidale received the second (Site 2). The post-induction traces (black) illustrate potentiation over the pre-induction traces (colored), and the degree of potentiation varied depending on location. Potentiation can manifest as increases in the initial spike-like component and slower synaptic component, but the difficulty of accurate quantification made it impractical to analyze their relative contributions. Taking the peak response in a measure window after the end of the initial spike (3.5–23.5 msec after stimulation) focused on potentiation of the synaptic component of the response.

To display the spatial distribution of potentiation, difference maps were prepared from peak I/I maps such as those in Fig. 1D. Subtracting the pre-induction map from the post-induction map produced a potentiation map. Fig. 4A displays potentiation maps from two experiments with stratum radiatum stimulation first; Fig. 4B presents potentiation maps from two experiments with stratum pyramidale stimulation first. The CCD images of slices (to the

left) aid in the registration of location and stimulation site. Each experiment produced a pair of maps for potentiation of responses to each of the two stimulation sites, with site 1 maps in the center and site 2 maps to the right in Fig. 4. The red patches in these maps represent the locations with the greatest potentiation, and the other colors reflect varying degrees of potentiation according to the color scale in the lower left corner of the bottom-middle panel in Fig. 4B. As a general trend with synaptic-somatic induction, potentiation was focused around the stimulation sites, even though prominent spikes at this location were largely excluded from the 3.5–23.5 measure window. Out of 18 experiments with stratum radiatum stimulation first, 15 had a focus around site 1 in the site 1 potentiation maps and 15 had a focus around site 2 in the site 2 potentiation maps. Similarly, in the 18 experiments with stratum pyramidale stimulation first, 15 experiments had a focus around site 1 in the site 1 potentiation maps and 14 had a focus around site 2 in the site 2 potentiation maps. Since spikes were generally large around the sites of stimulation (Figs. 1E1 and 1E2), the potentiation of the synaptic responses at these locations can be viewed as Hebbian. There is an association with action potentials.

Potentiation also sometimes occurred away from the site stimulated (the top row, right, Fig. 4A and the second row, middle, Fig. 4B), but the locations of these additional sites of potentiation showed no clear trends. For example, potentiation at the border with the dentate gyrus, as illustrated in the maps in Figs. 4A-top and 4B-bottom, was seen in 7 of 36 experiments. Potentiation at additional sites was seen more frequently in responses to stratum pyramidale stimulation; when this site was stimulated first there were 9 such instances and when this site was stimulated second there were 8 (out of 18 total in each case). There were only 5 such instances for responses to stratum radiatum stimulation, either first or second (out of 18).

In many studies of STDP in other brain regions, depression occurred when a synaptic input followed a post-synaptic spike (Bi and Poo, 2001; Caporale and Dan, 2008; Markram et al., 2012). Difference maps from experiments with 10 or 20 msec timing intervals generally failed to reveal regions where responses declined (data not shown). In some cases, regions were found where responses declined by ~10%, but this sometimes happened without induction. Thus, in the present study depression was not observed.

To view the time-course of potentiation, maps were used to locate 6 detectors reporting the highest potentiation in a given experiment (sites all within or bordering the red patches in the maps). The time course of peak I/I (in the 3.5–23.5 msec post-stimulus window) from these 6 locations was averaged within the experiment, normalized to the pre-induction baseline, and then averaged across experiments to generate time course plots displayed in Fig. 5. These plots showed that potentiation developed over the first ~5 minutes after induction and peaked within ~15 minutes. Similar slow timecourses have been observed in STDP studies (Markram et al., 1998; Bi and Poo, 2001). Stratum radiatum responses were potentiated ~45% following induction with stratum radiatum stimulation first (Fig. 5A). Stratum pyramidale responses in these experiments were potentiated ~30%, which is significantly less ($P=0.002$, $N=18$). The greater potentiation of responses to stratum radiatum stimulation offers a Hebbian perspective since spikes in the pyramidal cell bodies followed the synaptic inputs. The opposite induction sequence of stimulating the

stratum pyramidale first was less effective (Fig. 5B). With this order, responses to stratum pyramidale stimulation were potentiated by ~35%, while responses to stratum radiatum stimulation were potentiated by ~28%. This difference was smaller than with the opposite sequence, but still significant ($P=0.0035$, $N=18$). The potentiation of responses to stratum radiatum stimulation was greater when this site was stimulated first than when it was stimulated second (comparing squares in Fig. 5A with squares in 5B; $P<0.001$). The potentiation of responses to stratum pyramidale stimulation was only slightly greater when this site was stimulated first, but the difference was still significant (comparing filled circles in Fig. 5A and 5B; $P=0.031$). Thus, with synaptic-somatic induction, responses to the site stimulated first were potentiated more for either order.

Stimulation of both sites simultaneously at the same frequency used in sequential dual-site stimulation experiments was less effective in potentiating responses to either site. Simultaneous action potentials in two coupled CA3 pyramidal cells were previously reported to produce no LTP (Debanne et al., 1998). Responses to both stratum pyramidale and stratum radiatum stimulation were potentiated ~20% (Fig. 5C), with no distinguishable difference for the two sites of stimulation ($P=0.75$). All forms of sequential stimulation produced significantly more potentiation than simultaneous stimulation, except for the case of stratum pyramidale second (see legend of Fig. 5 for P values). Thus, timed pulses potentiate more effectively than the rate-based plasticity at the same frequency of 10 Hz. Timing between the stimulation of the two sites makes a difference, and serves as a potentiation cue.

Potentiation with Synaptic-Synaptic Induction—The above experiments used stratum pyramidale stimulation to test the role of timing between synaptic inputs and somatic action potentials. To test timing with two synaptic inputs, both electrodes were placed in the stratum radiatum (Figs. 6A1 and 6B1). Traces (colored according to the sites marked in Fig. 6A1) illustrate the responses to stimulation of site 1 (Fig. 6A2), site 2 (Fig. 6A3), or both with a 10 msec delay (Fig. 6A4). These traces, like those in Fig. 1E, show the rapid spike-like responses close to the site of stimulation, slower synaptic responses throughout, and summation to varying degrees at different locations. As with synaptic-somatic induction, analysis with a measure window that begins 3.5 msec after stimulation excludes the initial spike and focusses on the synaptic response component.

Potentiation was once again induced with 100 pairs of sequential paired stimulations of the two sites at 10 msec intervals. Traces in Figs. 6B2–6B3 (colored according to the sites marked in Fig. 6B1) show responses before induction. The responses after induction (black traces) show varying degrees of potentiation depending on location and which site was stimulated. To visualize the distribution of potentiation, potentiation maps were prepared in the same way as Fig. 4. Fig. 7 displays four examples. These maps failed to reveal the trends seen with induction by synaptic-somatic induction (Fig. 4). There were no foci of potentiation near stimulation sites, where response amplitudes were generally largest. The spatial distributions of potentiation varied widely from experiment to experiment. Within an experiment the potentiation maps often differed between the two stimulation sites.

As with synaptic-somatic induction, potentiation maps located sites with the strongest potentiation (the red patches), and timecourses from 6 detectors were averaged, normalized, and averaged across experiments. The time course plots in Fig. 8A exhibited a trend seen with synaptic-somatic induction (Fig. 5A). Responses to the site stimulated first were potentiated by ~45% and responses to the site stimulated second were potentiated by ~25%, which was significantly less ($P < 0.0001$). One hundred stimulations of both sites simultaneously produced very little potentiation, ~10% (Fig. 8B, squares), and this was significantly less than the potentiation seen in Fig. 8A of responses to site 1 ($P < 0.0001$) or site 2 ($P = 0.001$). However, stimulating a single site 100 times with pairs separated by 10 msec produced potentiation of ~20% (Fig. 8B, circles), which was less than site 1 in Fig. 8A ($P = 0.002$) but indistinguishable from site 2 in Fig. 8A ($P = 0.2$). The comparisons illustrate that stimulation of two distinct sites sequentially is more effective than stimulating either one site twice, or two sites simultaneously. This again illustrates the effectiveness of sequential dual-site stimulation above rate-based potentiation. Thus, as with synaptic-somatic induction, timing makes a difference, and is a cue for triggering plasticity. Furthermore, plasticity is greater with timed activation of non-identical populations of synaptic inputs than with activation of the same population at 10 msec intervals.

As noted with somatic-synaptic induction described above, depression was generally not observed with either 10 or 20 msec timing intervals (data not shown). Slight reductions could be found somewhere in the field of view in some experiments, but again similar reductions occurred over similar time periods in the absence of induction.

In an effort to identify response characteristics relevant to potentiation, the responses to successive stimulations of the two synaptic inputs were examined at the sites showing greatest potentiation. The dual-site timed responses (as in Fig. 1E3) from the 6 locations where potentiation was greatest were averaged across experiments. For the sites showing greatest potentiation in response to stimulation of the first site, the response to the first site was greater (Fig. 9A). The amplitude of the second response peaked at a slightly higher level due to temporal summation, but taking the difference from pre-onset to peak, the response to the first stimulation was 26% greater. For sites showing the greatest potentiation in response to stimulation of the second site, the response to the second site was 50% greater (Fig. 9B). Thus, potentiation of responses to stimulation of a particular site occurred where the response to stimulating that site dominated over the response to stimulation of the other site.

Antidromic action potentials

Synaptic-somatic induction potentiated responses with quite different spatial distributions compared to synaptic-synaptic induction. This is likely due to the greater effectiveness of stratum pyramidale stimulation in evoking somatic action potentials. However, action potentials initiated in CA3-CA3 collaterals by stratum radiatum stimulation (Fig. 1A2) can reach pyramidal cell bodies antidromically. To explore the potential impact of antidromic spikes, experiments were conducted with slices bathed in 200 μ M cadmium to block synaptic transmission. A slice was stimulated in the stratum radiatum and stratum pyramidale (Fig. 10A) and responses were displayed in Fig. 10B from the numbered locations marked in the image in Fig. 10A. In cadmium, stimulation of the stratum

pyramidale evoked large spikes near the site of stimulation (red traces 3 and 4 in Fig. 10B), and because synapses were blocked we could see smaller spikes further away (red traces 1 and 2 in Fig. 10B). Stimulation of the stratum radiatum also evoked responses at these locations (black traces in Fig. 10B), and they are presumed to be antidromic action potentials in pyramidal cells. Stimulation of this site will also activate cell bodies locally through their stimulated dendrites. These cell bodies will lie in the stratum pyramidale near a perpendicular line from the site of stimulation to that layer (roughly site #2 in Fig. 10A).

In general, the signals in the stratum pyramidale reflecting antidromic action potentials were much smaller than the signals reflecting action potentials evoked by direct stimulation of the cell bodies (as noted in Methods, stimulus pulses were negative so that positive optical signals are not direct effects of the stimulus pulse). The spikes evoked by stratum pyramidale stimulation were ~6-fold greater than the largest antidromic spikes evoked by stratum radiatum stimulation. The mean antidromic response was plotted versus distance from the largest response (Fig. 10C; note that distance was taken as the number of photodetectors, and their spacing was 67 μm). The dashed line in this plot at $I/I = 0.6 \times 10^{-2}$ reflects the average scaling between spikes evoked by stratum pyramidale and stratum radiatum stimulation. The results presented in Fig. 10C suggest that the number of cells that fire an action potential in response to stratum radiatum stimulation is less than 17% of the number of cells that fire an action potential in response to direct stimulation of cell bodies. These results help in comparing the two different induction protocols employed here, and provide an estimate of the relative effectiveness of synaptic versus somatic stimulation in triggering action potentials. Stimulating the stratum radiatum elicits some antidromic spiking in pyramidal cells, but this firing is more than 6-fold less than the firing elicited by stimulating the stratum pyramidale. Thus, the similar potentiation of responses to site 1 stimulation with synaptic-synaptic induction (Fig. 8A) versus synaptic-somatic induction (Fig. 5A) suggest that somatic action potentials are not a critical requirement for potentiation by timed stimulation.

Distances between sites

For synaptic-somatic induction, potentiation was usually focused at stimulation sites (Fig. 4), but for synaptic-synaptic induction, it was difficult to discern trends in the spatial patterns of potentiation. To look for more subtle features in synaptic-synaptic experiments, distances were determined between 1) the sites of maximal potentiation; and 2) the sites of stimulation and of maximal potentiation. These distances ranged from 374 to 548 μm (Fig. 11A). Site 2 is closest to the site of maximal potentiation of site 2 responses, and this distance was significantly less than two of the other distances presented in Fig. 11A (site 1 potentiation to site 2 and site 2 potentiation to site 1). The other distances were indistinguishable. Furthermore, on an experiment-by-experiment basis, the distances between the locations of maximal potentiation were not correlated with the distance between sites of stimulation (data not shown). Thus, examining stimulation sites and locations of maximal potentiation did not reveal trends or shed light on the spatial distribution of potentiation. However, the shorter distance between site 2 and the site of maximal potentiation of site 2 responses represents a weak parallel with the foci of potentiation around the sites of stimulation noted for synaptic-somatic stimulation (Fig. 4).

Comparison of spatial patterns with Euclidian distance

LTP induction with theta bursts can store complex spatial response patterns in the circuitry of the CA3 region in a hippocampal slice (Jackson, 2013), and computational models have explored the capacity of the CA3 region to perform the operation of pattern completion (Rolls, 2013). The spatial distributions of potentiation with synaptic-synaptic induction were complex and variable (Fig. 7), raising the possibility that sequential dual-site induction also stores patterns. With theta bursts, spatial maps of responses were compared quantitatively using the Euclidian distance (Jackson, 2013), and that analysis was implemented here. In a map denoted as vector a , a_i is taken as peak I/I at location i . To compare map a with map b , the Euclidian distance is calculated by summing over locations.

$$D_E = \frac{\sum_{locations} \sqrt{a_i^2 - b_i^2}}{N_{locations}}$$

$N_{locations}$ is the number of photodiodes from the 464 element photodiode detector. Sites in irrelevant regions (e.g. overlying cortex) were excluded so $N_{locations}$ was generally less than 464. If two maps are identical $D_E = 0$; nonzero D_E values quantify the degree to which two maps differ. The D_E values for pre- versus post-induction represent a global index of the degree of potentiation. The similar values for site 1 and 2 (Fig. 11B) suggest similar potentiation regardless of whether a site was stimulated first or second. This result differs from that seen with the locations of maximal potentiation, where responses to stimulation of the first site were potentiated more (Fig. 8A). This suggests that the greater differences in potentiation between the first and second sites were concentrated at the sites of maximal potentiation, but away from these foci potentiation was more similar.

To test the hypothesis of information storage, the D_E values representing the difference between response maps for stimulation of the two different sites were examined. If information is stored during induction, then site 1 response maps should become more similar to site 2 response maps. Furthermore, the single site maps should become more similar to the maps for the two sites in succession. Fig. 11C shows that the ratios of D_E values before and after induction were very close to one, indicating that the D_E values did not change (Fig. 10C). Thus, the response maps did not become more similar following induction, and this indicates little if any information storage. By contrast, with LTP induced by theta bursts this same test revealed ratios significantly less than one, suggesting that information had been stored (Jackson, 2013). The present study therefore suggests that sequential dual-site stimulation is less effective than theta bursts in storing patterns of activity in the CA3 region.

Discussion

This study investigated plasticity induced by sequential dual-site stimulation in the CA3 region of rat hippocampal slices using voltage imaging. Previous studies in hippocampal slices demonstrated that optical voltage signals can be potentiated by LTP-inducing protocols such as high frequency stimulation and theta bursts (Saggau et al., 1986; Momose-Sato et al., 1999; Hosokawa et al., 2003; Aihara et al., 2005; Chang and Jackson, 2006;

Wright and Jackson, 2014, 2015). These optical signals generally reflect both synaptic potentials and action potentials, and the potentiation observed with voltage imaging can reflect strengthening of excitatory synapses, increased spiking (both as a consequence of synaptic potentiation and through enhanced excitability, i.e. enhanced EPSP-spike coupling), and depressed inhibition. The present study showed that population responses can be potentiated with induction protocols that recapitulate previously employed single-cell STDP protocols (Markram et al., 1997; Bi and Poo, 2001; Caporale and Dan, 2008; Markram et al., 2012; Mishra et al., 2016). With maximal potentiation of ~45%, the plasticity seen here was not as robust as the LTP induced in the CA3 region by theta bursts, where responses were potentiated 60% with single site stimulation and 80% with simultaneous dual site stimulation (Jackson, 2013). The present study identified timing between stimulations of different sites as a factor. The greater potentiation seen with timed stimulation of two sites at intervals versus potentiation by simultaneous stimulations of different sites or paired stimulations of the same site establishes timed activation of different inputs as a significant factor that can potentiate more effectively than repetitive stimulation at the same rate. This work thus established that voltage imaging can be used to study the impact of sequential dual-site stimulation on potentiation at the population level. This provides results that may be relevant to studies of STDP at the single-cell level, and reveals aspects not readily seen in single-cell experiments such as the spatial distribution of potentiation, the relation to local spiking activity, and the capacity of sequential timing to store information.

Stimulation of pyramidal cell bodies in the stratum pyramidale was much more effective at evoking somatic action potentials than stimulation of Schaffer collateral axons in the stratum radiatum. This was evident in the magnitudes of spike-like components of responses, and was especially clear in experiments in which cadmium was used to block synapses (Fig. 10). These experiments indicated that direct stimulation of the cell bodies in the stratum pyramidale evoked population spikes 6-fold greater than the largest population spikes evoked by stimulation of the stratum radiatum. In light of this large difference in spike amplitude, it is significant that similar degrees of potentiation were observed either by synaptic-synaptic induction with stimulation of two sites in the stratum radiatum, or by synaptic-somatic induction with stimulation of sites in stratum pyramidale and stratum radiatum. Since synaptic-synaptic induction evokes much weaker somatic action potentials, the similar maximal degree of potentiation with the two protocols argues against a critical role for post-synaptic spikes. This relates to concerns about the biological role of STDP, which has been questioned because previous single-cell studies depended on spikes induced by the 'unnatural' procedure of direct current injection through an intracellular recording electrode (Lisman and Spruston, 2010). Stimulating the stratum radiatum does not induce spikes in this way, and compared to stratum pyramidale stimulation, was much less effective at evoking spikes, both locally and antidromically. The observation of comparable magnitudes of potentiation by these two induction paradigms thus argues against a Hebbian dependence on postsynaptic action potentials in the soma. Somatic action potentials may be sufficient for potentiation by timed stimulations, but they are not necessary. It will be interesting to test the activation of dual synaptic inputs in single-cell recordings, where the synaptic triggering of an action potential can be directly observed. However, the irregular

spatial distributions of potentiation with the synaptic-synaptic induction (Fig. 7) indicate locating the cells that are potentiated may prove challenging.

In contrast to synaptic-synaptic induction, synaptic-somatic induction produced foci of potentiation around the stimulating electrodes. Potentiation at the site of somatic action potentials implies that this induction paradigm engages a Hebbian mechanism (Fig. 4). Potentiation without cell body stimulation was not associated with large somatic spikes, but smaller dendritic spikes could play a role. This may indicate that action potentials in dendrites can support plasticity, perhaps as effectively as action potentials in the soma. The critical factor could be local calcium entry, activating signaling cascades in dendrites rather than in cell bodies (Kampa et al., 2007). With theta burst induction in the CA1 region, LTP was strongly correlated with local spikes, again consistent with a Hebbian mechanism (Chang and Jackson, 2006).

Dendritic calcium signals have been invoked in a study of STDP in the CA3 region, where similar potentiation occurred regardless of whether synaptic inputs preceded or followed the post-synaptic spike (Mishra et al., 2016). However, in organotypic slice culture depression occurred when presynaptic action potentials followed postsynaptic action potentials (Debanne et al., 1998), and this is consistent with studies in other brain regions. Mishra et al (2016) observed symmetrical potentiation with no depression. The present study with sequential dual-site stimulation found no depression with 10 or 20 msec intervals, but longer intervals were not tested and could prove effective. In contrast to the findings of Mishra et al (2016), the present study found some asymmetry, with unequal degrees of potentiation depending on whether synaptic inputs were activated before or after postsynaptic spikes. This may reflect a difference between STDP at the single-cell and population level, but it is also possible that animal age is a factor as the present study used older animals than those used in most patch clamp studies.

Synaptic-somatic induction potentiated the area around the site of stimulation, but synaptic-synaptic induction potentiated variable regions distributed widely. The complex spatial distribution of potentiation with synaptic-synaptic induction prompted a more detailed examination of these distributions to explore the idea that this sequential dual-site induction paradigm aids in the storage of response patterns in the circuitry of the CA3 network. One could envisage a result in which induction expanded the response map of the first stimulation to encompass regions activated by both stimulations. LTP induction by theta burst stimulation had been found to expand response maps and make them more similar, suggesting that this form of plasticity can store information in the CA3 network (Jackson, 2013). By contrast, the present results indicated that sequential dual-site induction did not make response maps evoked by stimulation of the two sites more similar. This may indicate that sequential dual-site stimulation is less effective than theta bursts in storing patterns of activity. Computation studies suggest that STDP is effective in storing temporally sensitive computational tasks such as sequence learning (Abbott and Nelson, 2000). Future experiments with more refined induction protocols may help clarify the role of timing in the storage of information in the CA3 region of the hippocampus. It may be that millisecond timing of inputs is less important for plasticity in the CA3 region. Timing of inputs is likely to be more important in brain regions where timing differences encode key features of the

environment. STDP has been reported in visual (Froemke and Dan, 2002), somatosensory (Feldman, 2000; Letzkus et al., 2006), and auditory (Vickers et al., 2018) cortex. Brain regions that receive primary or early stage sensory inputs often detect and process timing differences. It would be worthwhile to test the role of timing in information storage in other brain regions where timing of inputs has a clear role in encoding information.

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Fig. 1.

Imaging voltage changes induced by stratum radiatum and stratum pyramidale stimulation. **A1.** CCD image of the CA3 region of a hippocampal slice. The stimulation electrodes are visible and the tips are highlighted with double arrows. **A2.** A schematic based on the image in A1 presents the relevant anatomical structures targeted by the two stimulation sites. The electrode to the left in Site 1 is in the stratum radiatum (str rad) and the electrode to the right in Site 2 is in the stratum pyramidale (str pyr). The stratum pyramidale forms an arc from upper left to lower right, with the stratum oriens above and stratum radiatum below. The dentate gyrus is below and the edge is visible at the bottom of **A1**. Pyramidal cell dendrites extend into the stratum radiatum. Axons arising from pyramidal cells course through the stratum radiatum and form synapses on pyramidal cell dendrites. Colored *'s at two sites in the stratum pyramidale (red and yellow) and two sites in the stratum radiatum (green and purple) indicate locations of traces in E1, E2, and E3. **B** and **C.** The amplitudes of optical signals ($\Delta I/I$) at specific time points were encoded as color to show the dynamic spread of responses through this slice 2.5, 3.5, and 4.5 msec after stimulation of Site 1 (**B1–B3**) or Site 2 (**C1–C3**). **D.** The maximum response at each location (peak $\Delta I/I$ in the 3.5–23.5 msec window after stimulation) was encoded as color to illustrate the spatial extent of responses to stimulation of Site 1 (**D1**), Site 2 (**D2**), or Sites 1 and 2 with a 10 msec delay (**D3**). Color scales in **B1** and **D3** apply to all maps – dark blue signifies no response ($\Delta I/I = 0$), red signifies maximal response (100%). **E.** Traces of optical signal versus time from locations labeled with *'s of the same color in **A**. Responses to Site 1 stimulation (**E1**), Site 2 stimulation (**E2**), and Site 1 followed by Site 2 (**E3**). The vertical arrows below indicate times of stimulation. The response near Site 1 (purple) has a small spike component in **E1**. The response near Site 2 (yellow) has a large spike component in **E2**. Traces were averages of 4 trials at 30 second intervals, here and throughout this study.

Fig. 2.

Propagation velocity. **A.** An image of the CA3 region of a slice with two stimulating electrodes in the stratum radiatum highlighted with double arrows. Three sites are labeled with colored *'s. **B.** Optical traces from sites marked in **A** in the corresponding color. Above. Responses to stimulation of Site 1. The earliest response is in the purple trace close to Site 1. The onset of responses in the red and green traces report propagation away from Site 1 and toward Site 2. Below. Responses to stimulation of Site 2 show the opposite sequence. The arrows indicate the time of stimulation. **C.** Mean velocities from sequences of responses such as those in **B**. The time to half maximum was plotted versus distance with 3–7 points from each experiment to obtain a velocity from the slope of a linear fit. Velocities were determined from the site near the dentate gyrus toward and away from the dentate gyrus to give prox → prox and prox → dist velocities, respectively. Velocities were determined from the site away from the dentate gyrus in both directions to give dist → prox and dist → dist velocities, respectively. Separate experiments in 100 μ M cadmium were conducted with single sites of stimulation in the stratum radiatum. Values are means of 19–23 measurements $\pm$ standard error.

Fig. 3. Potentiation with sequential stimulation of the stratum radiatum and stratum pyramidale. **A.** An image of a slice with the two stimulating electrodes (highlighted by double arrows), and 4 sites marked with colored *'s. **B1.** The colored traces show responses at the corresponding locations to stimulation of Site 1 in the stratum radiatum. The black traces show the responses after induction by 100 sequential stimulations of Site 1 and Site 2 with a 10 msec delay. The largest potentiation was seen in the red trace near the site of stratum radiatum stimulation. **B2.** Responses at the same locations to stimulation of the stratum pyramidale before and after induction. The largest potentiation was seen in the yellow trace near the site of stratum pyramidale stimulation.

Fig. 4. Potentiation maps for somatic-synaptic induction. **A.** Two examples with stratum radiatum stimulated first. **B.** Two examples with stratum pyramidale stimulated first. Images of slices on the left show the positions of the stimulating electrodes, highlighted with double arrows. In each case Site 1 is to the left and Site 2 is to the right. The maps are color-coded differences (scale bottom center) between peak I/I responses (in the 3.5–23.5 post-stimulus time window) before and after induction. The middle maps are potentiation to Site 1 stimulation and the right maps are potentiation to Site 2 stimulation. All 8 maps have a focus of potentiation at or near the site of stimulation for that map, as can be seen by the proximity of the white double arrows to a colored focus. Some maps show potentiation at other locations. For example, in the top row both maps have a potentiation focus at the extreme right edge near the boundary with the dentate gyrus. The bottom row shows a focus only in the Site 1 map in the stratum oriens.

Fig. 5.

Potentiation time course plots with somatic-synaptic induction. Each plot represents peak I/I response amplitude (in the 3.5–23.5 post-stimulus time window) normalized to the pre-induction baseline, averaged over 18 experiments. Potentiation maps as in Fig. 4 were used to find the 6 detectors with the highest potentiation. Peak responses at each time point from these 6 locations were averaged and normalized, and these averages were averaged across experiments. Vertical arrows below indicate time of induction. **A.** Potentiation time course with the stratum radiatum stimulated first. **B.** Potentiation time course with the stratum pyramidale stimulated first. **C.** Potentiation time course with simultaneous stimulation of both sites. In all panels the circles are responses to stratum pyramidale stimulation and the squares are responses to stratum radiatum stimulation (indicated by the legend in **A**). The potentiation of responses to stratum radiatum stimulation was significantly greater than of responses to stratum pyramidale stimulation ($P = 0.00019$, $N = 18$). In **B** the potentiation of responses to stratum pyramidale stimulation was significantly greater than of responses to stratum radiatum stimulation ($P = 0.0035$, $N = 18$). In **C** the potentiation of responses to stratum radiatum and stratum pyramidale stimulation were indistinguishable ($P = 0.75$, $N = 18$). The potentiation of responses in **A** to stratum radiatum stimulation was significantly greater than of stratum radiatum responses in **C** ($P < 0.0001$), but the potentiation of responses to stratum pyramidale stimulation was indistinguishable between **A** and **C** ($P = 0.3$). The potentiation of responses in **B** to both stimulation sites was significantly greater than in **C** ($P = 0.028$ for stratum pyramidale and 0.027 for stratum radiatum).

Fig. 6.

Responses and potentiation with synaptic-synaptic induction. **A1.** Image of a slice with two stimulating electrodes in the stratum radiatum and four sites marked with colored *'s. **A2.** Responses to stimulation of Site 1 on the left at the locations indicated by colors. **A3.** Responses to stimulation of Site 2. **A4.** Responses to stimulation of Sites 1 and 2 with a 10 msec delay. Vertical arrows indicate time of stimulation. **B1.** Image with stimulating electrodes and labeled sites. **B2.** Responses to Site 1 stimulation at locations marked by colors. Potentiation was induced by 100 timed stimulations of Site 1 and 2. The colored traces were recorded before induction and the black traces after. **B3.** Responses to Site 1 before and after induction with colors as in **B2**.

Fig. 7.

Potentiation maps for synaptic-synaptic induction (as in Fig. 4). Images of slices on the left have two stimulating electrodes in the stratum radiatum. Potentiation was induced with 100 timed pulses, with Site 1 stimulated first and Site 2 stimulated 10 msec later. The middle images are maps of potentiation of Site 1 responses and the right images are maps of potentiation of Site 2 responses. In each pair of maps the scale is normalized to the maximum of the map with the greater potentiation maximum; this illustrates the trend of greater potentiation of responses to Site 1 (dark blue – no response, red - maximal response, following the scale bottom center). These experiments illustrate the absence of trends in the spatial distribution of potentiation with this induction paradigm.

Fig. 8.

Potentiation time course plots for synaptic-synaptic induction. Plots were constructed as in Fig. 5. **A.** Responses to Site 1 (stimulated first during induction) are represented by filled circles; responses to Site 2 stimulation are represented by filled squares. **B.** The potentiation of responses by induction with 100 simultaneous pulses to the two sites (filled squares) and with 100 pairs of pulses applied to the same site (filled circles). The potentiation of Site 1 responses is significantly greater than of Site 2 responses ($P < 0.0001$, $N = 21$). The potentiations of Site 1 and Site 2 responses in **A** are greater than the potentiation with simultaneous induction in **B** ($P < 0.001$ for both Site 1 and Site 2, $N = 25$ for simultaneous). The potentiation of Site 1 responses in **A** was greater than of the pair responses in **B** ($P = 0.002$, N for pairs = 15), but the potentiation of site 2 responses in **A** was indistinguishable from the potentiation of the pair responses ($P = 0.2$).

Fig. 9.

Responses to Site 1 and Site 2 stimulation with a 10 msec delay. With synaptic-synaptic induction, the six detectors with greatest potentiation were found in maps such as in Fig. 7. The responses at those locations to stimulation of Site 1 and Site 2 at 10 msec delays were then averaged. These averages were averaged again across the 21 experiments used for the time course plots from Fig. 8. **A.** The average response to timed dual-site stimulation at the site with maximal potentiation of Site 1 responses. The first spike was 26% greater than the second (taking temporal summation into account). **B.** The average response to timed dual-site stimulation at the site with maximal potentiation of responses to site 2 stimulation. The second spike was 50% greater than the first.

Fig. 10.

Direct and antidromic spikes in stratum pyramidale. 200 μM cadmium was added to block synaptic transmission. **A.** An image of a slice with stimulating electrodes in the stratum radiatum and stratum pyramidale. Numbered locations refer to traces shown in **B.** **B.** Traces from the locations in **A** in the stratum pyramidale. The red traces are spikes evoked by direct stratum pyramidale stimulation and the black traces are antidromic spikes activated by stratum radiatum stimulation. **C.** Plot of antidromic spike amplitude versus diode number from the peak antidromic spike (fields of diodes separated by 67 μm) along the arc of the stratum pyramidale. The dashed curve is the maximal response to stratum pyramidale stimulation with the same stimulus current ($-75 \mu\text{A}$, 200 μsec). Note that stimulus pulses were negative so the direct effect was opposite to the active response. The maximal antidromic spikes were $1/6^{\text{th}}$ the amplitude of action potentials evoked by direct stratum pyramidale stimulation.

Fig. 11.

A. Distances between sites in synaptic-synaptic induction. The stimulus electrodes were separated by $461 \pm 79 \mu\text{m}$ (mean $\pm$ SD, $N = 21$). Bars are means $\pm$ SE. The first bar represents the mean distance between the foci of highest potentiation of responses to Site 1 and Site 2. The next four distances are between sites of stimulation and sites of greatest potentiation. The dashed horizontal lines indicate statistically significant differences. The distance from Site 2 to the site of maximal potentiation of responses to Site 2 was the shortest and significantly less than the distance between Site 2 and maximal potentiation of Site 1 responses ($P = 0.018$), and between Site 1 and the maximal potentiation of responses to Site 2 ($P = 0.013$). **B.** Euclidian distances between response maps before and after synaptic-synaptic induction. These distances reflect a global measure of potentiation for the entire slice; the distances for Site 1 and Site 2 are indistinguishable. **C.** Euclidean distances between the Site 1 response map and the map of responses to Site 1 and Site 2 with a 10 msec delay (dual) were determined before and after induction. The ratio of these distances was near one, indicating that the response map of Site 1 did not become more like the dual-site response map. Likewise, the ratio of the pre- and post-induction distances between the Site 2 map and the dual-site map, as well as the distance between the Site 1 and Site 2 maps were near one, indicating no shift in the maps towards one another following induction. Thus sequential dual-site induction failed to store information.