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Interlamellar CA1 network in the hippocampus

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To understand the cellular basis of learning and memory, the neurophysiology of the hippocampus has been largely examined in thin transverse slice preparations. However, the synaptic architecture along the longitudinal septo-temporal axis perpendicular to the transverse projections in CA1 is largely unknown, despite its potential significance for understanding the information processing carried out by the hippocampus. Here, using a battery of powerful techniques, including 3D digital holography and focal glutamate uncaging, voltage-sensitive dye, two-photon imaging, electrophysiology, and immunohistochemistry, we show that CA1 pyramidal neurons are connected to one another in an associational and well-organized fashion along the longitudinal axis of the hippocampus. Such CA1 longitudinal connections mediate reliable signal transfer among the pyramidal cells and express significant synaptic plasticity. These results illustrate a need to reconceptualize hippocampal CA1 network function to include not only processing in the transverse plane, but also operations made possible by the longitudinal network. Our data will thus provide an essential basis for future computational modeling studies on information processing operations carried out in the full 3D hippocampal network that underlies its complex cognitive functions.

longitudinal axons | CA1 association fibers | sequence memory | DHR

The hippocampus is widely used to study functional connectivity of the brain with the hope that principles that operate within its relatively simple architecture may be extended to more complex cortical structures. Its manageable number of cell types also provides an attractive opportunity to examine fundamental issues in neuroscience such as the relationship between network circuitry and function. For example, considerable effort has been devoted toward elucidating the circuitry supporting episodic memory—a property closely linked to the hippocampus. CA3 pyramidal neurons form extensive recurrent connections with each other (1). Such connections are able to learn to associate components of an input pattern with each other (2), which, in turn, has greatly influenced thinking on the mechanisms of memory formation and recall (3). Under appropriate conditions, computer simulations reveal that recurrent neural networks have the capacity to learn temporal sequences and to carry out pattern completion (4, 5). Interestingly, although they are quite near to the CA3 region, CA1 pyramidal neurons reportedly form remarkably few associational connections (6, 7). This distinctive difference in network architecture might suggest that, although area CA1 could serve to decode the output of CA3, it would not possess the intrinsic ability for autoassociational computations. This idea would imply that the ability of CA1 to carry out independent information processing operations may be more limited than that of CA3. However, even after removal of all input from area CA3, CA1 pyramidal neurons still have the capacity to transform location-modulated signals from the entorhinal cortex into accurate spatial firing patterns (8). In addition, deficits in temporal sequence learning are more severe after selective lesions to CA1 than to CA3 (9). Finally, CA1 is more closely linked to memory of temporal order of visual objects and especially over long intervals (10). Thus, area CA1 appears to have a greater ability for intrinsic information processing than would be expected based on current understanding of its circuitry. Intrinsic processing could represent autoassociational computations through direct excitatory synaptic contacts among the CA1 pyramidal cells, but as noted, there is little evidence for such connectivity within CA1. This puzzle led us to reexamine the apparent sparseness of associational synaptic connections between CA1 pyramidal neurons using experimental techniques that were not previously available for this investigation.

The "trisynaptic circuits" (dentate gyrus: CA3–CA1) oriented transversely to the hippocampal long axis, the basis of the "lamellar hypothesis" (11), has greatly influenced thinking about the structure-function relationships of this structure. This hypothesis suggests that the hippocampus is organized as a stack of parallel, trisynaptic circuits. Although this view has been challenged by the observation of fibers running across lamellae, especially in dentate gyrus and CA3 area (12, 13), the hypothesis supported an explosion in the use of the transverse slice for electrophysiological studies of the hippocampus. However, axons oriented along the longitudinal axis are unavoidably severed in the preparation of the transverse slice, meaning that these studies are heavily weighted in favor of conclusions based on fibers traveling within the transverse plane. We used a whole hippocampus preparation, as well as longitudinal and transverse slice preparations, to obtain a more accurate picture of synaptic connections among CA1 pyramidal neurons in three dimensions. Remarkably, we found prominent associational connectivity along the longitudinal axis. Furthermore, synapses of the longitudinal network possess the capacity for synaptic plasticity that

Significance

It has generally been thought that CA1 cells form only negligible connections with each other along the longitudinal axis of the hippocampus. But if CA1 cells were interconnected in an effective autoassociational network, this information would add a critical new dimension to our understanding of cellular processing within this structure. Here, we report the existence of a well-organized, longitudinally projecting synaptic network among CA1 pyramidal neurons. We further show that synapses of this network are capable of supporting synaptic plasticity, including long-term potentiation, and a short-term memory mechanism called "dendritic hold and read." These observations will contribute to the construction of more realistic models of hippocampal information processing in behavior, memory, and other cognitive functions.

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The authors declare no conflict of interest.

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includes a novel memory mechanism we recently described called dendritic hold and read (DHR) (14). These findings may help to explain the intrinsic ability of area CA1 to process information transfer and provide novel data that will lead to more realistic models of hippocampal function in three dimensions.

Results

Longitudinal Connections Between CA1 Pyramidal Neurons. The axonal arborization of individual CA1 pyramidal neurons was examined by two-photon fluorescence imaging of cells filled with Alexa Fluor 594 dye using the mouse whole hippocampal preparation. This preparation maintains intact axonal processes and, thereby, permits following and reconstructing axonal processes in all dimensions. We found that the axonal processes were multiply branched and that, in fact, all CA1 pyramidal neurons examined (n = 9/9) had a distinct projection along the longitudinal axis. These processes are oriented perpendicularly to the transverse hippocampal lamellae (marked in red and with arrows in Fig. 1 *A* and *B*) and are readily distinguished from previously described local associational processes that ramify with a transverse orientation (6, 7, 15) (marked in green in Fig. 1). The longitudinal branch arises from the thick proximal axon that gives rise to the transversely oriented axons (Fig. 1 and Fig. S1*B*). All of the cells send a projection toward the subiculum; a branch heading to the fimbria, first described by Cajal (1911) (16), was observed in 8 of the 9 cells (Fig. 1 *B* and *C*). These subicular and fimbrial branches are consistent with the lamellar organizational principle (11); however, the extensive and organized projection of longitudinally projecting branches may be at variance with it (Fig. 1*C*), depending on the actual physiological influence that these branches exert on their target cells (see ref. 13 for discussion).

If these morphologically identified structures are part of an operationally significant associational network of CA1 pyramidal cells, they should form excitatory synapses onto other CA1 pyramidal cells. To examine the morphology and electrophysiology of the longitudinal network in more detail, a separate set of studies was conducted by filling single cells with dye in the longitudinal hippocampal slice preparation (Fig. 2*A*). With two-photon imaging, we observed that the CA1 pyramidal neuron sends a thick primary axon (PA; Fig. 2*B*, red arrow and Fig. S1) toward the alveus, presumably heading for either the subiculum or the fimbria. A thin collateral arose from the primary axon and projected longitudinally (Fig. 2*B*, red arrow and Fig. S1). The branch point was located on average 154 ± 30 μm from the cell body (n = 7), and the thin branches were followed in the longitudinal plane for up to 375 ± 22 μm (n = 7). Both PAs and LAs were studded with varicosities presumed to represent presynaptic terminals. The longitudinal axon (LA) either projected toward the ventral-temporal (referred to as "temporal") end (n = 2/7) or toward the dorsal-septal ("septal") end (n = 5/7). Similar results were obtained with reconstruction of biocytin-labeled cells (Fig. 2C and Fig. S2). The thick PAs were truncated in the longitudinal slice and appeared as short stumps. They were best observed in slices made at an angle of 45° with respect to the longitudinal axis (Fig. S1). LAs were significantly thinner than PAs, whereas the varicosity density per unit length of LAs was higher than that of the PAs (Fig. S3). If the varicosities actually represent sites of synaptic contact, then they should show prominent voltage-gated calcium signals when the axons are stimulated. To test this prediction, we imaged the varicosities in calcium-sensitive, dye-filled cells and measured action potential-evoked calcium responses within varicosities on longitudinal branches (white arrows in Fig. 2 *B* and *D*). The calcium responses were reliably propagated along the varicosities for up to 456 μm from the cell body without substantial decrement, demonstrating that the responses were induced by propagating action potentials and not by passive depolarizations from the neuronal somata (in which case the responses would have declined monotonically because of the electrotonic decay of the depolarization) (Fig. S4). Hence, it is likely that the axonal varicosities are presynaptic transmitter release sites.

It has been proposed that the previously described sparse associational projections to neighboring transverse lamellae in CA1 are primarily directed onto inhibitory interneurons (17, 18). To investigate whether the associational axonal processes described here represent excitatory-excitatory or excitatory-inhibitory connections, we looked for immunohistochemical evidence of contact with synaptic spines of pyramidal neurons. Spinophilin is exclusively localized at spine heads of hippocampal pyramidal neurons (19), and spines are rarely found on inhibitory interneurons (some numerically small interneuronal subtypes are exceptions).

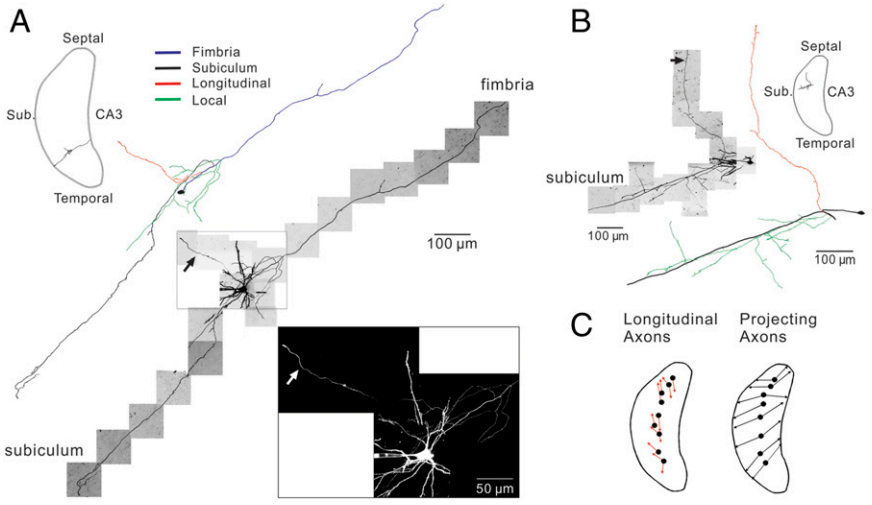

Fig. 1. Two-photon imaging of longitudinal axons in the whole hippocampal preparation. (*A*) The CA1 pyramidal cell region is viewed from above the hippocampus in the axis of the main apical trunk. A Neurolucida drawing of the axonal arborization of a CA1 pyramidal neuron is placed adjacent to the raw inverted fluorescence image. Higher magnification of parts of the fluorescence image is provided in *Lower Inset*. The longitudinal axons in the fluorescence images are identified by arrows. The relative position of the cell within the hippocampus is shown in *Upper Inset*. The four distinct parts of the axonal arbor are color coded. The longitudinal axon (red) can be distinguished from the local associational branches (green) by its perpendicular orientation to the main external projecting axons of the cell. (*B*) Example of another CA1 pyramidal neuron with a prominent longitudinal axon. The portion of its axonal process that projects to the fimbria in this neuron was not found. (*C*) Summary of two-photon imaging demonstrating the organized pattern of longitudinal and projecting axons.

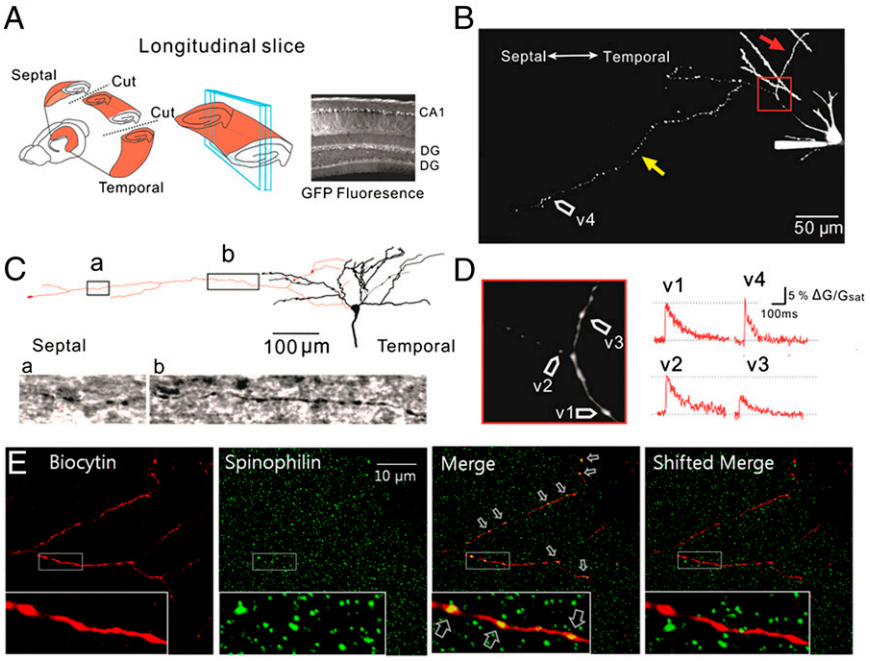

Fig. 2. Excitatory to excitatory longitudinal connections observed in longitudinal slice preparation. (*A*) Longitudinal hippocampal CA1 slices were prepared by removing the curved regions at the septal and temporal ends of hippocampus. The remaining portion was further sectioned longitudinally. Longitudinal slices typically contained one layer of CA1 pyramidal neurons and two layers of dentate granular cells. In initial experiments, we made longitudinal slices from hippocampi obtained from *thy1*-GFP–labeled mice (27), in which GFP labeling was restricted to subgroups of pyramidal neurons, and we verified that our cutting method preserves longitudinal axonal projections in the slices. (*B*) CA1 neurons from longitudinal slices typically produced a long thin axon (yellow arrow) that comes off a thick axon (red arrow). The image is a composite from a z stack of 2-P images of Alexa 594 fluorescence. (*C*) A CA1 longitudinal axon was filled with biocytin and reconstructed by using Neurolucida. The axons emerged from the somata of CA1 pyramidal neurons and coursed from the temporal to the septal regions in the stratum oriens (s. oriens), giving off collateral branches along the way. Varicosities were shown in proximal axon (a) and distal axon (b) along longitudinal direction. (*D*) Action potential-evoked calcium transients were observed from the dispersed axonal varicosities shown in Fig. 1*B*. (*E*) Morphological evidence of excitatory CA1 to CA1 synaptic contacts. When the spinophilin (green) and biocytin-labeled (red) images of a double immunostaining cell are merged, the varicosities on axons coregistered with the locations of spinophilin staining on excitatory pyramidal spine heads (yellow with arrows). Shifting the two images to simulate random superposition produced no overlap.

We asked whether longitudinally oriented CA1 axons labeled with biocytin terminated on spinophillin-positive postsynaptic elements (Fig. 2*E*). The extensive colocalization of presynaptic varicosities with spinophilin puncta provided morphological evidence for excitatory pyramidal cell-to-pyramidal cell synaptic connections.

Functional evidence of excitatory synaptic connections was confirmed by patch clamp recording of postsynaptic neurons combined with photolysis of caged glutamate in presynaptic CA1 pyramidal neurons in the longitudinal slice preparation. Precise and efficient targeting of presynaptic neurons was made possible by a recently developed 3D-holographic photolysis technique (Fig. S5) (20–22). Focal photolysis of caged glutamate onto neuronal somata located hundreds of microns away from the recorded cells frequently led to multipeaked postsynaptic depolarizations, indicative of excitatory synaptic responses triggered by burst firing of presynaptic neurons (Fig. 3*A*). This inference was tested by bath application of 50 μM cadmium, which blocks synaptic transmission (Fig. 3*D*). Cadmium completely abolished the responses, confirming that they did not reflect retrograde propagation of action potentials to the recorded cell body. Taken together, the results shown in Figs. 2 and 3 suggest that the longitudinal associational projections provide excitatory synaptic connections between CA1 pyramidal neurons.

We next compared the CA1 synaptic connectivity of longitudinal CA1 slices (i.e., cut at ~90° with respect to the transverse axis) with that of differently angled slices (cut at angles of 15° or 45° with respect to the longitudinal axis). We recorded from a CA1 pyramidal cell and then uncaged glutamate at distances of up to 600 μm away along the longitudinal axis on either the temporal or septal sides of the cell. Stimulation along the longitudinal axis produced considerably larger EPSPs in CA1 cells than those recorded in transverse

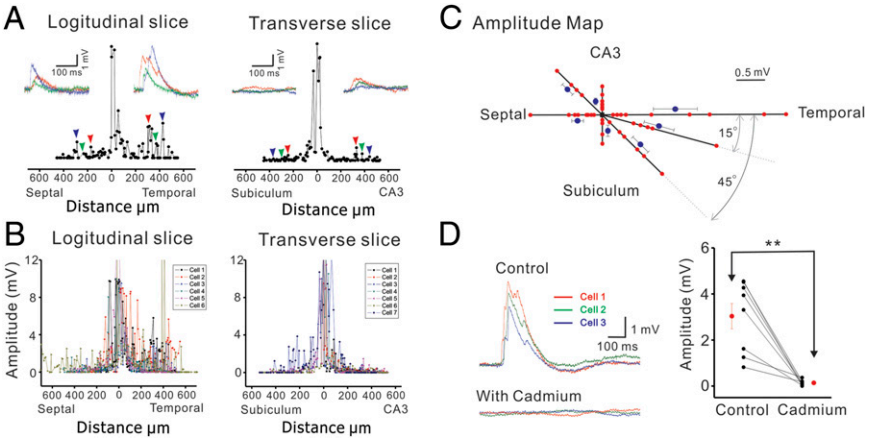

Fig. 3. Electrophysiological evidence of CA1 excitatory to excitatory synaptic contacts in differently angled slices. (*A*) Focal photostimulation of pyramidal neurons located up to 500 μm away in the longitudinal slice can evoke EPSPs. The probability and amplitude of the responses are greater when stimulation is positioned along the longitudinal orientation in the longitudinal slice (*Left*), which contrasts with the lower amplitudes of responses obtained from pyramidal cells in the transverse slices (*Right*). Colored arrows indicate the uncaging position and neuronal responses (traces shown above) recorded at that position in response to uncaging of glutamate at point zero. (*B*) Group data of the focal photostimulated responses for longitudinal and transverse slices. (*C*) Hippocampi were also prepared at different degree angles with respect to the longitudinal axis (i.e., the horizontal line) and the transverse axis (the vertical line), and we compared the averaged amplitudes of responses at distances ranging from 250 to 350 μm away from a recorded cell. Red dots indicate averaged amplitudes from a single cell. Blue dots indicate averaged amplitudes of all cells tested from all slices cut in the indicated plane. The response amplitude is represented by distance from zero along each axis; see calibration bar above. Error bars represent SEM. (*D*) Cadmium blocks the observed synaptic events consistent with the idea that the measured EPSPs are due to presynaptic release. Error bars represent SEM. $^{**}P < 0.01$.

slices with glutamate uncaging given on either the transverse side (Fig. 3 *B* and *C*; longitudinal, 0.94 ± 0.23 mV, $n = 19$ slices vs. transverse, 0.27 ± 0.05 mV, $n = 10$ slices, $\chi^2 = 6.78$, $P = 0.009$, Kruskal–Wallis ANOVA). The mean amplitudes of the responses evoked by photolysis at different distances are plotted as a function of the slice orientation in a group of cells (Fig. 3*C*). In Fig. 3*C*, response amplitudes are represented by distances along the given axis (either 0° = longitudinal, 15°, 45°, or 90° = transverse). A red dot represents the mean amplitude of a number of responses from a single cell located from 250 to 350 μm away from the uncaging spot in a slice of the indicated orientation. A blue dot indicates the mean amplitude of the responses from all of the cells (all 250–350 μm away from the uncaging spot) recorded in slices of the given orientation. Comparison of the blue dots shows that the largest responses are elicited in the longitudinal slices (horizontal line in Fig. 3*C*).

To examine signaling along the longitudinal axis at the network level we used voltage-sensitive dye imaging in the whole hippocampus preparation (Fig. 4*A*). A stimulating electrode was positioned in the superficial alvear field of the CA1 region. A single stimulation evoked the expected early voltage signal (within 10 ms), propagating within the transverse lamina toward the fimbria (Fig. 4*B*) and the subiculum (Fig. 4*C*). A slower longitudinal signal emerged that peaked at ∼25 ms after stimulus and was gone by ∼35 ms. Importantly, the longitudinal signal in CA1 was not secondary to longitudinal activation in CA3. A stimulus train (five stimuli at 100 Hz) evoked a similar early depolarization within the transverse lamina but evoked a stronger and longer lasting depolarization in the longitudinal axis between laminas. The group data for the spatial activation profile is shown in Fig. 4*C* ($n = 5$). These optical recordings further demonstrate the existence of a robust and functional associative excitatory network in region CA1.

Cellular Memory Mechanisms of the Interlamella CA1 Network. The potential functional roles carried out by the longitudinal pathway will depend in part on the physiological properties of the synapses formed on the target cells. We first examined their ability to support plastic mechanisms such as long-term potentiation (LTP). Application of high frequency stimulation (HFS) in longitudinal slices resulted in a robust LTP to 210.7 ± 3.6% of the control value ($n = 5$ slices; Fig. 5*B*) along the course of the longitudinally oriented fibers. This data suggests that LTP can strengthen interlamellar CA1-to-CA1 connections as well as the well-established CA3-to-CA1 connections in the transverse plane.

We next examined whether the synapses of the interlamellar network can express DHR (14). DHR was previously demonstrated to occur on apical oblique dendrites (14). Because the interlamellar network forms synapses mainly on basal dendrites, we tested for DHR on basal dendrites in the longitudinal slice preparation (Fig. 5*A*). Photolysis of caged glutamate was directed onto select CA1 basal dendrites to create the bound-but-blocked state of the NMDAR (blue arrow and blue circles in Fig. 5 *A, i*). This uncaging simulates the effects of temporally and spatially dispersed EPSPs arriving on the dendrite, which are unable to produce a depolarization large enough to remove the Mg^{2+} block of the NMDARs. Next, a stronger second stimulus ("gating stimulus", purple arrows and circles) that, by itself, would produce only a modest depolarization, was delivered to the same dendrite. However, if the gating stimulus is given within a several hundred-millisecond delay after the priming stimulus, its response is markedly potentiated, because the NMDARs put into the bound-but-blocked state by the priming stimulus become unblocked by the gating stimulus and initiate a regenerative local dendritic spike (Fig. 5 *A, i*). In essence a molecular memory of previous synaptic activity is stored in the bound-but-blocked state and is "read out" by the gating stimulus in the form of a larger gating response.

Previously, DHR was only demonstrated with photolysis of caged glutamate to prime the dendrite. We next sought to determine whether synaptically induced EPSPs produced by the longitudinal axons could also act in the priming role. To test this, we placed a stimulating electrode in s.oriens temporal to a CA1 neuron recorded under whole-cell current clamp conditions in a longitudinal slice (Fig. 5 *A, ii*). A moderately strong photolysis-mediated gating stimulus (red circles) was directed onto a target basal dendrite alone or 200 ms after priming by synaptic stimulation (Fig. 5 *A, ii Top*). The photolytic gating response was significantly potentiated if it occurred after the priming EPSP (control: 4.27 ± 0.54 mV vs. DHR: 7.17 ± 0.73 mV; paired *t* test; $n = 5$) (Fig. 5 *A, ii Middle*). The potentiated local dendritic spike could subsequently drive a somatic action potential even when the gating depolarization alone without priming could not (Fig. 5 *A, ii Bottom*). This data are consistent with the idea that the CA1 interlamellar network can support DHR mediated memory mechanisms.

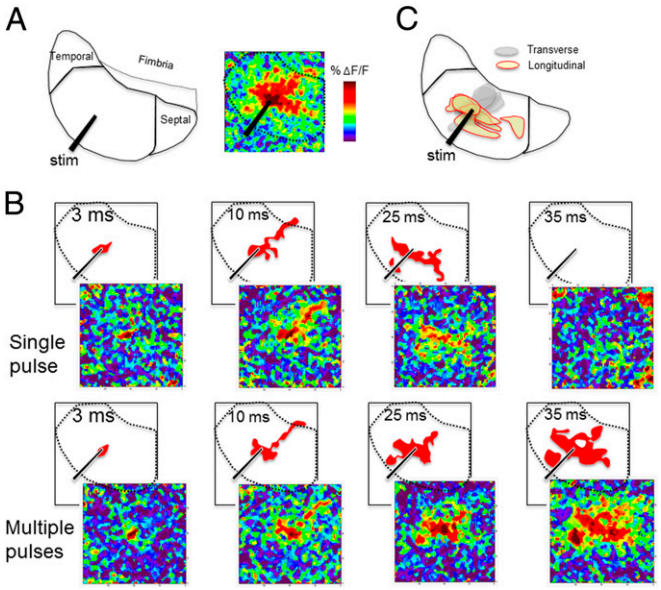

Fig. 4. The longitudinal propagation of electrical signals in area CA1 in the whole hippocampus preparation. (*A*) The stimulating electrode was placed in the alveus over area CA1. The image of voltage-sensitive dye (VSD) signals is shown in *Right* and represents the average of 500 individual frames. (*B*) VSD responses to a single stimulation (*Upper*) and multiple stimulation (five stimuli at 100 Hz) (*Lower*) are shown as snapshots of activation at 3, 10, 25, and 35 ms. (*C*) Group data of the spatial imaging profile of VSD to CA1 activation illustrate signal propagation along transverse and longitudinal direction.

Discussion

The experiments described here provide anatomical, electrophysiological, and electro-optical evidence of the existence of well-organized associational connections among CA1 pyramidal neurons. Two-photon imaging of intracellularly labeled CA1 pyramidal neurons shows that every CA1 pyramidal neuron that was examined sends out a longitudinally oriented axon that branches off, and is perpendicular to, the thick, well-established projection that goes toward the subiculum and fimbria. The subicular and fimbrial axonal projections agree with established data (17), but the longitudinally projecting collateral has not been described. Note that although we use the term "longitudinal" as others have, that this term was done partly for convenience: The most favorable orientation for excitatory effects was in the longitudinal plane; however, stimulation at angles oblique to the longitudinal plane was also quite effective in activating CA1 pyramidal cells. The delineation between transverse and longitudinal is not sharp, but rather a matter of degree in CA1, as it is in CA3. The synaptic contacts made by the longitudinal projections are excitatory, as evidenced by immunostaining and electrophysiology. It is likely that the thinner axons

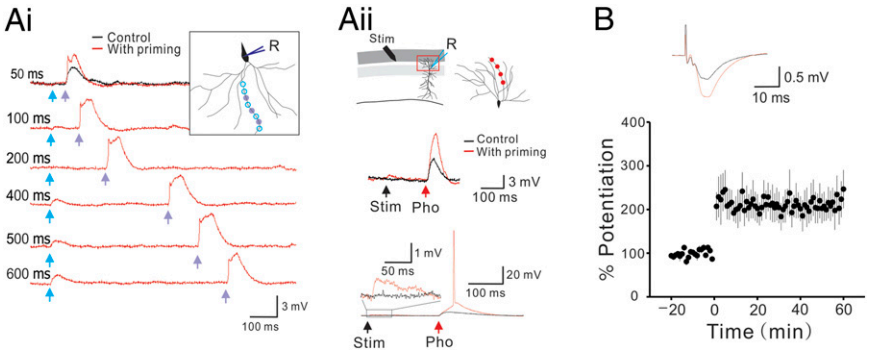

Fig. 5. Short-term and long-term memory mechanisms of the CA1 longitudinal network. (*A, i*) Basal dendrites of CA1 pyramidal neurons can support DHR. A low level of glutamate is first diffusely released by photolysis on a single basal dendrite to create a population of NMDA receptors in the "bound-but-blocked" state. The timing and location of this "priming" stimulus are marked by light blue arrows and circles, respectively. A second independent stimulus is provided to produce a moderate local depolarization. The timing and location of this "gating" stimulus are marked by the purple arrows and circles. If the gating stimulus was coupled to the priming stimulus within an interval of hundreds of milliseconds, a local dendritic spike was triggered. (*A, ii*) Electrical stimulation of the longitudinally oriented axons can also prime basal dendrites for DHR. The local dendritic spike produced by DHR of a single dendrite can then trigger a somatic action potential (lower trace). (*B*) LTP can be induced in synapses of the longitudinal network in response to a brief high frequency stimulus (100 Hz).

and their orientation with respect to the canonical transverse organization of hippocampal architecture led to this pathway being overlooked in earlier studies. Despite its more limited arborization, its ability to affect potent excitatory signaling is clearly evident from voltage sensitive dye imaging. The imaging results show that longitudinal spread of excitation in CA1 does not require preceding CA3 excitation. Furthermore, the CA1 associational network supports forms of synaptic plasticity, such as LTP, as well as DHR. These properties suggest that this system may be an integral component of the larger 3D information processing network of the hippocampus.

Comparison of CA1 and CA3 Associational Connection. Although we show here that CA1, as well as CA3 and the dentate gyrus, possesses associational connections, substantial differences among them suggest that they probably serve different functions. The CA3 and dentate mossy cell projections are extensive, and their signals, which propagate relatively rapidly (e.g., Fig. S6), could recruit large populations of target cells (either inhibitory or excitatory) and affect wide areas of the hippocampus in concert. The extensive CA3 recurrent connections may be suitable for dealing with large information transfer and mediate feedback memory mechanisms.

The relatively restricted projection of the CA1 autoassociational network suggests a more focused targeting, perhaps involving coordinated activity within smaller modules of cells. The CA1 associational axons are shorter in length than the CA3 axons and their signals propagate with significant time delays (Fig. 4). These characteristics would allow the CA1 system to participate in information processing functions that the larger CA3 and mossy cell longitudinal association systems are not capable of carrying out. For example, during exploratory behavior or rapid eye movement sleep, traveling theta waves propagate longitudinally throughout the entire extent of the CA1 region of the intact hippocampus, but with a 180° phase shift from the septal to the temporal poles (23). Similar phase precession is a hallmark of hippocampal place cell behavioral learning in rats learning to run a maze (24). Although the anatomical substrate for traveling theta wave propagation is unknown, Patel et al. (23) speculate that a longitudinal chain of "weakly coupled oscillators" could account for their observations. This idea would be an example of the type of processing that the slower and more spatially restricted axonal connections in the CA1 longitudinal system seem well suited to serve; whether this network is actually involved in propagation of traveling theta waves is of course unknown at this point.

Alternatively, the limited CA1 associational connections could theoretically form a delay-line network comprising a linear chain of serially connected, nearly identical CA1 pyramidal neurons (14). A unique feature of such linear chains is that they can transform a time sequence into a spatial sequence as information is iteratively passed from one cell to its downstream neighbors. The resulting "time-to-space" transform is effectively an efficient sequence memory mechanism, like the shift register that exists at the input stage of processors of all modern computers to serve as input memory buffers (25) Computers need memory buffers because information typically arrives as a time sequence over a finite interval and its meaning can only be revealed if each sequence is viewed as a whole and in proper order. Whether the brain uses a delay-line type circuit for sequence memory is not known. However, behavioral studies have suggested that area CA1 possesses the capacity for sequence memory (9, 10). The results here suggest that the CA1 longitudinal associational network possess two necessary requisites for a delay-line type memory. First, it contains linear chains of similar CA1 neurons. Second, the basal dendrites that participate in this associational network possess a robust newly described short-term memory and conditional retrieval mechanism, DHR (Fig. 5*A*). DHR operating on basal dendrites can function in a manner similarly to digital flip-flops in the shift registers of a computer. Direct demonstration of delay-line memory in the brain will have to wait for video imaging studies involving large populations of fluorescently labeled neurons in behaving animals.

Which of these or other phenomena are mediated by the CA1 longitudinal system will have to be addressed in future work. Perhaps most importantly, it should be kept in mind that the associational systems in all of the hippocampal subregions are part of a "three-dimensional series of connections," in which divergence and convergence are major organizational principles (18). Amaral and Lavenex (18) suggest further that the prominent associational connections within hippocampal subregions constitutes a substrate that allows each subregion to act "semi-independently from, as well as in concert with" other regions. The deeper significance of our observations may be that the CA1 region, previously thought to be incapable of taking part in septo-temporal processing operations, can now, by virtue of its own, distinctive longitudinal associational network, be recognized as a potential key partner in them.

Relevance for the Lamellar Hypothesis of Hippocampal Architecture. As noted, longitudinal interconnections are well known to exist among CA3 pyramidal cells and dentate gyrus mossy cells, but the likelihood of significant interconnections among CA1 cells has been considered "negligible" (13). Does their existence necessarily conflict with the lamellar hypothesis of hippocampal organization put forward by Andersen et al. (11)? Amaral and Witter (12) argue that, indeed, the longitudinal connectivity directly contradicts the lamellar hypothesis. In contrast, Sloviter and Lømo (13) argue that it is what the longitudinal connections actually do, their functional impact, that is the most important factor, and that a lamella should not be defined in strictly anatomical terms. For example, if the longitudinal projections were mainly targeted to inhibitory

interneurons (26), then excitation of principal cells would create zones of lateral inhibition that would, in effect, sharpen the functional borders of the lamellae and support, rather than contradict, the lamellar hypothesis (13). Alternatively, they suggest that longitudinal connections could simply serve to coordinate activity across lamellae. Although not part of the original lamellar hypothesis, such coordination would be in keeping with it, necessitating only that this local mechanism be added to the overall hippocampal model.

Clearly, data on the actual physiological influences of axonal projections, and their anatomical arrangements, are crucial for resolving such issues. For instance, our results essentially disconfirm the postulate that the longitudinal associational CA1 network primarily contacts interneurons. Stimulation of these fibers caused significant excitation, not inhibition, in their downstream target cells. In addition, the CA1 longitudinal system demonstrates considerable capacity for the kinds of synaptic plasticity thought to be important for information storage. If this pathway simply acted as a relay to coordinate the actions of lamellae, simple excitation of the downstream target cells would seem to be sufficient; there is no obvious role for synaptic plasticity if that is all they do. Hence, although we cannot rule out that coordination across lamellae is a function of the CA1 longitudinal pathway, its capacity to undergo significant and subtle synaptic plasticity permits it to play more sophisticated roles in hippocampal information processing.

Regardless of how the physiological details are resolved, our findings have important implications for the understanding of the hippocampus as a region capable of processing information throughout its full 3D extent. Indeed, as suggested (12), it may no longer be productive to view the evidence as being either "for" or "against" the lamellar hypothesis. This hypothesis, although highly important and influential, leading as it did to the massive and productive output of work done on the transverse hippocampal slice, is simply too limited to encompass the richness of the anatomical and physiological data.

Methods

Brain Slice and Whole Hippocampus Preparation for Physiology. Sprague–Dawley rats (postnatal age: 4–6 wk) for brain slices and C57BL/6 mice (postnatal day 10–12) for whole hippocampus imaging were used. Additional details are provided in *SI Methods*.

Whole-Cell Patch Recording. Whole-cell patch recordings were obtained by using an Axon instruments Axoclamp 700B Amplifier (Molecular Devices), and pClamp Version 10.2 software (Molecular Devices) or Igor Pro (WaveMetrics) was used for data acquisition. Additional details are provided in *SI Methods*.

Three-Dimensional Digital Holography Uncaging. The procedures for digital holographic photolysis have been described in detail in an earlier methods paper (20). Digital holographic photolysis is a method that readily permits flexible and accurate uncaging of glutamate and, therefore, stimulation of cells at multiple discrete dendritic sites. Unlike conventional uncaging techniques, the holographic method permits glutamate photolysis to be directed precisely at multiple sites and depths simultaneously. Additional details are provided in *SI Methods*.

Two-Photon Imaging. The laser was tuned to 810 nm for Ca^{2+} imaging. Epifluorescence and transfluorescence signals were captured through a 60×, 1.0 N.A. objective and a 1.4 N.A. oil immersion condenser (Olympus). Fluorescence was split into red and green channels by using dichroic mirrors and band-pass filters. Green fluorescence (Fluo-5F) and Red fluorescence (Alexa 594) were captured with H10440PA and R9110 PMTs, respectively . Data were collected in linescan mode (2.5 ms per line, including mirror flyback). For Ca^{2+} imaging, data were presented as averages of 10 events per site, and expressed as $\Delta(G/R)/(G/R)max \times 100$, where (G/R)max is the maximal fluorescence in saturating (2 mM) Ca^{2+}. Ca^{2+} transient peaks were calculated from the peak of the fluorescence rise after stimulus onset.

VSD Imaging. Whole hippocampi from rats (p7–p12) were stained with 0.125 mg/mL JPW3031 in artificial CSF for 20 min and imaged in an oxygenated interface chamber (34 °C) by using an 80 × 80 CCD camera (NeuroCCD; RedShirtImaging). Epi-illumination was provided by a custom LED illuminator. Compared with the more commonly used photodiode array, the CCD chip well size (215,000 e^-) requires use of relatively low light intensities, which minimized photodynamic damage. A 4× objective lens (0.28 N.A.; Olympus) imaged a 2.5 × 2.5-mm region in the hippocampal area CA1 (32 × 32-μm region imaged per pixel).

Extracellular Field Recording. Extracellular multiple unit recordings were obtained by using glass pipettes filled with ACSF (3-7 MΩ). Synaptic responses were evoked with 15–60 μA, 0.2-ms current pulses delivered through a concentric bipolar stimulating electrode (FHC, 100 μm o.d.). Baseline responses were recorded by using half-maximal stimulation intensity at 0.033 Hz. HFS for LTP was induced by 100 Hz stimulation (1-s duration).

Biocytin and Spinophillin Histochemistry. At the end of an electrophysiology experiment, the slices injected with biocytin were reacted in avidin–biotin–peroxidase complex. Labeled neurons were reconstructed in 3D by using a motorized microscopy interfaced with the Neurolucida software. For spinophilin histochemistry, tissue with biocytin was rinsed and placed into rabbit anti-spinophilin serum overnight at room temperature, and then incubated with a Cy2-conjugated goat anti-rabbit. Additional details are provided in *SI Methods*.

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