Mnemonic representations of transient stimuli and temporal sequences in the rodent hippocampus in vitro

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A primary function of the brain is the storage and retrieval of information. Except for working memory, where extracellular recordings have shown persistent discharges during delay-response tasks, it has been difficult to link memories with changes in individual neurons or specific synaptic connections. We found that transient stimuli are reliably encoded in the ongoing activity of brain tissue in vitro. Patterns of synaptic input onto dentate hilar neurons predicted which of four pathways were stimulated with an accuracy of 76% and performed significantly better than chance for >15 s. Dentate gyrus neurons were also able to accurately encode temporal sequences using population representations that were robust to variation in sequence interval. These results demonstrate direct neural encoding of temporal sequences in the spontaneous activity of brain tissue and suggest a local circuit mechanism that may contribute to diverse forms of short-term memory.

A fundamental property of the CNS is the ability to encode and retrieve information. In mammals, declarative memory function is typically divided into behavioral tasks that promote short- or long-term storage of items, such as names, places and specific temporal sequences. Although the specific cellular origin of individual long-term declarative memories has remained elusive, previous work highlighted the importance of several critical brain regions, including the prefrontal cortex and the hippocampal formation, for encoding short-term, or working, memories. Extracellular unit recordings in these brain areas often display periods of persistent spiking activity at elevated frequencies when animals are required to retain transiently presented sensory information. This delay-period activity typically lasts for seconds and is extinguished when the animal initiates a behavioral response to indicate whether it remembered the correct transient stimulus. The reduction in persistent spiking during error trials, trials in which the animal made an incorrect behavioral response following the delay period, argues that delay-period activity in those specific neurons reflects activity in neural circuits encoding that short-term memory. Some types of CNS neurons also contain constellations of intrinsic conductance that enable them to maintain simple memories by firing persistently for several seconds following transient depolarizing stimuli, even when pharmacologically isolated from all other neurons. Computational models that combine individual simulated neurons with intrinsic persistence and recurrent excitatory connections are capable of generating persistent firing modes without the high connection weight tuning precision required to enable simple associative network models to fire persistently. Although biological experiments have revealed examples of both intrinsic persistence and recurrent excitatory connections in different brain regions, the absence of a tractable in vitro system capable of short-term information storage has limited the opportunities to determine which specific mechanisms are required to generate short-term mnemonic representations of transient stimuli.

We found that four distinct patterns could be reliably encoded in the spontaneous synaptic activity in conventional rodent hippocampal slice preparations. Each pattern was evoked by briefly activating a different subset of entorhinal input axons (the perforant path) using an array stimulation electrode; information was read out by recording intracellularly from two or three dentate hilar neurons, which sample the activity of both dentate granule cells and semilunar granule cells (SGCs; Fig. 1a), a recently discovered excitatory cell type that responds to perforant path stimulation with graded depolarizing plateau potentials. We found that synaptic barrages evoked in downstream hilar neurons by persistent firing in SGCs reliably encoded both the identity of individual stimuli and temporal sequences of perforant path stimuli. Decoding both individual stimuli and temporal sequences relied on population representations of synaptic inputs to dentate hilar cells. Short-term encoding of sequences was robust to perturbation of sequence interval, suggesting that contextual coding in these experiments arises from stimulus order rather than the delay between stimuli. Although several in vivo recording...
studies have suggested that neurons in the hippocampal formation are involved in short-term memory function⁴⁻⁷, our results provide, to the best of our knowledge, the first demonstration that neural networks in the dentate gyrus are capable of encoding both multiple transient inputs and context-dependent information such as the order of temporal sequences.

**RESULTS**

**The dentate gyrus supports multiple neural representations**

Transient stimulation of the perforant path (two 200-μs shocks) led to sustained increases in the frequency of spontaneous excitatory postsynaptic potentials (EPSPs) recorded in dentate hilar neurons over multiple seconds (Fig. 1b), as described recently²⁰. That study found that the circuitry contained in a conventional hippocampal slice preparation could encode two distinct activity patterns. We asked whether more than two patterns could be encoded in the dentate gyrus and what factors govern coding accuracy.

Hilar cell responses to stimuli at different perforant path locations were recorded after placing an array electrode (four contacts, 115-μm spacing) in the middle molecular layer of the dentate gyrus. Activation of each electrode contact (A, B, C or D) individually typically elicited synaptic barrages (average EPSP frequency of spontaneous EPSPs in barrage, 18.7 ± 0.5 Hz; immediately before perforant path stimulation, 3.0 ± 0.1 Hz; P < 0.0001, n = 33 cells). The 3.5-min interval between episodes was substantially longer than synaptic barrages triggered by perforant path stimuli (decay tau ~8 s)²⁰ and we did not observe steady changes in baseline EPSP frequency during experiments. Although responses evoked by different electrodes in this experiment had different mean EPSP frequencies, the range of EPSP frequencies in these responses overlapped (Fig. 1c,d) and were only infrequently statistically separable (see below).

We next asked whether the separation of responses evoked by stimuli at different locations could be increased by sampling the hilar network more densely using multiple simultaneous intracellular recordings (Fig. 2a and Supplementary Fig. 1). Using this approach, responses to each stimulus in an experiment could be plotted in the three-dimensional space formed by the frequency of EPSPs recorded in each of three hilar neurons during the initial 4 s after the stimulus (Fig. 2b). In most experiments, responses to each stimulus type were clustered in different regions in the three-dimensional plot of EPSP frequency in each hilar cell (Fig. 2b). By analyzing synaptic responses of hilar networks in EPSP frequency space, we can directly apply standard statistical and clustering methods along biologically meaningful dimensions without requiring dimensionality reduction techniques such as principal component analysis. As the presentation order was randomized, response clustering was unlikely to represent short-term plasticity associated with repeated stimulation at the same location.

**Figure 1** Persistent synaptic activity evoked by multiple perforant path stimuli. (a) Experimental configuration. A four-position stimulating electrode was used to activate different perforant path segments while recording synaptic input in hilar neurons. GCL, granule cell layer; ML, molecular layer; GC, granule cell; MC, mossy cell. (b) Example of a hilar mossy cell synaptic barrage evoked by perforant path stimulation (arrowheads). The timing of the onsets of individual EPSPs is indicated by vertical lines. Inset, enlargement of region indicated by the horizontal bar. (c) Experimental protocol and EPSP analysis windows. (d) Responses to three repetitions of each of the four stimulus positions (A–D) in one hilar mossy cell. Vertical lines indicate EPSP onset times. Mean EPSP frequency and range for each stimulus type are indicated on the right.

**Figure 2** Short-term hilar representations of multiple perforant path stimuli. (a) Triple hilar cell recording configuration. PP, perforant path. (b) Stimulation at different sites evoked distinct synaptic barrages in hilar networks. Shown is a summary plot of mean EPSP frequency during the initial 4 s of barrages following perforant path stimulation in one experiment. Black dots represent 14 consecutive single trials and crosses represent centroids of each trial type (stimulus A, B, etc.). Shaded zones represent bounding ellipsoids used to illustrate variance associated with repeated responses to the same stimulus (60% confidence intervals). (c) Histogram of experiments with two, three and four statistically separable responses over 12 experiments. (d) Plot of the number of statistically significant (P < 0.05) planes obtained by LDA in the nine triple recording experiments (black bars) and following shuffling stimulus identity (gray bars). Results are presented from LDA computed on data from all three simultaneous recordings (right set of bars, n = 9) and when only one (n = 27) and two neurons (n = 27) were considered. **P < 0.01. Data are presented as mean ± s.e.m. (e) Plot of probabilities associated with the OVL for the same 12 experiments. Dashed line indicates statistically significant OVL range (P < 0.05). See Supplementary Figure 3 for details.
Although many experiments yielded visually distinct response clusters from each stimulus location (Fig. 2b), linear discriminant analysis (LDA) offers a rigorous method to determine whether specific pairs of responses to different stimulus locations (A/B, B/C, etc.) are statistically separable. Statistical significance on six pairwise LDA tests is required to completely separate the responses to all four stimuli ($P < 0.0083$ for each LDA test, reflecting a Bonferroni correction for multiple comparisons). This criterion was met in 50% of the experiments that we analyzed (6 of 12, EPSP frequencies assayed in initial 4 s of each response; Fig. 2c), indicating that the ability of the dentate gyrus to generate short-term representations of four transient inputs is robust. Responses to three stimuli were statistically separable in half of the remaining six experiments (3 of 12 experiments). On average, we obtained 4.8 ± 0.5 out of the six separation planes required to separate four response patterns, compared with the 0.83 planes expected by chance ($P < 0.001, n = 12$ experiments, 9 triple recordings and 3 paired recordings; Online Methods). Our ability to statistically separate four patterns was greatest (5.6 ± 0.7 significant planes) when we only considered the nine experiments with simultaneous triple recordings. Eliminating data from one or two neurons in the nine triple recording experiments substantially degraded the statistically separate responses to different stimuli (Fig. 2d); synaptic barrages recorded from one hilar neuron typically were only able to discriminate between two of the four response patterns (1.7 ± 1.4 statistically significant planes; Fig. 2d).

The ability of hilar networks to create mnemonic representations of transient stimuli was relatively independent of the window duration over which EPSP frequency was analyzed (Supplementary Fig. 2), with only a minor (13.8%) reduction in the number of significant LDA planes obtained with 1-s analysis windows, compared with the 4-s windows used in the initial analysis. We next conducted a separate set of triple recordings at relatively depolarized membrane potentials ($−60$ mV) using two stimulus locations to determine whether stimulus identity also was represented in the spike output of hilar cells. In each experiment in which responses to A and B stimuli were separable using LDA of EPSP frequency across the three cells ($n = 3$), LDA $P$ values ranged between $2.0 \times 10^{-9}$ and $2.2 \times 10^{-4}$, we found stimulus-specific suprathreshold responses on the basis of spike frequency assayed in the same 4-s window (LDA $P$ values ranged between 0.002 and 0.016; mean spike frequency following perforant path stimuli, 3.0 Hz; control conditions, 0.06 Hz). These results indicate that both the synaptic input to hilar cells and the resulting spike output can represent stimulus identity. We also found significantly more variation between responses from different stimulus locations than from responses evoked by the same location using an omnibus measure of response separation (OVL, 12/12 experiments with $P$(OVL) < 0.05; Fig. 2c and Supplementary Fig. 3), which is used to assay overlapping distributions. Responses to the four stimuli were not separable on the basis of LDA of baseline (pre-stimulus) EPSP frequencies (mean 1.0 plane; significantly different from the number of planes from stimulus responses, $P < 0.0001$). The stimulus intensities used to evoke synaptic barrages were not significantly different on each contact ($F = 1.23, P > 0.05$, one-way ANOVA, $n = 180$).

We next assayed the accuracy of recall by comparing individual responses to the average response recorded at each stimulus location. We first tested the accuracy of response classification by predicting stimulus identity on the basis of the nearest average response (centroid) computed from the first 4 s of each response (Fig. 3a). In this decoding method, if a response triggered by the A stimulus was closest to the average response to all A stimuli, the analysis would generate a correct A prediction. In contrast, if the response triggered by that stimulus was closest to the average response of all C trials in that experiment, then an incorrect prediction (that the response was triggered by C instead of the correct answer, A) would be recorded. Over all 12 experiments, this method predicted which one of the four possible stimulus locations was activated with an accuracy of 76.1% ($n = 180$ trials with 137 correct and 43 incorrect predictions). This prediction accuracy was significantly greater than the 25% accuracy expected by chance (s.d. = 3.2%, $P < 0.0001$; also significantly greater than the accuracy obtained following shuffling stimulus identities, $P < 0.0001$; Fig. 3b). We did not observe a correlation between the physical distance between pairs of stimulating electrodes tested and the Euclidean distance between the resulting synaptic barrages (Fig. 3c). Neither was there a correlation between the inter-electrode distance and the accuracy differentiating hilar responses (for example, A/B was differentiated as accurately as A/D; Fig. 3d). These results indicate that the dentate gyrus can generate distinct and repeatable population responses patterns even when nearby stimulation electrodes are activated, arguing that separation of network states in EPSP frequency space is not related to the physical separation between the stimulating electrodes used.

Origin of recall errors

We examined the 43 trials with classification errors to determine the factors that govern accuracy of short-term information storage in the dentate gyrus. We plotted the distance between response pairs that were always correctly classified and those that were misidentified at least once in an experiment (Fig. 3e). Although the range of mean Euclidean distances between responses that were accurately predicted spanned 50 Hz, all classification errors arose from experiments in which average responses between pairs of stimuli were separated by less than 30 Hz. The distance between average responses that were misidentified (10.2 ± 1.0 Hz, $n = 43$) was significantly less than the distance between responses that were always correctly classified (21.6 ± 1.3 Hz, $n = 44, P < 0.002$). Half of all errors occurred when discriminating between two average responses separated by less than 7 Hz (Fig. 3f).

These results suggest that one factor governing mnemonic coding accuracy is the separation of responses to different stimuli in EPSP frequency space. Although restricting recall tests to trials with well-separated average responses improved accuracy (data not shown), there was a relatively poor correlation between the distance between pairs of average responses and the accuracy differentiating between those responses ($R^2 = 0.13, P > 0.05$; Fig. 3g). This model of variation in accuracy also did not predict the average separation between centroid pairs that were always classified correctly. However, scaling the distance between responses by the pooled variance of each response improved the correlation with accuracy ($P < 0.0001, F = 33.1$; Fig. 3h). Moreover, this model, based solely on imperfectly classified trials, predicted the scaled distance between perfectly classified trials. These results suggest that much of the variability in recall accuracy (62% of the variance) can be explained by a simple two-component model using the Euclidean distance between average responses and the scatter around those average responses.

Time course of response separability

Synaptic barrages evoked by perforant path stimuli decayed with an average tau of ~8 s (ref. 20) and decreased to 39.8% of the initial frequency after 18 s ($n = 12$ cells; Fig. 4a). When displayed in three-dimensional EPSP frequency space (Fig. 4b), average responses evoked by different stimulating electrodes followed distinct trajectories toward the origin and did not coalesce, even though overall EPSP frequency decreased continuously in all responses. We next asked how long hilar
We examined the ability of the rodent dentate gyrus to encode four representations following transient molecular layer stimulation (Supplementary Fig. 5). Each possible stimulus location is represented by a character (A–D) whose contrast is a function of the distance to the average response (Online Methods). A trial with a population response very close to the mean response to stimulus B would be represented by a dark B superimposed on dim A, C and D characters. This method illustrates both the high accuracy of dentate representations of stimulus identity (only 1 of 14 consecutive trials was incorrect) and the persistence of the population coding in the dentate hilus. This visual analysis was cross-validated (Fig. 5); half the data set was used to compute mean response centroids and the other half used to generate the character display.

Dentate gyrus circuits encode temporal sequence order
Besides representing individual stimulus positions, hilar networks reliably encoded different temporal sequences. Distinct hilar up-states

**Figure 3** Prediction of stimulus identity from hilar population responses. (a) Analysis method based on computing Euclidean distance from each response (black diamond) to the four centroids (filled circles) that represent average responses to each type of stimuli. (b) Plot of mean accuracy in predicting stimulus identity (A–D) based on the nearest average response centroid, compared with accuracies expected by chance. **P < 0.0001. (c) Plot of the average Euclidean distance between pairs of response centroids evoked by adjacent (115-μm separation) and nonadjacent (230- and 345-μm separation) stimulating electrode contacts. n.s., not significant, P > 0.05. (d) Plot of accuracy of predicting stimulus identity based on nearest centroid versus stimulus electrode separation. Pairs of stimuli evoked on adjacent electrode contacts were classified as accurately as stimuli evoked by nonadjacent pairs. Accuracy expected by chance was 50% in this pairwise analysis. (e) Distribution of inter-centroid distances between pairs of responses that were correctly (black symbols) and incorrectly (red symbols) classified. The number of errors associated with each inter-centroid distance is plotted on the y axis. Mean ± s.e.m. distance for correctly (black circle) and incorrectly (red circle) classified response pairs was plotted next to each distribution. (f) Cumulative distribution of distances associated with classification errors. Half of the classification errors results from comparisons between response centroids separated by less than 7 Hz (arrow on x axis). (g) Plots of accuracy classifying response pairs with occasional errors versus distance/s.d. ratio. Solid lines represent linear regression fits in both g and h. Black symbols represent the mean Euclidean distance (in g) and distance/s.d. ratio (in h) associated with response pairs that were always classified correctly. Error bars represent s.e.m.

**Figure 4** Time course of hilar population responses. (a) Plot of decay of average EPSP frequency during synaptic barrages in 12 experiments (thin lines represent each experiment, symbols represent overall mean ± s.e.m.). Arrow on y axis indicates average baseline EPSP frequency. (b) Plot of centroid position as response evolves over time in one experiment (18 sliding 4-s windows). Each point reflects mean EPSP frequency in three hilar cells over a 4-s window. (c) Plot of the number of statistically separable responses (out of four possible) versus time. Symbols represent mean ± s.e.m. (n = 12 experiments). Red and blue lines represent the best and worst three experiments, respectively. (d) Plot of classification accuracy over time using the entire data set. The arrow along the y axis indicates accuracy expected by chance. (e) The initial response OVL was negatively correlated (R² = 0.62, P < 0.005, F = 15.4, n = 12 experiments) to the duration that responses remained significantly distinguishable. Error bars represent s.e.m.
Figure 5 Visual display of cross-validated data. Shown is a graphical representation of response classification using only untrained data. Half of the episodes were used to compute response centroids; character plots were generated from the remaining 50%. All of the episodes from the same experiment were plotted using the display algorithm used in Supplementary Figure 5.

Figure 6 Short-term representations of temporal sequences in hilar neurons. (a) Experimental configuration. (b) Responses to three trials of forward (ABCD) and reverse (DCBA) temporal stimuli sequences recorded intracellularly from three hilar cells. Vertical lines represent EPSP onset times. (c) Plot of individual responses to forward and reverse sequential stimulation (black circles) in EPSP frequency space (five forward and six reverse, acquired in pseudo-random order; 4-s analysis window after final stimulus in each sequence). All points were contained in bounding ellipsoids (60% confidence interval, as in Fig. 2b) centered on response centroids (black asterisks). (d) Plot of probabilities that forward and reverse points are significantly different by LDA. Forward and reverse sequences were significantly different (P < 0.05) in 10 of 11 experiments (filled symbols) and not different in one experiment (open symbol). (e) Plot of the effect of MK801 (10 μM) on the mean EPSP frequencies in three sequential stimulation experiments. Population responses to forward and reverse sequential stimulation were separable using LDA in control conditions in each experiment (before MK801, all P < 0.05). Sequential stimulation triggered a mean increase of 17.8 Hz in control conditions in these three experiments, which was not statistically different than the 16.1-Hz increase observed in the larger set of 12 sequential stimulation experiments. Sequential stimulation failed to trigger an increase in EPSP frequency in MK801 (P > 0.05). *P < 0.05. (f) Plot of mean EPSP frequency across a triple recording for all forward and reverse sequences tested in one experiment. Horizontal bars indicate mean EPSP frequency for baseline period and for 4-s windows following each stimuli in the sequence. (g) Vector representation of average population response to forward and reverse sequences. Responses to each stimuli were combined head-to-tail and failed to converge for fast (5-s intervals, left) sequences, but converged for slow (120-s intervals, right) sequences in a different experiment. (h) Plot of average separation in head-to-tail vector representations of forward and reverse sequences (similar to g) over 11 experiments. Black bars represent results from 5-s sequences; purple bar represents forward/reverse separation at the end of 120-s sequences. **P < 0.0005. Data are presented as mean ± s.e.m.
Figure 7 Sequence representations are robust to perturbation of stimulus interval. (a) Example of EPSP responses to forward and reverse sequential stimuli at 5-s intervals (ABCD, DCBA) and reverse stimuli at 4-s intervals (DCBA short) recorded in three hilar mossy cells. (b) Plot of forward (black symbols in green ellipsoid), reverse (black symbols in orange ellipsoid) and reverse short (white points in purple ellipsoid) responses in EPSP frequency space in one experiment. Response centroids indicated by asterisks (reverse and reverse short centroid symbols overlap); colored ellipsoids represent 60% confidence intervals. (c) Plot of scaled distance between forward (green symbols) and reverse (orange) 5-s interval sequence responses in one experiment. Responses to 4-s reverse responses (purple symbols) overlapped the 5-s reverse responses. Over five experiments, the mean scaled distance of the 4-s reverse points was 0.72, which was significantly different from 5-s forward responses (P < 0.005) and not significantly different from 5-s reverse responses (P > 0.05). (d) Plot of classification accuracy for experiments with forward (green bar; n = 11) and reverse (orange bar; n = 11) 5-s interval sequences, and reverse sequences with 4-s intervals (purple bar; n = 5). Mean accuracies not statistically different from each other (P > 0.05). Data are presented as mean ± s.e.m.

of responses were added head to tail (Fig. 6g) and when responses to the same stimuli were plotted from the same origin (Supplementary Fig. 6). Over 11 triple recording experiments, hilar sequence responses significantly diverged (P < 0.05) following the second stimuli (Fig. 6h) and failed to converge at the end of the sequence (convergence would be expected if responses to sequential stimuli were commutative). Increasing the interstimulus interval from 5 to 120 s eliminated most of the history dependence (Fig. 6g,h), suggesting a temporal limit to the ability of the dentate gyrus to encode sequential stimuli in vitro. We also found statistically significant sequence-specific hilar responses when forward and reverse sequential stimuli were repeated at 8-s intervals (three of three experiments with LDA P values < 0.05; Supplementary Fig. 7), but only infrequently when using 2-s intervals (one of three experiments with P < 0.05; Supplementary Fig. 8).

Sequence classification accuracy (forward or reverse) was robust to small perturbations of interstimulus interval. Intracellular responses of hilar cells to shortened DCBA stimulus trains (4 s instead of the standard 5-s interval) more closely resembled responses to standard DCBA than to ABCD stimulus trains (Fig. 7a). Population responses to shortened (4 s) DCBA trains also overlapped with responses to 5-s DCBA stimuli in EPSP frequency space (Fig. 7b) and were closer to the centroid of the 5-s DCBA response than to the 5-s ABCD response (Fig. 7c). In five experiments tested, the plane generated by LDA of sequences with 5-s interstimulus intervals accurately classified 4-s reverse sequences (85.4 ± 9.0% correct; not significantly different than the overall accuracies classifying 5-s interval stimulus trains, P > 0.05; Fig. 7d). These results indicate that synaptic responses recorded in small groups of hilar neurons in vitro can encode both stimulus and sequence identities and are robust to variation in sequence interval.

Population representations of information in the hilus

Hilar representations of stimulus information could arise from a simple coding strategy based on the magnitude of the distinct EPSP frequencies across hilar cells. Alternatively, stimulus location and sequence

Figure 8 Population representations of stimulus and sequence identity in the dentate gyrus. (a) Diagram illustrating rate and population coding strategies. (b) Plots of responses to four different stimulus positions in normalized EPSP frequency space from two experiments. (c) Plot of all responses from 11 experiments in normalized EPSP space. Gray regions represent surface of sphere with unit radius. (d) Plot of the number of statistically significant LDA separation planes (P < 0.05) from all experiments with four different stimulus locations (actual, n = 12), the number of planes in the same data set after normalizing vector magnitudes (normalized) and after eliminating vector direction information (magnitude only). Dashed line indicates mean number of significant LDA planes after shuffling stimulus identities in all 12 experiments. Data are presented as mean ± s.e.m. **P < 0.0001 from shuffled stimulus identities. Both actual and normalized were significantly different from magnitude only (P < 0.01); actual and normalized were not significantly different (P > 0.05). (e) Plot of the number of statistically significant LDA separation planes (P = 0.05, out of six possible) over time. Blue symbols indicate mean ± s.d. number of significant planes after shuffling the stimulus identities. Both actual (black symbols) and normalized responses (red symbols) remained significantly greater than shuffled throughout the time period examined (P < 0.05). (f) Plot of accuracy of predicting sequence identity over time in control conditions (actual, black symbols), following response normalization (red symbols) and using only vector magnitude information (green symbols). Both control (actual) and normalized conditions were significantly different from the magnitude only condition at all time points. *P < 0.002.
information could be encoded by diverse population activity patterns reflecting differential responses in individual hilar neurons (Fig. 8a). We first asked whether eliminating the information associated with differences in vector magnitude impaired information encoding. Responses to each of four stimulus locations remained well clustered following vector normalization (Fig. 8b). The wide distribution of vector directions over nine triple recording experiments (Fig. 8c) suggests that there was minimal systematic bias to generate clusters in one particular region. Eliminating response magnitude information only slightly reduced the number of significant LDA separation planes (from 4.8 ± 0.5 to 3.9 ± 0.7, $P > 0.05$, $n = 12$, Mann-Whitney; Fig. 8d), whereas eliminating vector direction information (retaining only vector magnitudes) significantly impaired response discrimination (1.4 ± 0.4 planes, significantly less than both actual and normalized, $P < 0.01$).

Normalized population responses also tracked actual discrimination performance well over time (Fig. 8e) and remained significantly above chance performance over the time period analyzed. Hilar responses to sequential stimuli were similarly robust to normalization (10 of 11 experiments with separable forward and reverse responses following magnitude normalization, $P < 0.05$ from LDA), implying that the dentate gyrus uses primarily distributed population codes to represent stimulus information in these experiments. Sequence identity was classified accurately in both control and normalized conditions over 6 s and prediction accuracy remained significantly better ($P < 0.005$ for both conditions) than expected by chance over the time window tested (Fig. 8f).

**DISCUSSION**

We found that dentate hilar neurons reliably encode information as distinct patterns of spontaneous synaptic activity that persist for seconds and that resemble the persistent activity patterns recorded in nonhuman primates $^3$ and rodents $^7$ during cross-modality working memory tasks. The same cellular mechanism that encodes isolated stimuli appears to be able to generate reliable contextual representations of stimuli presented in temporal sequences. Responses of dentate hilar neurons accurately predicted the identity of sequences of stimuli, were distinct from responses to the final stimulus presented in isolation in most experiments and were robust to perturbation of sequence interval. Our results suggest that both sequential and nonsequential information were represented by population codes in the dentate gyrus.

**Information representation in the dentate gyrus in vitro**

Although hilar synaptic barrages have been reported previously in response to perforant path stimulation $^2$, our results are, to the best of our knowledge, the first to show that changes in the ongoing synaptic input to dentate gyrus neurons can encode both the identity of more than two stimuli presented individually and in temporal sequences. Stimulus-evoked synaptic barrages reliably represented information that could be decoded using a standard classification method $^{28}$ assessing the Euclidean distances between a response in a single trial and the four centroids that reflect average responses to the four stimuli. The classification accuracy we found using this unbiased decoding approach (~76% correct trials) was significantly greater than that expected by chance; high classification accuracy was maintained when we constructed a naive decoder using centroids computed from half of the data set (Fig. 5). The success of this simple, distance-based decoder was unexpected given the small number of nearby hilar cells sampled and argues that the ability of the dentate gyrus to generate multiple distinct activity patterns is robust and is preserved in acute hippocampal slice preparations. Although experimental limitations restricted these experiments to assaying closely spaced groups of neurons, asking whether widely separated populations of hilar cells can also represent information should be possible in the future using optical activity probes. The success of our decoding method suggests that the separation between different population response patterns is typically large enough to overcome noise and any potential synaptic plasticity associated with repeated trials in the same experiment. Most decoding errors occurred in experiments in which pairs of response centroids occupied similar locations in EPSP frequency space (Fig. 3e). A simple model that included both the separation between centroids and the dispersion of individual responses around each centroid $^{28}$ predicted the minimal inter-centroid distance required for reliable (error free) decoding (Fig. 3h).

In addition to assessing accuracy using a Euclidean distance-based decoder, we used two independent methods (OVL and LDA) to test whether hilar population responses evoked by different stimuli were distinct. These methods revealed that responses evoked by individual stimuli or temporal sequences of stimuli were statistically separable in most experiments. We used intracellular recordings to examine the underlying synaptic events that excite hilar neurons, rather than inferring synaptic drive from extracellularly recorded spiking activity. One tradeoff with this approach, however, is the limited time over which three simultaneous intracellular recordings could be maintained (typically 60–90 min). This limitation restricted our ability to gather the hundreds of repetitions typically required for rigorous information-theoretic analysis.

Previous studies have found that perforant path–evoked synaptic barrages are unlikely to result from recurrent excitatory connections. The majority of mossy cell axonal projections are to contralateral and distal ipsilateral granule cells $^9$, projections that are largely absent in the transverse hippocampal slices that we used. The local projections of glutamatergic hilar mossy cells are primarily to GABAergic interneurons and only infrequently (0.5% connection probability) form recurrent monosynaptic connections onto other nearby mossy cells $^{30}$. Instead, hilar synaptic barrages appear to result from persistent spiking activity in SGCs, a newly discovered excitatory cell type in dentate molecular layer $^{25,26}$. SGCs in the molecular layer respond to transient perforant path stimuli with depolarizing plateau potentials that require L- and T-type voltage-gated calcium channels $^{20}$. Granule cells, in contrast, are typically inhibited by perforant path stimuli that trigger hilar synaptic barrages. Both granule cells and SGCs are polarized neurons whose axonal arbors are excluded from regions containing their own somatodendritic compartments $^{5,26}$, indicating that neither cell type forms recurrent excitatory connections. Our ability to trigger statistically separable hilar population responses may result from recruitment of persistent spiking activity in different subsets of SGCs that project diffusely to hilar neurons. Alternatively, the distinct patterns that we observed may result from stimulus-specific graded plateau depolarizations $^{20}$ in a uniform subset of SGCs or from differential activation of inhibitory local circuits. Further experiments monitoring larger subsets of dentate gyrus neurons will be required to determine the extent of the SGC network excited by different stimuli to distinguish among these hypotheses.

Previous in vitro work in neocortical slices $^{31}$ revealed that proportionally balanced excitation and inhibition enables self-sustaining activity mediated by recurrent excitatory connections, termed up-states. Cortical up-states occur spontaneously in acute brain slices bathed in solutions that enhance excitability and can be triggered by synaptic stimulation $^{31,32}$. Although these up-states can form representations of a single stimulus (whether a stimulus was presented or not), these network states are not synapse specific $^{31}$. In contrast, perforant path–evoked hilar synaptic barrages are highly synapse specific and are capable of representing at least four distinct patterns. Cortical networks
also exhibit spontaneous neuronal avalanches that persist for tens of milliseconds\textsuperscript{33}, a much shorter timescale than that of the persistent activity that we observed.

In addition to representing specific stimulus locations, we found that hilar synaptic barrages accurately represented temporal sequences of stimuli. Rather than simply triggering stereotyped, stimulus-specific activity patterns, stimulus sequences typically triggered new activity patterns that were distinct from those evoked by the final stimulus in the train (ABCD was different from D presented alone; \textit{Supplementary Fig. 8}). This context dependence of stimulus representation, in which the response to a specific stimulus was dependent on prior stimuli, was apparent when synaptic inputs during sequence trains were analyzed (Fig. 6c). Notably, classification accuracy for decoding temporal sequences was robust to a perturbation in sequence interval (Fig. 7d), suggesting that context dependence arises primarily from the relative order of the stimuli. Semilunar granule cells often receive inhibitory postsynaptic responses following hilar and molecular layer stimulation\textsuperscript{25} that could provide a cellular substrate for context dependence.

Although sequence memory often involves the hippocampal formation\textsuperscript{2} and has been demonstrated in computational models of cortical networks\textsuperscript{34–37}, reliable short-term storage of temporal information has only been infrequently observed at the cellular level. Although a recent study\textsuperscript{38} used focal glutamate uncaging at multiple sites along one dendrite to generate sequence-dependent intracellular responses, this mechanism presumably operates primarily through rapid cell-autonomous mechanisms. In contrast, the dentate gyrus circuits that we activated reliably encoded temporal sequences with substantially longer time intervals and could be decoded accurately only by comparing activity across multiple simultaneously recorded hilar cells. Multiple groups have identified state-dependent network responses \textit{in vitro}\textsuperscript{39} and \textit{in vivo}\textsuperscript{40} that reflect the history of previously presented stimuli. Although the contextual responses we observed in the dentate gyrus \textit{in vitro} likely reflect state-dependent processes, our results differ from previous findings in several respects. We found that hilar networks represented information over longer time periods (many seconds) compared with previous state-dependent studies\textsuperscript{39,40}. Hilar networks can encode the order of temporal sequences, not only immediately following the sequence, but also for several seconds afterward. Finally, our results demonstrate rapid encoding of information, whereas previous \textit{in vitro} work required many repeated presentations of stimuli\textsuperscript{39}, implying a gradual entrainment or learning process.

Relationship to delay-period activity

Much of our understanding of the cellular basis of short-term information storage comes from classic extracellular recording studies that found persistent spiking of neocortical\textsuperscript{5,6,8,27} and hippocampal\textsuperscript{17} units during the delay period of short-term memory tasks. These studies showed that the particular units recruited during the delay period depended on the stimulus type (for example, spatial location) and that delay-period activity was typically absent or reduced in trials in which the preferred stimulus was presented and the subject made an incorrect behavioral response\textsuperscript{5,27}. Extracellular recording studies have demonstrated elevated firing frequencies for as long as 20 s during working memory tasks\textsuperscript{5,41}, arguing that cortical networks must contain mechanisms that support persistent activity over this timescale. However, neurons with a diversity of persistent firing durations typically contribute to delay-period activity during working memory tasks, and only a fraction are typically active during the entire delay phase. The subset of delay-period neurons with prolonged persistent activity may have a critical role as ‘summatoms’ that integrate activity from neurons that are active over different timescales\textsuperscript{6,8}. Although it is difficult to determine the kinetics of the mechanisms underlying persistent activity during working memory tasks, as the learned motor output typically extinguishes persistent firing\textsuperscript{5}, our results indicate that the circuitry contained in transverse hippocampal slices, including SGCs with intrinsic persistence, are sufficient to represent information over these long behavioral timescales. Multiple studies\textsuperscript{8,42} have found that compact representations based on population decoding approaches can accurately predict behavioral responses in working memory tasks. Similarly, preserving the heterogeneity in hilar cell responses while ignoring differences in the overall amplitude of responses (that is, normalization) maintained accurate decoding in our experiments. In contrast, average rate-based coding schemes that ignored the differential contribution of each neuron to the population response failed to classify accurately either location or sequence stimuli.

One proposed function of dentate gyrus neurons \textit{in vivo}\textsuperscript{13,44} is to transform highly overlapping entorhinal input patterns into more distinct output patterns. Although this potential functional role has only recently begun to be tested experimentally\textsuperscript{45,46}, our observation that multiple, reproducible population responses can be generated in dentate gyrus neurons is consistent with the hypothesis that the neural networks in this brain region can stably represent multiple population patterns. Semilunar granule cells may be important during pattern decorrelation by generating a ‘latch’ function that enables hilar neurons to maintain their activity following the transient presentation of a particular entorhinal input pattern. Future \textit{in vivo} studies will be required to follow the downstream consequences of hilar persistent activity, as most of the axonal projections of mossy cells extend to distant ipsilateral and contralateral granule cells\textsuperscript{29,47}, which are not present in transverse hippocampal slices.

The largely feedforward local circuits that appear to support short-term storage of information in the dentate gyrus suggest a fundamentally different mechanism than the recurrent excitatory networks commonly employed in artificial neural network models of working memory\textsuperscript{10–13}. Despite extensive efforts to identify biological persistent activity modes mediated by recurrent excitatory synaptic connections (for example, ref. 48), very little experimental evidence supports the classic hypothesis that reverberant network activity functions to encode and maintain information\textsuperscript{9}. Our finding that sequential stimulation did not reset hilar persistent activity to a new state corresponding to the most recent stimulus is also inconsistent with the predictions from theoretical studies employing attractor-based networks with recurrent connectivity\textsuperscript{49}. At the other extreme, multiple groups have found neuron cell types that can fire persistently following transient stimuli through cell-autonomous intrinsic mechanisms\textsuperscript{16,17} and several theoretical studies have observed that combining intrinsic persistence with recurrent excitatory connections relaxes the requirements for precise tuning of synaptic weights\textsuperscript{11,14,18,19}. The alternative intrinsic/feedforward mechanism we hypothesize enables hilar neurons to represent stimulus and sequence identity is related to a recent theoretical study\textsuperscript{37} arguing that purely feedforward circuits can generate prolonged network activity when the effective network time constant is extended by creating multiple serial processing stages. Presumably, the number of stages required to generate delay-period activity would be reduced if some stages of the feedforward network employed neurons with intrinsic persistence, such as SGCs.

The dentate gyrus is unusual in the high frequency of spontaneous synaptic activity recorded in hilar neurons and because of SGCs, a subtype of excitatory projection with multistability under standard physiological conditions. Populations of hilar neurons were presumably able to encode stimulus and sequence identity in our experiments because at least some divergent SGC-to–hilar neuron synaptic connections are preserved in conventional horizontal brain slices. Revealing persistent activity modes in the dentate gyrus was facilitated...
by the anatomical separation of multistable excitatory neurons (SGCs) and downstream hilar neurons. However, other examples of bistability and multistability of excitatory projection neurons in more heterogeneous brain regions have been reported following bath application of modulatory neurotransmitters, raising the possibility that the hybrid intrinsic/feedforward mechanism that we propose in the dentate gyrus may be generalizable. For example, a subclass of neurons in entorhinal cortex are capable of persistent firing following transient stimulation in the presence of muscarinic receptor agonists. To date, there have been no reports as to whether activation of different synaptic inputs to entorhinal cortex leads to mnemonic encoding. Transient inputs also can trigger cell-autonomous persistent firing in neocortical neurons when intrinsic excitability is enhanced following cholinergic receptor activation, providing a potential connection between the intrinsic and downstream persistent mechanisms that we propose in the dentate gyrus and classic delay-period activity recorded in prefrontal cortical neurons in vivo.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.A.H. and B.W.S. designed the experiments, analyzed the data, prepared the figures and wrote the paper. R.A.H. performed all of the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Horizontal slices (300 µm thick) of the ventral hippocampus were prepared from postnatal day 14–25 Sprague–Dawley rats anesthetized with ketamine, as described previously20,33. Slices were incubated at 30 °C for 30 min and then maintained at 20–24 °C until needed. All experiments were carried out under guidelines approved by the Case Western Reserve University Animal Care and Use Committee.

Electrophysiology. All intracellular recordings were performed in a submerged recording chamber maintained at 30 °C and perfused with an extracellular solution containing 124 mM NaCl, 3 mM KCl, 1.23 mM NaH2PO4, 1.2 mM MgSO4, 26 mM NaHCO3, 10 mM dextrose and 2.5 mM CaCl2, equilibrated with 95% O2/5% CO2 (pH 7.3). Whole-cell patch clamp recordings were made using Axopatch 1D amplifiers (Molecular Devices) and borosilicate glass pipettes (3–10 MΩ). Recording electrodes contained 140 mM potassium methylsulfate, 4 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM MgATP, 0.3 mM Na2GTP and 10 mM phosphocreatine, adjusted to pH 7.3 and −290 mOsm. Individual neurons were visualized under infrared differential interference contrast video microscopy (Zeiss Axioskop FS1) before patch-clamp recording. We typically restricted our experiments to a set of two or three nearby hilar cells with inter-soma distances of less than 100 µm. Intracellular recordings were low-pass filtered at 2 kHz (FLA-01, Cygnus Technology) and acquired at 5 kHz using a simultaneously sampling 16-bit data acquisition system (ITC-18, Instrutech) operated by custom software written in VB.NET (Microsoft) and Matlab (MathWorks). Intracellular voltages were not corrected for the liquid junction potential. A matrix microelectrode consisting of four sharpened tungsten monopolar electrodes (115-µm spacing, FHC) was used for extracellular stimulation. Stimulus intensity was controlled by a custom-built constant-voltage stimulus isolation unit. Hilar barrages were evoked most reliably by paired stimuli (500-ms interstimulus intervals), as described previously30. All of the population responses reported reflect paired stimuli at this interval; responses to sequential stimuli (for example, ABCD) reflect paired shocks at each matrix electrode contact at the indicated interval (paired shocks every 4–120 s). Hilar recordings and population responses were typically stable over 10–15 paired stimuli or temporal sequences of different stimuli (3.5-min delay between different stimuli in both experiment types, except for 120-s sequential stimuli experiments). We collected the data set of experiments using different stimuli (3.5-min delay between different stimuli in both experiment types, except for 120-s sequential stimuli experiments). We collected the data set of experiments using different stimulus locations in one continuous series after an initial set of pilot studies to optimize stimulus and recording conditions. Only experiments in which hilar cell recordings degraded or in which we obtained fewer than three responses from each stimulus type were excluded from the analysis.

Data analysis and statistics. Hilar cells were identified by their intrinsic properties (mean spike time, spike clustering, and spike afterhyperpolarization) following 4-s-duration depolarizing and hyperpolarizing current steps, as described previously30. Most of the recordings were from presumptive hilar mossy cells. Spontaneous EPSPs were identified automatically using a custom detection algorithm30. Except where noted, all data are presented as mean ± s.e.m. Statistical significance was determined using Student’s t test, unless otherwise specified.

Analysis of hilar population responses. EPSPs were typically detected in a 4-s window that began 0.5 s after the last shock of each paired stimulus. The mean EPSP frequency in this window in each recorded hilar cell was used to analyze population responses. We approached the question of whether population responses evoked by different stimulus electrodes were distinct using three complementary methods. First, we assessed the separability of population responses by calculating the overlap coefficient (OVL)1,52, Supplementary Fig. 3. In this method, we computed the distribution of Euclidean distances from individual population responses (the mean EPSP frequencies in the same 4-s analysis window applied to each simultaneously recorded hilar cell) with the centroid calculated from all responses evoked by the stimulus electrode in the same experiment (cis distances). The distribution of these cis distances was then compared with the distribution of Trans distances calculated from dissimilar data point/centroid pairs (for example, A points to the centroid of all B points). The overlap coefficient OVL was computed from Gaussian fits of cis and Trans distributions. The statistical significance of the OVL coefficient was determined by bootstrap methods (20,000 randomizations of cis/Trans labels on each actual dataset). All location experiments analyzed in this study yielded statistically distinct cis/Trans distributions (all OVL calculated reflected $P < 0.05$; Fig. 2e).

Second, we used LDA53–55 to determine the number of statistically separable population responses in each experiment. Complete classification of population responses to all four stimulus locations (A–D) required six statistically significant pairwise LDA tests (A/B, B/C, etc.), each with $P < 0.0083$ reflecting a Bonferroni correction for multiple comparisons. Plots of the number of discrimination planes in different conditions (Figs. 2d, 8d and Supplementary Fig. 2) reflect the number of pairwise LDA tests with $P < 0.05$. We estimated the number of planes expected by chance by randomly reassigning EPSP frequencies in each hilar cell to different stimulus identities. We recalculated the number of statistically significant LDA planes following 30 iterations to generate an estimate of the number of planes expected by chance. A statistically significant OVL coefficient combined with six of six possible LDA tests (with $P < 0.0083$) represents a conservative test for response separability, a criterion that was met in 50% of the experiments that we analyzed. If one experiment failed to generate four separable responses, it was re-analyzed to determine whether three (three appropriate planes with $P < 0.016$) or two (one plane with $P < 0.05$) responses could be isolated. We calculated the time course of response separability using a sliding 4-s window and repeated the LDA at 1-s intervals. Times indicated reflect the beginning of each 4-s time window. We also used LDA based on the mean EPSP frequency in 4-s windows that began 0.5 s after the last stimulus in each sequence (for example, 0.5 s after the D in ABCD and after the D in DCBRA) to determine whether population responses to different temporal sequences were significantly different. We employed the same time windows when analyzing sequences with both 4- and 5-s intervals.

Finally, we assessed the accuracy of hilar population responses in predicting stimulus identity, assayed by nearest stimulus centroid. Classification accuracy was calculated for the entire data set from the ratio of trials classified correctly to the total number of trials tested. Sequence identity was predicted from the sign of the response distance to the LDA plane.

Character intensity plots (Fig. 5 and Supplementary Fig. 5) reflect the relative Euclidean distances between each population response and the four stimulus centroids, recomputed at 1-s intervals. Each location reflects the superposition of four characters (A, B, C, and D) whose intensities are scaled to reflect their relative distance to stimulus centroids. The scaling function was normalized by the sum of the scaled distances to all four centroids:

$$\text{Intensity} = e^{-d_{ij}} + e^{-d_{i2}} + e^{-d_{i3}} + e^{-d_{i4}}$$

where $d$ represents the Euclidean distance between a single response and a response centroid and $x$ is the stimulus identity being tested. This transformation resulted in one very dark (high contrast) character superimposed on three light (low contrast) characters if a response was very close to a stimulus centroid. We then iterated through all four possible centroids at each time step

$$P_{jk} = \frac{1}{e} \sum_{i=1}^{4} e^{-d_{ijk}}$$

$$d_{ijk} = |E_{ijk} - E_{ij}^{*}|$$

where $p$ is character intensity, $i$ represents stimulus identity, $j$ represents the time bin, $k$ represents the trial and $F_e$ represents the centroid vector for one stimulus identity at one time bin. The relative distance between forward and reverse responses in Figure 7c was calculated as the signed length of the projection of the population responses onto the difference vector

$$D_{ij} = \frac{E_{ij} - E_{ij}^{*}}{|E_{ij} - E_{ij}^{*}|}$$

where $d$ represents the difference vector of the mean population responses for forward and reverse sequences and $F$ is the population response vector. Data analysis was performed in Matlab (MathWorks) and Origin (OriginLab).
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