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Differential responsivity of neurons in perirhinal cortex, lateral entorhinal cortex and dentate gyrus during time-bridging learning

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Abstract

Many studies have focused on the function of hippocampal region CA1 as a critical site for associative memory, but much less is known about changes in the afferents to CA1. Here we report the activity of multiple single neurons from perirhinal and entorhinal cortex and from dentate gyrus during trace eyeblink conditioning as well as consolidated recall, and in pseudo-conditioned control rabbits. We also report an analysis of theta activity filtered from the local field potential (LFP). Our results show early associative changes in single-neuron firing rate as well as theta oscillations in lateral entorhinal cortex (EC) and dentate gyrus (DG), and increases in the number of responsive neurons in perirhinal cortex. In both EC and DG, a subset of neurons from conditioned animals exhibited an elevated baseline firing rate and large responses to the conditioned stimulus and trace period. A similar population of cells has been seen in DG and in medial, but not lateral, EC during spatial tasks, suggesting that lateral EC contains cells responsive to a temporal associative task. In contrast to recent studies in our laboratory that found significant CA1 contributions to long-term memory, the activity profiles of neurons within EC and DG were similar for conditioned and pseudoconditioned rabbits during post-consolidation sessions. Collectively these results demonstrate that individual subregions of medial temporal lobe differentially support new and remotely acquired memories. Neuron firing profiles were similar on training trials when conditioned responses were and were not exhibited, demonstrating that these temporal lobe regions represent the CS-US association and do not control the behavioral response. The analysis of theta activity revealed that theta power was modulated by the conditioning stimuli in both the conditioned and pseudoconditioned groups and that although both groups exhibited a resetting of phase to the corneal airpuff, only the conditioned group exhibited a resetting of phase to the whisker conditioned stimulus.

Keywords

Trace eyeblink conditioning; Medial temporal lobe; Hippocampus; Memory consolidation; Single neuron activity

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INTRODUCTION

Many studies have focused on the function of hippocampal region CA1 as a critical site for associative memory, yet hippocampus draws on multiple nodes within medial temporal lobe to communicate with diverse regions of cortex and form associations among stimuli in the environment, and neurons within the human medial frontal cortex and medial temporal lobe have exhibited memory related persistent activity (Kaminski et al., 2017). Before reaching dentate gyrus (DG) and CA1, multimodal information from prefrontal and secondary sensory cortices, converges in perirhinal cortex (PR) and entorhinal cortex (EC) (Furtak et al., 2007b; Kerr et al., 2007), and these regions become increasingly engaged in long-term retrieval of consolidated associative memories (Hattori et al., 2014b; Takehara-Nishiuchi et al., 2012), when hippocampus-independent retrieval occurs for trace eyeblink conditioning (EBC) (Kim et al., 1995).

Acquisition and retention of trace EBC has been used to model declarative memory formation, since it is hippocampus-dependent for acquisition, but not post-consolidation retention (Kim et al., 1995; Moyer et al., 1990; Weiss et al., 1999), and because trace EBC in humans requires an awareness that the conditioned stimulus (CS, typically a tone) predicts the onset of the unconditioned stimulus (US, typically an airpuff to the eye) (Clark and Squire, 1998; Knutten et al., 2001). There is general agreement from lesion and recording studies that perirhinal and lateral entorhinal (latEC) cortices are implicated in object and complex feature processing, while the parallel hippocampal input pathway of postrhinal and medial entorhinal cortex (MEC) subserves context and orientation processing. Although the layer 3 MEC to CA1 pathway may also subserve temporal bridging (Jochems et al., 2013; Kitamura et al., 2014; Knauer et al., 2013; Suh et al., 2011), the PR and latEC cortices both support persistent spiking in vitro (Egorov et al., 2002; Hasselmo et al., 2009; Navaroli et al., 2011; Tahvildari and Alonso, 2005; Tahvildari et al., 2007) and episodic memory encoding may rely on persistent spiking (Hasselmo et al., 2010). In MRI-BOLD studies, conditioned stimulus (CS) responses in EC and PR during training predict later performance on delayed match-to-sample tasks in humans (Nauer et al., 2015); and PR and latEC show inter-stimulus activity specific to paired stimuli in primates (Naya et al., 2003).

Cortical input to the hippocampus proceeds from layer II of entorhinal cortex via the perforant path to the dentate gyrus (DG) granule cell layer (Dolorfo and Amaral, 1998), while return projections target deep layers of EC. Thus electrophysiological recordings from the hippocampus and its afferents may help to identify the neuronal processing that occurs throughout this intertwined network. Given that single neuron responses from DG revealed increases to both the CS and US during acquisition of trace fear conditioning (Gilmartin and McEchron, 2005), and recordings from PR during trace fear conditioning revealed an increase in the percent of responsive neurons (Furtak et al., 2007a), we hypothesized that EC and PR should be involved in acquisition and perhaps consolidation of trace EBC. Multi-unit recordings in DG during delay eyeblink conditioning found that responses to the unconditioned stimulus (US) increased by several hundred percent by the tenth trial of training (Berger et al., 1976) and increased DG field potential responses to perforant path stimulation, consistent with long-term potentiation of EC-to-DG synapses, were also

observed (Berger et al., 1976; Weisz et al., 1984). Furthermore, single-neuron recordings revealed learning-related changes in dorsal CA1 in trace EBC, (Hattori et al., 2014a; McEchron and Disterhoft, 1997; McEchron et al., 2001; Weible et al., 2006; Weiss et al., 1996). However, there are no such reports describing how perirhinal cortex, lateral entorhinal cortex or dentate gyrus neurons are involved in forebrain dependent trace EBC.

In addition to single neuron data, more global signals in the form of local field potentials (LFPs) are descriptive of the changes that occur throughout the network. LFPs were recorded during non-hippocampal dependent delay EBC in the rabbit (Thompson et al., 1980; Berger et al., 1983). Those LFPs were found to have an inter-burst interval equivalent to an 8 Hz theta oscillation, and the oscillation continued into the intertrial period. Furthermore, a greater ratio of theta to non-theta power during a pre-training session was found to correlate significantly with faster learning of delay EBC. Single neurons recorded simultaneously with the LFP revealed two classes of neurons with conditioning-specific changes in activity, one class increased its firing rate while displaying the theta rhythm, and another class decreased its firing rate following onset of the CS.

Theta oscillations are also important in acquisition and retention of hippocampal-dependent tasks (Takehara-Nishiuchi et al., 2012, Hoffmann, Cicchese, Berry, 2015). In fact, Berry and colleagues reported that presenting trials when theta power is high results in faster learning of trace (or delay) EBC as compared to presenting trials when theta is not present (Seager et al., 2002; Cicchese et al., 2015). Failures to replicate this effect may be due to presentation of the conditioning stimuli at a sub-optimal phase of the theta cycle (Nokia et al., 2015), hence an analysis of theta phase and phase resetting is important. Regardless, stimulus-specific theta modulations in CA1 remain strong throughout acquisition and consolidated performance of trace eyeblink conditioning (Hattori et al., 2014a). In contrast, dentate gyrus theta rhythm is less well explored. Theta-frequency stimulation of EC stellate inputs to DG induce LTP in CA1 (Stepan et al., 2012). Theta-gamma coupling in DG plays a role in spatial learning (Bott et al., 2016) and in an associative task, cue presentation results in a decrease in theta amplitude (Rangel et al., 2015). However, whether hippocampal subregions express similar theta modulation during trace eyeblink conditioning remains unclear.

The present study compared responses of multiple single-neurons in PR, latEC and DG between Conditioned and Pseudo-conditioned animals during whisker-signaled trace EBC. We show that DG and latEC contain high-firing rate cells during trace EBC that represent learned information, and that medial temporal lobe (MTL) neuronal activity at the single-cell and ensemble levels encode the CS-US association. However, MTL does not encode the conditioned blink response per se, because single-neuron activity is similar on trials with or without a conditioned response. We find that association-induced neuronal activity at the single-neuron and LFP level occurs early during learning and is markedly attenuated in these brain regions after memory consolidation has occurred.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 10 female New Zealand White rabbits (*Oryctolagus cuniculus*), approximately three to six months of age at onset of study. All subjects were individually housed with ad lib access to food and water and maintained on a 12 hour light/dark cycle.

Surgery and training

All surgeries were performed in accordance with NIH guidelines and approved by the Northwestern University Institutional Animal Care and Use Committee, as described previously (Hattori et al., 2014a). In brief, rabbits were implanted with a microdrive fashioned from a custom mold and assembled to allow chronic recordings using 24 independently moveable tetrodes. Each tetrode consisted of four, 12.7 μ m, heavy-formvar insulated Stablohm 800 wires (California Fine Wire, Inc) with the tips gold-plated to lower the impedance to 200 k Ω at 1kHz. The drive was angled 10 degrees laterally and was stereotactically positioned so that tetrodes ranged from 3 to 6mm posterior and 3 to 7mm lateral to bregma (foot plates were either ca. 3mm diameter circle or 1mm by 4mm oblong); lateral tetrodes recorded from PR (Fleischhauer et al., 1980), medial tetrodes from latEC, and center tetrodes from hippocampus (dorsal DG). Recordings were from the left hemisphere, contralateral to whisker vibration CS and air puff US application.

After >5 days of recovery, tetrodes were advanced daily for several weeks, relying on turn counts and firing characteristics to locate targets in granule cell layer of dorsal dentate gyrus, or the superficial and deep layers of PR and lateral EC, and animals were trained as described below. Each tetrode was adjusted after each session, if needed, to maximize number of cells recorded. After conclusion of all recording activities, electrolytic marking lesions and histological analyses were done as described previously (Hattori et al., 2014a).

Task and behavioral analysis

As illustrated in Fig. 1D, Conditioned animals received 80 trials per session of paired CS (whisker vibration, 250ms) and US (corneal airpuff, 150ms) separated by 500ms trace interval; the mean intertrial interval was 45s (30–60s range). Animals were trained in a yoked pair design with Pseudo-conditioned rabbits that received 80 CS-only and 80 US-only trials in random order per session (15–30s iti). Behavioral response (extension of nictitating membrane) was assessed using an infrared reflective sensor. After tetrodes were advanced to target areas, and rabbits were habituated to recording apparatus over several days, (Pseudo-) Conditioning was initiated and continued with one session per day. Training was done in the middle of the day and the rabbits remained alert throughout the conditioning session. Conditioned Responses (CRs) were defined as extension of the nictitating membrane (third eyelid) to >4SD above pre-CS baseline for >15ms during the final 20ms of the trace period (just prior to US onset). Rabbits attained behavioral criterion when CRs were produced on 8 out of 10 trials in a sliding window, and this was maintained for 2 days in a row.

After reaching criterion, rabbits were trained and recorded for an additional 5 sessions. They were then left in their home cages for at least 30 days, before returning to tetrode adjustment and 5 days of ‘post-consolidation’ training using the same paradigm.

Data were grouped into pre- and post-consolidation sessions (D1 to C+5 vs R1 to R5) for statistical analysis of single-neuron activity. For theta activity, 3 phases of learning were used: Early acquisition (first day “D1” to day of criterion “C”), Late acquisition (day after criterion “C+1” to 5 days post-criterion “C+5”); and post-consolidation retention “R1” to R5”. Data from a total of 5 Conditioned and 5 Pseudo rabbits are included in this study. One Conditioned rabbit was not evaluated post-consolidation: Pseudo N=5; Conditioned N=4 to 5. In Figure 1, percent CR was compared between groups using rm-ANOVA with group as a factor and learning phase as a repeated measure.

in vivo Electrophysiology and Single-Neuron Isolation

Data were collected and filtered, and clusters of single-unit activity were isolated offline as previously described (Hattori et al., 2014a): Electrical signals recorded from the tetrodes were amplified x10,000, digitized, band-pass filtered at 600–6000 Hz, and saved for offline analysis using Cheetah Data Acquisition Software (Neuralynx, Inc). Clusters of single-unit activity were isolated offline based on waveform characteristics (peak, valley) using Spikesort 3D (Neuralynx, Inc). Waveforms of isolated clusters were subjected to a custom written signal-to-noise (SNR) program in MATLAB (Mathworks, Inc). For each cluster, the waveform of each spike was divided into 32 points with the peak of the waveform forced to the 8th point. Then, the mean waveform and variance waveform was computed for each neuron. Noise, N , was defined as $N = \sqrt{\frac{1}{n} \sum_{i=1}^n \sigma_i^2}$, where $n = 32$ points and σ^2 = variance waveform. The signal, S , was defined as the difference between the peak and the trough in the mean waveform. The quotient of S and N yielded the SNR for each cluster and all clusters with $SNR < 5$ were excluded from further analyses.

Recorded dentate gyrus neurons were classified as principal rather than interneuron based on baseline firing rate (DG <10Hz granule cells (Neunuebel and Knierim, 2014)). Given the difficulty of distinguishing DG granule cells from dentate mossy cells or hilar cells when neither place field or sleep/wake activity data are available (GoodSmith et al., 2017; Senzai and Buzsaki, 2017), we simply refer to these cells as dentate neurons. Neurons in latEC were grouped into high-firing and low-firing with a cutoff of 3Hz, because no high-firing cells were reported in latEC in previous publications. A total of 4 cells were found in latEC and 5 were found in PR with baseline firing >10Hz; these cells had comparatively large spikewidths; nonetheless they were excluded as putative interneurons, following Deshmukh (2012). Cells recorded from each day were conservatively treated as unique (independent) neurons; a subset analysis was also performed to exclude the possibility of repeated sampling of identical neurons, see below.

Data Analysis

Neural data from each rabbit were aligned on day of behavioral criterion to allow comparisons among animals that learned at different rates. Due to low firing rates in latEC, comparisons were performed by pooling neurons recorded from all rabbits in a group and

statistical comparisons were performed among “phases” of training, rather than among individual days of training., e.g. for a given comparison, if 5 conditioned rabbits contributed 3 neurons each, the N for the comparison would be 15 for the conditioned group.

Several different measures of neural activity were used as each one provides a unique characterization of the neuronal population: baseline firing rates, number of significantly modulated cells, stimulus and trace-period responsivity. Responsivity was analyzed as follows: for each neuron, peri-stimulus time histograms encompassing 1 s pre-CS onset and 2 s post-CS onset were generated for each training day using 10 ms bins. Raw baseline firing rates (Hz) were compared between groups using ANOVA with group and training phase (acquisition/post-consolidation) as factors; main effects with $p < .05$ were followed with Scheffe’s post-tests.

Comparisons of neuronal responses during task intervals were performed by first comparing the average firing rate during the CS interval (250ms) to the baseline firing rate. Cells were classified as increasing (INC) if the CS interval showed a greater mean firing rate than during baseline, or as decreasing (DEC) if the CS interval showed a lower mean firing rate than the baseline for that cell.

To determine the percent of recorded neurons that responded to the task, Mann-Whitney U (MWU) non-parametric test comparing 1s pre-CS baseline activity to activity during the CS and Trace intervals of the trial was used to define statistically significant rate-increasing (RI) or rate-decreasing (RD) neurons with $p < .05$.

Because baseline firing rates are frequently increased in conditioned as compared to pseudo rabbits (see Figs. 3 & 4), responses were normalized by calculating z-scores for each bin as compared to a 1s baseline firing rate immediately prior to CS onset, as described previously (Hattori et al., 2014a). Conditioned and Pseudo-conditioned groups were compared using rm-ANOVA with group as a factor and 6 levels of 250ms time windows as a repeated measure, i.e., baseline, CS, first half of trace interval (tr1), second half of trace interval (tr2), US window, and post-US window. CS-trace and US-post-US intervals were evaluated separately because in the Pseudo-conditioned group, the CS and US data are from separate trials. Significant results were followed by post-hoc unpaired t-tests. Acquisition and post-consolidation responses were additionally compared for the Conditioned group only by rm-ANOVA as above, but with training phase (pre- vs post-consolidation) as a factor.

To address the possibility that the results were affected by recording from identical neurons on successive days of training, which could lead to an artificially depressed variance of the data, we performed a supplemental analysis using only data from a single day for each rabbit. This excludes the possibility of repeat sampling of a neuron. We selected the single day within a training phase (acquisition and post-consolidation) for each rabbit on which the maximal number of neurons was recorded. If two days had the same maximal number of recorded cells, the earlier day was chosen. As for the inclusive analysis above, we then performed a 2-way rm-ANOVA on the interval z-scores of this subset of data to test for a group and pre/post-consolidation difference.

To compare differences in the number of statistically significant RI and RD neurons from each group relative to the total number of increasing or decreasing neurons, a chi-square goodness of fit test was used with training group (Conditioned, Pseudo) and response type as factors. For purposes of illustration, graphs are shown as percent RI or RD cells.

To compare neuronal activity in Conditioned animals on trials with an adaptive behavioral response (CR trials) versus failed trials (no-CR trials), data from peristimulus time rasters for each neuron were separated based on the behavioral outcome of each trial (CR or no CR). Data from post-criterion days, when percent CR trials approached 50 percent, were used to maximize the statistical power for detecting differences between CR and no-CR trials. Separate mean CR and no-CR session histograms were calculated for each neuron. Population means for each interval were compared using rm-ANOVA with CR/no-CR as a factor.

For slow wave data analysis, continuously sampled data were processed as described previously (Hattori et al., 2014a). Data collected from one wire of each tetrode from each trial of each session for each animal was band-pass filtered for activity in the rabbit theta range (4–8Hz, (Green and Arduini, 1954)) and instantaneous amplitudes calculated by Hilbert transform of each trial were normalized to baseline (1s pre-CS), then averaged to obtain the mean amplitude of a session. Mean amplitudes of a session were averaged across animals to obtain a group response. For examination of phase-resetting, circular statistics were used to obtain the mean resultant vector (MRV) of each animal per session. MRV yields the mean angular direction (phase) of the signal. The circular mean of the MRVs of animals within a session was then taken to get a group response for the session. The Harrison-Kanji test (Harrison et al., 1986), an analog of a two-way ANOVA for circular statistics, was used to determine whether the MRV changed significantly over sessions and between groups.

RESULTS

Rabbits implanted with tetrodes for simultaneous recording in PR, latEC and DG received daily training sessions of 500-msec trace EBC with paired (Conditioned) or unpaired (Pseudo-conditioned) presentation of CS and US (Figure 1D). Conditioned animals reached criterion (8 trials in a sliding block of 10 consecutive trials with a conditioned response (CR; blink during the 20ms preceding the US)) on average during the eighth session (mean 8.2, SEM 2.7 days; Figure 1D, bottom panel, note that the X axis does not show data for the variable number of sessions between the first session and the criterion session). After criterion, rabbits received an additional 5 sessions of training to help maximize learning. Yoked Pseudo-conditioned rabbits received the same number of sessions as the Conditioned rabbits. Percent CR per session became significantly different between groups starting on the day of criterion (rm-ANOVA, $p=0.0013$). After a training-free consolidation period of one month, rabbits were trained again for 5 sessions. Percent CR remained significantly different between groups, indicating that the CS-US association was successfully established and maintained in all Conditioned rabbits.

Conditioning increases baseline firing rates

2173 well-isolated neurons were recorded from sites in PR (Cond N=358, Pseudo N=75), latEC (Cond N=235, Pseudo N=196), and dorsal DG (Cond N=499, Pseudo N=810) over the course of 17 sessions. Although some of the data may represent the activity from the same neurons across days, a secondary analysis that avoided repeated sampling of individual neurons revealed similar results to those below and are summarized in the section: Subset Analysis To Avoid Resampling of Individual Neurons.

Figure 2 shows response profiles for each neuron recorded during conditioning in latEC and DG, the regions where conditioning-specific enhancements were observed during learning. The majority of latEC neurons were recorded from rabbits A, B and C; the majority of DG neurons were recorded from rabbits A and E. The “largest” latEC response in the raster plots is seen from rabbit A, although it is clear that latEC neurons from rabbits B and C were similar in overall shape. The DG neurons in rabbit A showed the largest increase in firing in the raster display, and the activity of DG neurons from rabbit E mirrored the activity of rabbit A. There were relatively few neurons recorded from rabbits C and D. Note that no single rabbit or single neuron dominated the average response profiles. More studies will be necessary to determine if there is a spatial localization of neurons that show large response changes in latEC or DG during trace EBC; and whether responsive neurons can be demonstrated in all or most animals during training.

Average peristimulus time histograms (bin size 10ms) of normalized firing rates (z-scores) for cells in each region during acquisition (top row) and post-consolidation (bottom row) in trace EBC are shown in Figures 3 and 4. Neurons with increased firing (INC) in response to CS (Fig. 3) were analyzed separately from those that show a decrease (DEC) in rate (Fig. 4); that is, the average firing rate during the baseline was simply subtracted from the average firing rate during the CS and a positive result defined an INC neuron, while a negative result defined a DEC neuron. Note that Pseudo plots show the initial 1.75sec of CS-only trials plus the final 1.25sec of US-only trials to facilitate comparison with Conditioned (CS + US-trial) results. Insets in each panel represent baseline firing rates of Conditioned (black bar) and Pseudo groups (open bar).

Baseline firing rates: effects of conditioning

PR and latEC showed very low baseline firing rates in both Conditioned and Pseudo animals (combined means $\pm$ SEM: PR, 0.803 Hz $\pm$ 0.124; latEC, 1.299 $\pm$ 0.238). As indicated in the insets of Fig. 3, INC neurons from Conditioned rabbits showed increased baseline firing rates compared to neurons from pseudoconditioned control animals during acquisition (Figure 3, insets): baseline rates of INC cells were increased significantly in the Conditioned group as compared to Pseudo in both latEC ($p=0.0018$) and DG ($p<0.0001$), but only in DG post-consolidation, i.e. consolidated sessions ($p<0.0001$, Figure 3 insets). INC neurons from PR did not show learning-specific baseline changes, but DEC PR neurons from conditioned rabbits showed increased baseline firing as compared to neurons from pseudoconditioned rabbits during acquisition sessions ($p=0.0371$, Figure 4 inset). Baselines of DEC neurons were increased in DG both pre- ($p<0.0001$) and post-consolidation ($p<0.0001$, Figure 4 insets). No significant changes in baseline firing were found between pre- and post-consolidation

training in Conditioned rabbits. A subset of cells in latEC and DG with increased baseline firing rates is further discussed in Figure 5.

To compare neuronal responses within the trial presentations, we normalized responses by calculating per-bin z-scores of the firing rate histogram for each cell (as compared to baseline firing rate). Because z-scores cannot be calculated for any cell with a baseline firing rate of 0Hz, “silent cells” that did not fire during the 1-second baseline of at least one trial per session were excluded from analysis. The percent of silent cells remained relatively constant across areas (PR Conditioned 11.2%, Pseudo 6.7%; latEC 14.5% and 6.6%; DG 16.6% and 9.9%), but was higher in Conditioned than Pseudo in each region, reaching statistical significance in latEC and DG (chi-square test $\chi^2=6.753$, $p=.0094$ latEC; $\chi^2=12.931$, $p=.0003$ DG).

PR does not show a conditioning-specific response change during trial presentation

Despite the increased baseline firing in DEC cells noted above, PR did not show a conditioning-specific change in neuronal response during the trial period in either INC (Fig. 3) or DEC cells (Figure 4; rm-ANOVA of z-scores with group as factor; Scheffe post-test). Neurons in PR did respond to presentation of the CS and US stimuli but their responses were equivalent during conditioning and pseudoconditioning. The percentage of significantly responsive PR neurons was however greater in conditioned rabbits as compared to pseudoconditioned rabbits (see subsequent section and Fig. 5).

EC and DG neurons encode temporal association

In contrast to high-fidelity stimulus representations in PR, i.e. no significant difference between neuronal responses from conditioned and pseudoconditioned rabbits, latEC neurons showed a *conditioning-specific* increased magnitude of the CS response (Fig. 3), as well as of the US response, in INC cells during acquisition, i.e. there was a significant difference in firing rate between neurons from Conditioned as compared to the Pseudo group (rm-ANOVA with group as factor: group $p=.0045$, interaction of group and task interval $p=.0001$; post-test comparing groups during: CS $p<.0001$, first half of the trace period (tr1) $p=.0207$, US $p=.0010$, other intervals NS). Note that in Conditioned animals, the magnitude of the entorhinal CS-response is greater than the response to the US, whereas the US representation predominates in neurons from Pseudoconditioned animals. An interaction between group and task interval for z-scores in both INC and DEC cells indicated different shapes of the neuronal response profiles between groups ($p<.0001$). This interaction gave statistical quantification to the visually apparent fact in Fig. 3 that latEC neurons showed a larger effect of the CS than of the US as compared to those in Pseudoconditioned rabbits, suggesting altered processing, rather than simply amplification, of stimuli.

latEC DEC neurons demonstrated a prolongation of the CS response throughout the trace interval in Conditioned as compared to Pseudoconditioned animals, with a group difference in early and late trace periods (main effect $p<.0001$ with interaction $p=.0046$; post-test comparing groups: CS interval NS, tr1 $p=.0001$, tr2 $p=.0006$).

In DG INC cells, the Conditioned US response is more prominent than the response to the CS. DG neurons of both INC and DEC type have a conditioning-specific CS response, and

notably the response is maintained throughout the trace period in Conditioned animals, completely bridging the time between associated stimuli (Fig. 3: INC neurons by rm-ANOVA with group as factor: $p < .0001$, interaction $.0445$; post-test for group effects: CS $p = .0011$, tr1 $p < .0001$, tr2 $p = .0011$; Fig. 4: DEC neurons by rm-ANOVA $p = .0002$, interaction NS; post-test for group effects: CS $p = .0014$, tr1 $p = .0037$ and tr2 $p = .0033$). Conditioned INC neurons additionally maintain a prolonged US response as compared to the brief, stimulus-locked US response in Pseudoconditioned animals, both pre- and post-consolidation (rm-ANOVA for US: acquisition $p = .0023$, consolidated $p = .0169$, interaction NS; post-tests for group effects: US NS, post-US interval $p < .0001$ at both time points).

Subset Analysis To Avoid Resampling of Individual Neurons

Because recordings from several days were combined for analysis and because not all tetrodes were moved every day, it is possible that the same neuron was occasionally re-sampled on two or more days. If so, this would violate the assumption of independence of sampling for statistical testing and over-represent a given response profile. To confirm that our main results are significant when this possibility is excluded, we also performed the conditioned-pseudoconditioned comparison using data from only a single day per animal pre- and post-consolidation. The number of cells in this analysis was: PR Pre-consolidation, Cond $N = 13$, Pseudo $N = 3$; Post-consolidation Cond $N = 13$, Pseudo $N = 9$. latEC Pre-consolidation, Cond $N = 17$, Pseudo $N = 13$; Post-consolidation Cond $N = 4$, Pseudo $N = 7$. DG Pre, Cond $N = 16$, Pseudo $N = 24$; Post-consolidation Cond $N = 13$, Pseudo $N = 20$. These data came from eight of the rabbits in the study. The results were heterogeneous among rabbits with at least two of the rabbits contributing robust activity for each region.

In this subset analysis, rm-ANOVA with group and acquisition/post-consolidation as factors showed a highly significant difference between groups during acquisition in DG ($F_{1,31} = 12.005$, $p = .0016$) and approached significance in latEC ($F_{1,9} = 4.407$, $p = .0652$), with no difference in PR ($F_{1,14} = 2.223$, $p = .1581$). Conditioned animals showing increased trace activity compared to pseudo in both latEC and DG during acquisition (rm-ANOVA:EC $F_{1,4} = 5.610$, $p < .0004$, DG $F_{1,4} = 6.441$, $p < .0001$) and post-criterion (EC $F_{1,4} = 7.164$, $p < .0002$, DG $F_{1,4} = 4.978$, $p = .0009$). These results exclude the possibility of resampling and confirm the main results of the summative analysis presented above: In summary, there is a conditioning-specific increase in firing during the trace period in latEC and in DG, but not in PR. In this subset analysis, group differences persist post-consolidation.

MTL activity is decreased after memory consolidation in trace EBC

Overall, MTL neurons showed markedly reduced group differences after a one-month period of memory consolidation (Fig. 3 & 4, lower panels). Post-consolidation, PR maintains only stimulus-locked responses to CS and US, with no significant difference in response profiles between groups. LatEC conditioning-specific activity is decreased post-consolidation, as described above, with no significant difference remaining between groups. Both latEC and DG show similar magnitude of CS and US responses post-consolidation, whereas pre-consolidation the CS predominated in latEC and US in DG. DG is uniquely active in that it maintains some learning-specific effects post-consolidation, maintaining both a post-US

increase in Conditioned versus Pseudo INC cells ($p < .001$), as well as a group difference in baseline firing rate in both INC and DEC cells, as described above.

Task-Modulated cells are increased in number in Conditioned animals

An important measure of computational effort is the number of neurons recruited during a task, in addition to the degree of firing rate modulation and baseline characteristics. Figure 5 demonstrates the percentage of cells that showed a statistically significant change in rate during the CS and/or trace periods, as compared to baseline, according to the Chi-square test. PR, which did not show conditioning-specific effects in mean firing rate, did recruit a greater number of RI cells in Conditioned pre-consolidation as compared to Pseudo ($p = .0176$, $\chi^2 = 8.0797$), as did latEC ($p = .0292$, $\chi^2 = 7.0672$) and DG ($p = .0153$, $\chi^2 = 8.3598$; $df = 2$ for all comparisons in this section).

RD cells were recruited in higher numbers in Conditioned than Pseudoconditioned animals in latEC both pre- and post-consolidation ($p = .0010$, $\chi^2 = 9.2103$ and $.0012$, $\chi^2 = 13.4509$). In Pseudoconditioned animals, fewer neurons were responsive post-consolidation than pre-consolidation ($p = .0430$, $\chi^2 = 6.2931$). In DG, RD neurons were more prevalent in Conditioned than Pseudo during acquisition ($p = .0012$, $\chi^2 = 13.4509$). The maintenance of increased RD neuron numbers in latEC of Conditioned animals both pre- and post-consolidation suggests a continued assignment of computational resources during trace EBC even in the absence of an increase in the magnitude of neuronal responses as demonstrated by histograms plots.

Trace conditioning recruits high-firing cells in latEC and DG that carry associative signal

Figure 6 demonstrates that during trace EBC, latEC exhibits a subset of high-firing rate neurons (baseline $> 3\text{Hz}$; Cond $N = 9/90$, Pseudo $N = 0/84$) similar to those found in medial EC, but not in latEC on a spatial task (Neunuebel et al., 2013). No neurons with baseline $> 3\text{Hz}$ were active in Pseudo-conditioned animals. Additionally, latEC cells were sorted based on recording site in superficial layers that project into DG and CA1, or deep layers that receive CA1 output. All of the high-firing rate cells were located in superficial layers of latEC. DG also showed a group of high-firing rate cells (baseline $> 2\text{Hz}$, as described in (Neunuebel and Knierim, 2014)) that were active primarily in Conditioned animals (Cond $N = 26/150$, Pseudo $N = 3/273$). The lower panels show peristimulus time histogram z-scores of Conditioned animals' high-firing cells (solid line) and non-high-firing cells (dotted line) for latEC (A) and DG (B). Note that stimulus and trace-period activity is contributed mostly by high-firing rate cells in each region, even after normalization as z-scores. Trial period responses are significantly different between high- versus low-firing cells ($p = .0172$ by rm-ANOVA of z-scores) and varies over the trial (interaction z-score $\times$ group $p < .0001$).

The somewhat elevated firing rate of these neurons raises the possibility that they may be (inhibitory) interneurons; however all cells that were included for analysis fulfill the standard criteria used in the field to exclude interneurons, i.e. they fire at the relatively low rate of $< 10\text{Hz}$ (Neunuebel and Knierim, 2014; Neunuebel et al., 2013). The observed spike widths, in the range of roughly 0.2–0.3ms, are indeterminate in regards to cell type classification (Becchetti et al., 2012).

Single-Neuron activity in MTL encodes stimulus associations, but not behavioral responses

Because MTL regions showed changes specific to associative learning, we tested whether neuronal firing in each region differs between behaviorally successful (CR) trials and unsuccessful (no-CR) trials. Figure 7 shows PSTH z-scores of activity during CR trials (solid line) and no-CR trials (dotted line) in Conditioned rabbits at a point in training when there was a large number of each trial type performed, i.e. after the behavioral criterion of 8 CRs within 10 consecutive trials was achieved. The similarity between CR- and no-CR neuronal activity is visually apparent throughout PR, latEC and DG, and no statistically significant differences were found between the two trial types during post-criterion sessions when the percent of CR trials was near 50%.

Theta activity in DG and latEC, but not PR, encodes association

We analyzed LFP activity in the theta band for rabbits (4–8Hz, (Green and Arduini, 1954)) from PR, latEC and DG. Theta activity was initially evaluated separately for superficial and deep layers of latEC (data not shown), however no significant differences were seen between these layers, so the data were combined. As shown in Figure 8, theta power (top set of panels) differed as a function of conditioning session and region. In the PR cortex paired presentations of the CS and US led to an increase in theta power during most sessions, except for the initial presentations of the US. The increase was followed by a notable decrease in theta power during the ITI. In contrast, CS alone trials led to a prolonged, but modest increase in theta power that was followed by a burst of power in response to presentation of the US during initial sessions at the start of training and initial sessions at the second start of training after the 30 day hiatus. Also in PR, theta activity from both conditioned and pseudoconditioned rabbits exhibited a phase reset to presentation of the US, as evidenced in Fig. 8A by the blue-yellow-red striping aligned at US onset. US alone trials exhibited phase resetting to US offset in post-criterion sessions. There was also significant phase resetting to CS presentation during pre-criterion sessions.

In the EC, the CS and US during conditioning trials led to a brief and robust increase in power and then a prolonged decrease, just as observed in the PR cortex. The response to the CS during pseudoconditioning was minimal, and although the US was associated with a lower amount of power than when presented in combination with the CS, the US did induce a large increase in theta power. Phase resetting was also evident in EC. The Conditioned group exhibited an early onset of CS and US phase resetting (lower graphs), which developed later in pseudoconditioned animals (interaction CS $p < 0.001$, US $p < 0.001$; group difference CS $p < 0.001$, US $p < 0.001$). While phase resetting continued for the US in the conditioned group, the CS response decreased (session effect $p < 0.001$).

In the DG, the most robust responses were observed during US only trials where the theta power was maximal during the US. This is not surprising given the large increase in hippocampal and entorhinal theta power during tone-signaled delay conditioning (Berger, Clark, Thompson, 1980). Theta power in response to CS only presentations was less and more diffuse in time. Theta power during conditioning trials increased for a shorter duration and was followed by a notable decrease in theta power following presentation of the US. In

DG, as in EC, both conditioned and pseudo animals exhibited phase resetting to the CS and the US (lower graphs). However, in DG conditioned animals show a decrement of phase resetting to both conditioning stimuli after the criterion session, whereas pseudoconditioned rabbits maintained a phase reset (especially to the US) throughout training (interaction cs $p < 0.01$, US $p < 0.01$; session cs $p < 0.01$, us $p < 0.001$).

DISCUSSION

We found that neuronal signals in trace conditioning are transformed into representations of individual stimuli in PR, association-specific stimulus representations in latEC, and stimuli connected by trace-bridging activity in DG. The signal is carried by both baseline and trial-period responsivity changes. These changes may be sequential in nature, through the classic tri-synaptic pathway, or happen during parallel processing due to direct projections from lateral entorhinal cortex to area CA1. We discuss each region in turn.

Perirhinal Cortex

PR showed responses to both CS and US stimuli, with no conditioning-specific group differences in response profiles during either early training or post-consolidation. PR is essential in forms of associative learning employing complex stimuli in primate (Murray et al., 2007) and rat (Bussey et al., 1999), in a muscarinic cholinergic-dependent manner (Tang et al., 1997), and during trace conditioning (Suter et al., 2013). However, Conditioned animals notably recruited an increased number of PR neurons in acquisition (see Fig. 5), consistent with previous observations during fear conditioning (Furtak et al., 2007a). Our findings are consistent with PR being required for this temporal associative learning task, perhaps by relaying required stimulus information to more central portions of MTL.

Lateral Entorhinal Cortex

Cortical input to the hippocampus proceeds from layers II/III of entorhinal cortex via the perforant path to the dentate gyrus (DG) granule cell layer (Dolorfo and Amaral, 1998), while return projections target deep layers of EC. Thus EC may function as a comparator between hippocampal input and output for novelty detection (Witter et al., 2000) and for stimulus associations during trace EBC. Specifically, latEC showed a conditioning-related increase in magnitude and duration of the CS response. LatEC also showed increased recruitment of neurons in both rate-increasing and rate-decreasing neuron populations, and increased baseline firing rates that may reflect reductions in the post-burst after-hyperpolarization, as seen in CA1 in acquisition of trace EBC (Moyer et al., 1996; Weiss et al., 2000).

LatEC supports persistent spiking activity in vitro, suggesting that this area may support trace-bridging mechanisms independent of hippocampal function (Egorov et al., 2002; Hasselmo et al., 2009; Tahvildari and Alonso, 2005; Tahvildari et al., 2007). In primates, entorhinal neurons have been shown to maintain firing during an interstimulus interval in matching tasks (Naya et al., 2003; Suzuki et al., 1997). Interestingly, while some neurons did show an increase in firing in the late trace period as compared to the CS period (see Figs. 3 & 4), the group difference in CS response, while prolonged beyond CS offset, did not

persist until the late trace period and is not, on average, trace-bridging. While the average neuronal response in latEC dipped during the trace period, a subset of cells showed increased activity in the second half of the trace period (tr2) as compared to the CS period.

Intrinsic and task-related properties of latEC are less well described than those of medial EC with its network of grid cells (Hafting et al., 2005). LatEC participates in spatial tasks in a cue-centered, rather than spatial coordinate-centered, manner (Deshmukh and Knierim, 2011; Hargreaves et al., 2005; Neunuebel et al., 2013; Tsao et al., 2013). LatEC neurons combine some spatial information with object representations into a *misplace*-like memory trace (Knierim et al., 2014), and control rate coding in CA3 place cells for environmental changes (Lu et al., 2013), but have not been explored in temporal tasks. During spatial tasks, DG and medial EC show a subset of high-firing rate cells, while latEC does not (Neunuebel and Knierim, 2014; Neunuebel et al., 2013). These neurons do not appear to be interneurons based on spike width and baseline firing rate. Here we report conditioning-specific high-firing rate cells engaged during a temporal task, which are exclusively located in superficial layers of latEC. The location in superficial latEC is significant because layers II/III project to DG and CA1 (as compared to deep layers, which receive projections from CA1), and as such may present an associative signal as input to hippocampus. The high-firing cells show stronger stimulus and persistent spiking activity than non-high firing cells, suggesting that they are the main source of associative information. The close similarity between high-firing cells in latEC and DG (see below) suggests that these latEC cells may project directly to DG, and thus may be layer III perforant path neurons or layer II stellate neurons (Kitamura et al., 2014).

We previously found no significant impairment in trace EBC acquisition following lesions of latEC, as opposed to PR lesions (Suter et al., 2013). A direct PR-to-hippocampus projection, though anatomically minor (Furtak et al., 2007b), was hypothesized to mediate the association in the absence of latEC input, a theory that is not supported by weak conditioning-specific responses in PR in the current work. A similar dissociation between lesion results and recordings is emphasized in a recent paper from our laboratory that found robust associative activity in CA1 post-consolidation (Hattori et al., 2014a), a time when hippocampal independence of trace EBC is well-established in lesion studies (Kim et al., 1995). This suggests that CA1 may permanently store a representation of the CS, as we suggested in a previous study (Hattori et al., 2014a) even if the CA1 is not required for expression of the consolidated CR. A similar result, was found with fear conditioning after even longer periods of retention (Lux et al., 2016), and may be related to the percentage and profile of post consolidation responses we found in latEC as shown in Figures 5 and 8.

Dentate Gyrus

DG neurons responded more strongly to the CS in Conditioned than in Pseudo animals, and the response was maintained throughout the trace period (Figures 3 and 4). This maintained response was not totally surprising given that DG neurons have been shown to exhibit persistent firing in vitro (Anderson and Strowbridge, 2014; Larimer and Strowbridge, 2010). Importantly, persistent firing represents a neuronal response that could bridge the temporal gap between the associated stimuli. DG neurons from Conditioned animals additionally

maintained a prolonged US response (Figure 3), possibly contributing to reflex facilitation of the blink response, which is known to be amygdala-dependent (Weisz et al., 1992). As in latEC, the DG changes occurred well before any behavioral evidence for learning. Though novel, this finding is consistent with multi-unit recordings in DG during delay eyeblink conditioning that found increased responses to the US very early in training, as well as long-term potentiation of EC-to-DG synapses (Berger et al., 1976; Weisz et al., 1984).

DG also showed increased baseline firing rates and increased recruitment of significantly responding cells, demonstrating effects on both an individual neuron and a population level. As in latEC, DG expressed a subset of high-firing neurons in the Conditioned group only; these neurons have been observed in DG on spatial tasks as well (Neunuebel and Knierim, 2012). Newborn granule neurons in young adult animals are more excitable (Mongiat et al., 2009; Marin-Burgin et al., 2012) and show facilitated associative long-term potentiation (Schmidt-Hieber et al., (2004). These features could facilitate their propensity to be involved in new learning. Additionally, Shors and Gould and colleagues demonstrated that elimination of newly generated neurons blocked trace but not delay eyeblink conditioning (Shors, 2004; Shors et al., 2001). It has also been demonstrated that newly born neurons tend to be more engaged in learning spatial pattern separation and context encoding (Clelland et al., 2009; Danielson et al., 2016a) and that neurogenesis is increased following trace EBC (Gould et al., 1999). Alternatively, the high firing rate cells we recorded from the dentate gyrus may actually be mossy cells of the hilus (GoodSmith et al., 2017; Senzai and Buzsaki, 2017). The facts that dentate granule cells tend to be inactive during wakefulness (GoodSmith et al., 2017), and our rabbits were being given training trials which included corneal air puff presentations on average every 45 seconds (which should have insured wakefulness), would also argue that our highly active neurons were likely to have been mossy cells. It is also interesting that persistent firing in dentate gyrus has been demonstrated to be a feature of dentate mossy cells (Larimer and Stowbridge, 2010; Anderson and Stowbridge, 2014). Importantly and somewhat unexpectedly, the high firing rate dentate neurons we recorded showed a striking capacity to bridge the temporal gap by persistently firing between CS and US onsets (Figs. 3 and 6). Whether the dentate neurons we recorded were newly born granule neurons and/or mossy cells will need to be pursued in future experiments aimed to differentiate these possibilities with molecular genetic approaches (e.g., Senzai and Buzsaki, 2017).

MTL as a driver of associative learning

MTL changes in PR, latEC and DG showed a characteristic CS-trace-US response of individual neurons (Figure 2). The responses are conditioning-specific and in line with development of associative neuronal firing underlying the developing conditioned behavioral response. Distinct brain regions have shown complementary timelines of learning-related changes during trace EBC: whisker barrels in deep layers of primary somatosensory cortex parallel behavioral changes (Ward et al., 2012), prelimbic cortex shows robust post-consolidation trace-period activity (Hattori et al., 2014b), and cholinergic interneurons in caudate nucleus, change post-criterion while medium spiny neurons respond during initial days of training (Flores and Disterhoft, 2009). Regions that change earliest include ventral hippocampus (Weible et al., 2006) as well as caudal anterior cingulate in prefrontal cortex,

with increased responsive neurons on training day one and high baseline firing rate within the first ten paired trials (Weible et al., 2003). Further studies will be required to determine the exact time that changes in MTL regions occurred in relation to the start of paired trial presentation.

In the context of nearly identical neuronal firing patterns during CR and no-CR trials (Fig. 7), the current findings suggest that MTL stores associations between environmental stimuli that are paired in time early in learning, rather than acting as an output-oriented center for behavioral coordination, or a detail-oriented sensory center.

Further supporting early association-oriented MTL function, latEC and DG show decreased encoding of the association post-consolidation, when behavior is optimized. Both latEC and DG showed strong characteristic firing rate profiles during acquisition, but mostly stimulus-locked profiles post-consolidation, with decreases in both stimulus response magnitude and in maintained trace-period firing. Post-consolidation, PR maintained stimulus-locked responses to CS and US, with no difference between groups. In contrast, we have previously shown that CA1 activity in Conditioned animals is enhanced post-consolidation (Hattori et al., 2014a) and maintains a US-predominant firing rate profile.

MTL is an associative, not a behavioral control, center

We observed that neuronal changes in latEC and DG, but not PR, during learning support association-specific firing and in DG, persistent trace-period activity. PR does however exhibit learning specific changes by an increase in the percentage of neurons responsive to the conditioning stimuli. MTL does not directly control behavioral responses, since conditioning-specific responses predated the appearance of conditioned responses, and were essentially identical on CR and no-CR trials after learning (Figure 7), suggesting that these regions mediate temporal associations rather than motor outputs. Our observations converge with those of Pilkiw, et al. (2017), who observed that lateral entorhinal cortex firing patterns of individual neurons were not correlated with the expression of conditioned eyeblinks on a trial-by-trial basis.

The associative activity in entorhinal cortex is of special relevance because EC is the site of earliest pathologic changes in Alzheimer's Disease, when initial difficulties in learning and memory are noted, and because overexpression of mutant human tau (P301L) in adult rats was associated with attenuated hippocampal-prefrontal coupling (Tanninen et al., 2017), although behavioral acquisition was normal. The mechanistic basis for high-firing cells and interactions between PR, latEC and DG that contribute to trace EBC acquisition remains to be determined. Importantly, it is not known whether the enhanced CS responses in latEC and DG are generated through feed-forward PR coincidence detection, intrinsically in latEC and DG, or recurrently due to DG or CA1 feedback. These questions can be readily addressed using trace EBC in further studies.

Ensemble activity agrees with single-neuron changes

The theta activity we recorded in DG as well as in PR and latEC differ from those in CA1 (Hattori et al., 2014a), showing decreased post-consolidation theta amplitude and phase reset, confirming that hippocampal theta is subregion-specific and differentially modulated

by trace-bridging learning. While PR regains some activity post-consolidation, that activity does not differ from the CS-US response in pseudoconditioned animals. DG and latEC theta post-consolidation most closely resembles activity at the end of training, rather than at the beginning of acquisition, reinforcing the concept that DG and latEC are preferentially acquisition-oriented; this in contradistinction to CA1, which maintains stable theta regardless of learning state, pre- and post-consolidation. Changes in activity over the course of learning and between conditioned and pseudo-conditioned animals support differential roles for these MTL regions in forming associations. Further studies of hippocampal and parahippocampal slow-wave activity in forebrain-dependent learning are suggested by these findings and will be of interest, especially in relation to an apparent variable oscillator in the medial prefrontal cortex that matches the interstimulus interval used for training (Carco-Martin et al., 2015).

Note also that our rabbits were comfortably secured in a cotton sac and an acrylic restrainer, all inside a sound- and light- attenuating chamber. This environment greatly minimizes spatial cues. Similarly, although the rats that we use in EBC experiments are freely moving about a darkened chamber, they often settle into a single location and remain there for the duration of the conditioning session (Weiss et al., 1999; Kuo et al., 2008; McKay et al., 2012; Curlik et al., 2014). The fact that our rabbits were head restrained and not moving through the environment likely minimized spatial information flowing through the MTL and accentuated the processing of stimulus associations that would otherwise occur when freely moving animals stop and attend to conditioning stimuli (Shan et al., 2016). In contrast, the context dependent environment that is sometimes used by the Takehara-Nishiuchi laboratory revealed that context can and does affect MTL neuronal responses during EBC experiments in freely moving rats with some lateral entorhinal neurons responding to stimuli in one environment and not another (Pilkew et al., 2017).

In summary, our results show early associative changes in single-neuron firing rate as well as theta oscillations in lateral entorhinal cortex (EC) and dentate gyrus (DG), and increases in the number of responsive neurons in perirhinal cortex. In both EC and DG, a subset of neurons from conditioned animals exhibited an elevated baseline firing rate and the largest responses to the conditioned stimulus and trace period, suggesting that lateral EC and DG contain cells that are responsive to a temporal associative task. However, during post-consolidation sessions, the activity profiles of neurons within EC and DG were similar for conditioned and pseudoconditioned rabbits. Collectively these results demonstrate that individual subregions of medial temporal lobe differentially support new and consolidated conditioned blinks.

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Significance Statement

Area CA1 of hippocampus has an important role in memory formation for spatial and temporal associations, and an analysis of neurons upstream of the hippocampus (grid cells and island cells) has recently been reported for spatially demanding tasks. Here we report the responsiveness of neurons upstream of the CA1 hippocampus in the perirhinal/lateral entorhinal cortex and dentate gyrus during acquisition of a temporally demanding task, and during recall of the remotely acquired memory. We found differential responsiveness within the perirhinal, entorhinal and dentate regions of the medial temporal lobe that reflects a conversion of sensory signals to associative signals. Associative activity in entorhinal cortex is of special relevance because it is the site of earliest pathology in Alzheimer's Disease, when initial difficulties in memory are noted.

Figure 1. MTL anatomy and task.

A, circuit diagram: stimulus (CS and US) information converges at PR and superficial latEC and is conducted to both DG and CA1. CA1 projects to deep layers of latEC, which projects back to PR and cortical regions. B, lateral view of rabbit brain (schematic): blue region indicates hippocampus, red PR, purple latEC. C, 'P#' indicates distance caudal from bregma (mm). Red/blue dots represent recordings in Conditioned/Pseudo rabbits. rf, rhinal fissure. D (upper panels), Conditioned animals received 80 trials per session of paired CS (whisker vibration, 250ms) and US (corneal airpuff, 150ms) separated by 500ms trace interval. Pseudo-conditioned rabbits received 80 CS-only and 80 US-only trials in random order. Behavioral response (extension of nictitating membrane) was assessed using an infrared reflective sensor. Session average eyeblink response in a well-trained conditioned (blue) and a pseudo-conditioned animal (red) is shown. D (bottom panel), Percent Conditioned responses (CR) per daily session for Conditioned (solid line) and Pseudo (dashed line) rabbits. 'D1' denotes first day of training; 'C' day of criterion; 'P', post-consolidation. Gray boxes represent sessions during acquisition (five recording sessions preceding and five sessions following behavioral criterion: successful CRs on 8 out of 10 consecutive trials), and post-consolidation (following 30 days without training). Data represent mean $\pm$ SEM. N=4–5 animals per group (one conditioned rabbit did not go through post-consolidation testing).

Figure 2. Response profiles of individual neurons segregated by subject.

Each row within a panel represents the activity of a unique single neuron averaged across the trials of a training session. The color code represents standardized Z scores of the firing rate for each 10ms bin. Black vertical lines represent the timing of the whisker vibration (CS) and airpuff to the eye (US). The presented data show a one second preCS baseline and two seconds of data after CS onset. Data presented in the left and right panels were not necessarily recorded simultaneously. Instead, data are organized by the overall activity for each neuron, i.e. the most CS-responsive neuron is represented in the top row of each panel. Note the robust response to the CS in several latEC neurons and the robust response to the US in many DG neurons. Data from perirhinal cortex are not shown because there were no conditioning specific changes in their response profiles (instead the number of responsive PR neurons increased with conditioning). Data were not recorded from latEC in rabbit E and were not recorded from DG in rabbit D, i.e. N=4 rabbits per region for the data presented in this figure.

Figure 3. Conditioning-specific responses in latEC and DG, but not in PR, during acquisition.

Peristimulus time histograms for cells with an increase in firing to CS are shown as average of individual neuron responses for Conditioned (solid line) and Pseudo (dotted line). Z-scores as compared to baseline for each neuron are used to compare task responses between groups, since baseline rates differ (see insets). Panels A and D show recordings in PR, panels B and E in latEC, and panels C and F in DG. z-scores of Pseudo-conditioned animals are shown as a composite line combining the CS and trace period (0ms to 1759ms) from CS trials, with the US and post-US period (1760ms to 3000ms) from US trials. Fewer significant group differences are present post-consolidation (D, E, F) as compared to acquisition (A, B, C). Gray bars represent duration of CS and US. Insets, baseline firing rates (Hz) for Conditioned (black bar) and Pseudo (open bar; mean $\pm$ SEM); stars indicate group difference in baseline firing by rm-ANOVA (* $p < .05$, ** $p < .01$, *** $p < .001$). For line charts, if groups differed significantly by rm-ANOVA, interval z-scores were compared using unpaired t-test. Group differences are indicated by stars. tr1, early trace period (first 250ms); tr2, late trace period (last 250ms); post, post-US interval (1100ms).

Figure 4. Conditioning-specific rate decreases in latEC and DG, but not in PR, during acquisition.

Figure is arranged as in Figure 3, for neurons with a decrease in firing to the CS interval. Panels A to C (Acquisition) and D to F (post 30-day consolidation) show z-scores of peristimulus time histograms for Conditioned (solid line) and Pseudo (dotted line). Insets, baseline firing rate in Conditioned (black bars) and Pseudo (open bars). Fewer significant group differences are present post-consolidation (D, E, F) as compared to acquisition (A, B, C). Gray bars represent duration of CS and US. Insets, baseline firing rates (Hz) for Conditioned (black bar) and Pseudo (open bar; mean $\pm$ SEM); stars indicate group difference in baseline firing by rm-ANOVA. For line charts, if groups differed significantly by rm-ANOVA, interval z-scores were compared using unpaired t-test. Group differences are indicated by stars. tr1, early trace period (first 250ms); tr2, late trace period (last 250ms); post, post-US interval (1100ms).

Figure 5. Percent significantly responsive cells is enhanced in Conditioned animals.

Top panels show percentage of significantly rate-increasing neurons (RI), bottom panels show percentage of significantly rate-decreasing neurons (RD) for PR, latEC and DG, with pre- and post-consolidation data in each panel. Numbers on each bar represent number of RI / total INC neurons (top) or number RD / total DEC neurons (bottom). Each region showed an increased percentage of RI or RD neurons in Conditioned animals during acquisition. Stars represent group differences by chi-square test comparing number of RI and non-RI cells between groups, as indicated. Black bars: Conditioned group; open bars: Pseudo-conditioned group.

Figure 6. LatEC and DG show a population of neurons with high baseline firing rate. Panel A, B Top Panels: Distribution of baseline firing rate and spike width in latEC and DG. Dotted lines indicate cutoff value (latEC 3Hz, DG 2Hz) used to evaluate high- and low-baseline neurons (bottom panels). High firing-rate neurons are present exclusively in superficial layers of latEC, and only in Conditioned animals. Bottom Panels: z-score PETHs for high (solid lines) and low (dotted lines) baseline firing rate neurons.

Figure 7. MTL activity does not differ between successful (CR) and unsuccessful (no-CR) trials. A-C, INC cells in PR, latEC and DG, respectively. D-F, DEC cells in PR, latEC and DG, respectively. Average z-score histograms are shown separately for CR trials (solid line) and no-CR trials (dotted line) from the same session for Conditioned animals. Data from post-criterion days, when percent CR trials approached 50 percent, were used to maximize statistical power between conditions. rm-ANOVA showed no difference in activity based on behavioral outcome.

Figure 8. LFP oscillations in the theta range (4–8Hz) are significantly modulated during task performance in an association-specific manner.

A. In PR, theta power (top graphs) is modulated by stimuli in both Conditioned and Pseudo groups. Conditioned and Pseudo show phase reset to US (mean resultant vector, lower graphs), however only LFP from Conditioned rabbits show phase reset to CS, which decreases after day of Criterion (Harrison-Kanji: interaction $p < 0.001$, group < 0.001 , session < 0.005). Interestingly, US phase resetting in LFPs from Pseudo rabbits develops later than LFPs from the Conditioned group (interaction $p < 0.001$ and session difference $p < 0.001$), despite a strong amplitude response from Day 1. B. Results from an analysis of EC

superficial and deep EC layers were combined; due to similarity of results. The Conditioned group shows early onset of CS and US phase resetting (lower graphs), which develop later in pseudoconditioned animals (interaction CS $p<0.001$, US $p<0.001$; group difference CS $p<0.001$, US $p<0.001$). While phase reset continues for the US in the conditioned group, the CS response decreases (session effect $p<0.001$). C. In DG, as in EC, both conditioned and pseudo animals show phase resetting to CS and to US (lower graphs). However in DG, conditioned animals show a decrement of phase reset after criterion, whereas pseudoconditioning maintains phase reset throughout training (interaction cs $p<0.01$, US $p<0.01$; session cs $p<0.01$, us $p<0.001$).