Segregation of cortical head direction cell assemblies on alternating theta cycles

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High-level cortical systems for spatial navigation, including entorhinal grid cells, critically depend on input from the head direction system. We examined spiking rhythms and modes of synchrony between neurons participating in head direction networks for evidence of internal processing, independent of direct sensory drive, which may be important for grid cell function. We found that head direction networks of rats were segregated into at least two populations of neurons firing on alternate theta cycles (theta cycle skipping) with fixed synchronous or anti-synchronous relationships. Pairs of anti-synchronous theta cycle skipping neurons exhibited larger differences in head direction tuning, with a minimum difference of 40 degrees of head direction. Septal inactivation preserved the head direction signal, but eliminated theta cycle skipping of head direction cells and grid cell spatial periodicity. We propose that internal mechanisms underlying cycle skipping in head direction networks may be critical for downstream spatial computation by grid cells.

Oscillations may coordinate neural assemblies to reliably influence and interact with downstream reader-integrators1. In the hippocampus and entorhinal cortex, the theta rhythm (4–12 Hz)2–4 appears to support spatial memory function5–7 and learning of associations8. Reduction in theta rhythm magnitude by pharmacological inactivation of the medial septum correlates with impairment in spatial memory tasks9,10. Theta rhythm may coordinate hippocampal and medial entorhinal networks via theta phase spiking relationships between cell types11 and subregions12 and the correlation between spiking phase and animal location, known as theta phase precession, in hippocampal place cells13,14 and entorhinal grid cells15. Models use this temporal organization to simulate spatial properties of place cells and grid cells16–19 and to support episodic memory function20–22.

The mechanisms of grid cell generation are debated, but input from head direction cells appears to be essential23–26. The head direction signal, or ‘internal compass’, is generated subcortically27, passed to the thalamus28 and terminates cortically in the dorsal presubiculum (postsubiculum)29, retrosplenial cortex30, parasubiculum31 and medial entorhinal cortex32. The latter two structures contain grid cells33,34. Head direction cells are often clustered in deeper layers and send projections to anatomically defined grid cell patches34. However, little is known about the temporal organization of head direction cells.

Spike-time autocorrelations of the majority of neurons in the presubiculum, parasubiculum and medial entorhinal cortex show temporal periodicity at theta frequency31. Some autocorrelations reveal theta cycle skipping35,36, in which the first side peak of the autocorrelogram is smaller than the second side peak, indicating that spikes are occurring on alternating theta cycles, a phenomenon that is more common in ventral than dorsal entorhinal cortex36 and that has been attributed to lower frequencies of intrinsic oscillations of neurons in ventral entorhinal cortex32, and lower frequency input from prefrontal cortex37,38. Theta cycle skipping has not otherwise been explored in detail.

We found that theta cycle skipping was predominantly exhibited by neurons with substantial head direction tuning and that cycle skipping facilitated temporal segregation of neurons with overlapping, but offset, directional preferences. We found that theta cycle skipping neurons tended to have tighter tuning curves than non–theta cycle skipping neurons, and the degree to which a neuron skips cycles was positively correlated with measures of head direction tuning. Theta cycle skipping was associated with strong input near the peak of the head direction tuning curve and center of spatial fields of conjunctive grid–by–head direction cells.

Co-recorded cells revealed that the alternating cycles (odd or even) preferred by a particular cell were not random. Cross-correlation analysis revealed that many cell pairs skipped theta cycles together (labeled as synchronous pairs, identified by high correlations at lags of 0 ms and ~250 ms, and a low correlation at a lag of ~125 ms), whereas other cell pairs were segregated on alternating theta cycles (labeled as anti-synchronous pairs, identified by low correlations at lags of 0 ms and ~250 ms, and a high correlation at ~125 ms). These cycle relationships were stable throughout each recording session and across days.

Simulations of cells with random head direction and theta cycle preferences revealed that, without additional network mechanisms, the head direction tuning differences between cells form equal distributions for both synchronous and anti-synchronous groups. In contrast with this expectation, we observed substantially different distributions and an absence of anti-synchronous pairs with similar head direction preferences. To the best of our knowledge, this is the

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first demonstration that neural content (that is, the head direction signal) can be segregated by oscillation cycles, supporting the hypothesis that oscillatory cycles can serve as periodic attractors that segregate discrete content. Analysis of a portion of our data set, in which pharmacological inactivation of medial septum disrupts grid cells, revealed elimination of theta cycle skipping in all head direction cells and conjunctive grid–by–head direction cells. Our results suggest network mechanisms that segregate head direction cell assemblies by alternating theta cycles and may be required for grid cell function and the temporal segregation of attractors in the hippocampus.

RESULTS

Theta cycle skipping in the head direction network

Our data set contained 2,313 putative neurons recorded from the dorsal medial entorhinal cortex (Fig. 1a) and parasubiculum in six male rats during 66 recording sessions in an open field arena. Consistent with prior studies, we observed normal theta rhythmicity (Fig. 1b), in addition to theta cycle skipping, in many cells, which appeared as a larger second peak, \( p_2 \) (~250 ms), than the first peak, \( p_1 \) (~125 ms), in the spike time autocorrelation (Fig. 1c,d, and Supplementary Fig. 1a). To quantify theta cycle skipping, we fit an equation (equation (3), Online Methods) to the autocorrelation and calculated the ratio of the peaks (theta skipping index, \( TS \)), defined as \( (p_2 - p_1) / \max(p_1, p_2) \). To minimize false positive detections, we designed strict criteria for identifying theta cycle skipping cells (Online Methods), which required a baseline theta power component and good fit between the model parameters and spike time autocorrelation measured by the coefficient of determination (\( R^2 \)). Autocorrelations for 1,294 (56%) neurons were well fit by model parameters (\( R^2 > 0.7 \)), and 806 of these cells (62% of neurons with valid fits) surpassed the threshold for theta rhythmicity. These cells represent the qualifying set of neurons for which the theta cycle skipping metric was valid; among them, 118 exceeded the threshold for theta cycle skipping (theta skipping index > 0.1), of which 13 were removed by visual inspection (as a result of a poor fit to equation (3)) to give 105 theta cycle skipping neurons.

Of the 105 theta cycle skipping neurons in our data set, 82 (78%) were head direction cells with mean resultant lengths greater than 0.2 (Online Methods). To estimate the percentage of head direction cells that exhibited theta cycle skipping, we used two methods that avoid the potential bias of including the same neuron over multiple recording sessions. First, in recording sessions, an average 17% of head direction cells displayed theta cycle skipping. Second, a conservative approach ensured that all head direction neurons were unique cells (Online Methods and Supplementary Fig. 2). Of 435 head direction cells in the full data set, 157 were determined to be unique. Of the 278 head direction cells not included in the analysis, 62 exhibited theta cycle skipping (22.3%). The analyses of head direction cells reported here were restricted to the 43 head direction cells that exhibited theta cycle skipping out of the 157 (27.4%) unique head direction cells.
The theta skipping metric (Fig. 1c) revealed a significant positive correlation between the degree of theta cycle skipping and the strength of head direction tuning, as measured by the mean resultant, \( R_m \) (\( P < 0.01 \), linear regression, t statistic; \( n = 43 \) cells, degrees of freedom \( = 41 \), \( R = 0.465 \); Fig. 1e). The cumulative distribution functions of \( R_m \) of theta skipping neurons was shifted to the right more than in the entire population of recorded cells (Fig. 1f). A receiver operating characteristic analysis confirmed the observation that head direction tuning (\( R_m \)) was a good predictor of theta skipping (area under the curve (AUC) = 0.802; Fig. 1g). By comparison, gridness and firing rate were not as effective at discriminating cycle skipping neurons (gridness, AUC = 0.638; firing rate, AUC = 0.391). Gridness may have some predictive qualities, as it is confounded with head direction in the case of conjunctive grid--by--head direction cells, whereas firing rate has some negatively predictive properties that are likely a result of the large number of fast spiking interneurons that are not theta cycle skipping cells.

**Theta cycle skipping occurs during increased drive**

For many theta cycle skipping neurons, a minority of spikes fired in a theta rhythmic manner (that is, some theta cycles were not skipped), as detected by the presence of a smaller peak at \( \sim 125 \text{ ms} \) in the autocorrelation and in spiking rasters of theta cycle skipping neurons (Fig. 2a). When does a theta cycle skipping neuron fail to skip a cycle and when is theta cycle skipping most pronounced? To answer this, we evaluated the contingencies of theta cycle skipping on a number of behavioral and neural states.

We examined how theta cycle skipping covaries with the network theta rhythm, and found that cycle skipping was most prevalent for spikes at a neuron’s preferred theta phase. We separated spikes during preferred phases of theta (±90 degrees of the peak) from spikes during non-preferred phases, during epochs of time where theta power was significant (Online Methods). The autocorrelation of spikes during the preferred half of the theta cycle exhibited robust theta cycle skipping, whereas spiking during the non-preferred half was theta rhythmic (\( P < 0.001 \), Wilcoxon paired signed rank test; all spikes, \( n = 36 \) cells, theta skipping index = 0.26 ± 0.02; spikes at preferred phases, \( n = 31 \) cells, theta skipping index = 0.21 ± 0.03; spikes at non-preferred phases, \( n = 15 \) cells, theta skipping index = −0.08 ± 0.02; Fig. 2b,c). Running speeds during periods of spiking in the preferred range of theta phases were significantly elevated relative to running speeds when spiking occurred during non-preferred theta phases (all, \( n = 40 \) cells,
49.0 ± 1.4 cm s⁻¹; preferred theta phase, \( n = 40 \) cells, 50.0 ± 1.4 cm s⁻¹; non-preferred theta phase, \( n = 40 \) cells, 42.2 ± 1.9 cm s⁻¹; \( P < 0.001 \), Wilcoxon paired signed rank test; Fig. 2d). The incidence of fast inter-spike intervals (<10 ms), that is, bursting, was elevated during epochs of spiking at preferred theta phase (\( P < 0.001 \), Wilcoxon paired signed rank test; all, \( n = 40 \), \( P_{\text{BLI}} < 10 \text{ ms} < 0.37 \pm 0.02 \); preferred theta phase, \( n = 40 \), 0.38 ± 0.02; non-preferred theta phase, \( n = 40 \), 0.21 ± 0.02; Fig. 2e), indicating that cycle skipping may occur more during Bursting modes, when the rat is moving at faster speeds.

We hypothesized that an increase in firing rate would correspond to an increase in theta cycle skipping, as short, bursting interspike intervals were less prevalent among the theta rhythmic spikes and theta cycle skipping was strongest during spiking in the preferred range of theta phases in which most spikes occurred. We summed the number of spikes in a 60-ms-wide sliding window (10-ms step size) to characterize spiking and bursting activity at theta timescales. From peaks in the resulting local spike rate function (local = 60 ms), we generated separate ‘event trains’ for spikes and bursts of 1–6 spikes. These event trains consisted of ones in which bursts of \( n \) spikes occurred and zeros at all other time steps, allowing spike and burst events with different spike counts to be separately compared with coincident neural and behavioral activity. We then looked for differences in theta cycle skipping activity (Fig. 2f), theta phase (Fig. 2g), and behavioral and spatial variables, such as running speed, head direction and position in the enclosure (Fig. 2h–j), each as a function of the strength of theta bursts. As firing increased, so did intensity of theta cycle skipping. The average cross-correlations of event trains for 1–6 spike bursts with the full spike train (bursts with more than six spikes were too rare to include) revealed that single spike events
Figure 4 Characteristics of synchronous and anti-synchronous theta cycle skipping pairs. (a) Left, analysis of the difference in preferred head direction between neurons in synchronous and anti-synchronous pairs. Synchronous pairs had a significantly smaller head direction tuning offset than anti-synchronous pairs (synchronous, $n = 58$ pairs, $35.5 \pm 42.4$ degrees; anti-synchronous, $n = 41$, $103.9 \pm 27.8$ degrees; $P < 0.001$ unpaired Wilcoxon rank sum test). Note that the difference was largely driven by the limited number of pairs with differences of less than 60 degrees in the anti-synchronous group. Right, the results of our statistical model revealed that the differences of preferred directions of synchronous and anti-synchronous cell pairs are expected to have similar distributions. (b) Schematic of statistical model used to demonstrate that the expected directional tuning offsets for synchronous and anti-synchronous cell pairs are equal and evenly distributed if cycle selection is random. The model produces anti-synchronous (top right) and synchronous (bottom right) firing patterns for all neuron pairs such that the degree of directional overlap has no bearing on whether pairs are synchronous or anti-synchronous. This contrasts with our finding that anti-synchronous cycle relationships were rarely seen in neurons with similar directional preferences. (c,d) Two experimentally recorded cell pairs with overlapping tuning curves (red and blue polar plot, normalized by peak firing rate) displayed synchronous (c) or anti-synchronous (d) theta cycle firing during periods of coactivity, evident in the cross-correlation (black histogram) and spike rasters. (one spike bursts) occurred during epochs of theta rhythmic firing and stronger burst events (with 2–6 spikes) corresponded to epochs of theta cycle skipping (Fig. 2f).

Next, we examined the absolute angular distance between the mean phase of bursts with different spike counts (Fig. 2g). This analysis captures the similarity between any two event trains in terms of spiking phase with respect to the network theta cycle. The angular distance was smallest between bursts with similar spike counts and greatest between bursts with large differences in spike count (Fig. 2g). This result is consistent with the preferred/non-preferred theta phase analysis, as it indicates that those spikes that occurred during theta cycle skipping (higher spike count bursts) were segregated in network theta phase from those that did not. Differences in spike counts in theta bursts corresponded to differences in running speed, head direction and location in the environment (Fig. 2h–j).

We found stronger theta bursting and cycle skipping at the preferred head direction and centers of grid fields. During periods of high spiking and high theta cycle skipping, running speeds were elevated (Fig. 2h), directional headings were close to the preferred tuning direction (Fig. 2i) and positions in the enclosure were near the centers of grid fields (in conjunctive grid cells; Fig. 2j). Consistent with the observation that running speeds were elevated during periods of spiking at the preferred theta phase (Fig. 2d), the theta rate function analysis revealed that strong theta bursts tended to occur when the rat was running faster (Fig. 2h). However, there was no difference in running speed across theta bursts of 1–4 spikes (Fig. 2h), whereas theta cycle skipping was greatly strengthened across this range (Fig. 2f), reflecting that running speed alone is not an effective predictor of theta cycle skipping. Low spike count theta bursts, which are associated with theta rhythmicity, occurred further away from the preferred head direction, whereas high spike count theta bursts occurred closer to the preferred direction (Fig. 2i). This emphasizes that cycle skipping is strongest at the preferred head direction.

A subset ($n = 10$) of theta cycle skipping cells were conjunctive grid–by–head direction cells. We compared the distribution of theta bursts of different strengths relative to the centers of grid fields (Fig. 2j). As expected, similar to the tuning of head direction, theta bursts with low spike counts occurred farther away from grid field centers than stronger bursts, suggesting that theta cycle skipping increases near the center of grid fields. These data demonstrate that theta cycle skipping occurs most robustly during elevated spiking activity driven by a rat’s behavior and location. A rat’s elevated running speed, heading at the preferred direction for a given head direction cell and proximity to grid cell firing fields are each associated with stronger theta-locked bursting and theta cycle skipping. These associations eliminate the possibility that theta cycle skipping is a byproduct of lowered synaptic drive, but rather indicate that theta cycle skipping is related to increased drive to these neurons.

Temporal segregation of head direction cell assemblies

To test whether theta cycle skipping is a result of cellular or network-level mechanisms, we examined whether theta cycle
skipping is coordinated between simultaneously recorded neurons. We calculated cross-correlations between theta cycle skipping neurons with a considerable amount \((N_{\text{latency}} \leq 0.6 \text{ s} > 500)\) of coincident spiking and analyzed whether theta cycle relationships between cells were fixed or varied over time. More than one-third of the cross-correlations between theta cycle skipping cells were

**Figure 5** Possible downstream functional implications of theta skipping head direction cells. Medial septum inactivation eliminated theta cycle skipping and grid cell spatial periodicity in conjunctive grid–by–head direction cells. (a) Four examples of cells that lost their theta skipping and spatial periodicity. Heat maps show neuron firing rate in the open field, with warmer colors corresponding to increasing rate (Hz) from zero (blue) to the peak firing rate (red). Two examples are shown that remained theta rhythmic during the inactivation (arrows 2 and 3). Mean (m) and peak (p) firing rates are provided above each rate map. (b) Cross-correlations of synchronous or anti-synchronous pairs before, during and after pharmacological inactivation of the medial septum. Although these cells remained highly active within 400 ms of each other, the theta cycle relationship was nearly completely absent during the inactivation. Theta cycle relationships recovered 3–6 h and 24 h after the muscimol infusion. (c-f) Inactivation of the medial septum by infusions of muscimol simultaneously caused a reduction of theta cycle skipping in the autocorrelations; pre, \(n = 20\), theta skipping index = 0.26 ± 0.02 (mean ± s.e.m.); infusion, \(n = 20\), theta skipping index = −0.11 ± 0.02; 3 h, \(n = 19\), theta skipping index = 0.25 ± 0.04; 24 h, \(n = 19\), theta skipping index = 0.23 ± 0.04; ***\(P < 0.001\), Wilcoxon paired signed rank test); the number of theta cycle skipping cross-correlations (white bars, d; number of \(T_{\text{sweep}} > 0.2\); pre, 23; infusion, 4; 3 h, 26; 24 h, 22), a reduction of grid cell spatial periodicity (e; pre, \(n = 32\), gridness = 0.73 ± 0.05; infusion, \(n = 32\), gridness = −0.07 ± 0.03; 3 h, \(n = 29\), gridness = 0.44 ± 0.08; 24 h, \(n = 23\), gridness = 0.63 ± 0.07; \(P < 0.001\), Wilcoxon paired signed rank test) and maintenance of the head direction cell signal (f; pre, \(n = 36\), \(R_m = 0.41 ± 0.04\); infusion, \(n = 35\), \(R_m = 0.43 ± 0.04\); 3 h, \(n = 29\), \(R_m = 0.41 ± 0.04\); 24 h, \(n = 21\), \(R_m = 0.40 ± 0.05\); *\(P > 0.05\), Wilcoxon paired signed rank test).
Figure 6 In vitro theta cycle skipping is reduced with stronger input. (a) We combined an 8-Hz sinusoidal current injection with variable levels of direct current. The proportion of cycles with spiking increased linearly with increased direct current, up to 1:1 locking to the stimulus, that is, 1 spike per theta cycle. Insets, spiking of eight layer V MEC neurons in response to the sinusoid stimulus (depicted below voltage traces) at direct current levels driving spiking on ~50% of theta cycles (filled circles and color voltage traces) and 1:1 locking (black voltage traces). (b) Example of one neuron’s spiking at different direct current levels. Stable ratios of spikes (top) to theta cycles (bottom). (c) Further increasing direct current drove spiking above 1:1 locking, and a second ratiometric regime appeared for direct current levels at which the ratio of spikes per theta cycle was >1:1 (top). Theta cycle skipping in vitro was strongest for lower input levels (bottom) that drove single spikes on alternating theta cycles (0.5 spikes per theta cycle). Weaker theta cycle skipping was also evident for direct current input levels that drove three spikes per two theta cycles (doublets alternating with single spikes). As expected, increased input to an isolated MEC neuron in vitro decreased the occurrence of theta cycle skipping and served to highlight the counterintuitive finding that increased input to MEC neurons in vivo increased the probability and strength of cycle skipping. These data suggest that network mechanisms may underlie the phenomenon of theta cycle skipping.

Clearly either synchronous (Fig. 3a and Supplementary Figs. 2a,b and 3a) or anti-synchronous (Fig. 3b and Supplementary Figs. 2c,d and 3b) (34.5% of pairs had a cross-correlation theta skipping index (TScore) > 0.2, of which 59% were synchronous and 41% were anti-synchronous) indicated that these between-neuron cycle relationships were fixed. Spiking rasters of simultaneously recorded theta cycle skipping head direction cells showed fixed synchronous or anti-synchronous theta cycle relationships (Fig. 3c).

Between-neuron cycle relationships of theta cycle skipping neurons (synchronous or anti-synchronous) could not be attributed to time lags between preferred head direction fields (Supplementary Fig. 4), and cycle relationships remained stable for hundreds of passes through directional fields during 20-min recordings, and, in one example, remained fixed over 6 d (Fig. 3d). Synchronous and anti-synchronous combinations co-occurred at the same recording locations, suggesting that membership to a particular set of theta cycles is not region specific (Fig. 3e). We also found some evidence that interneurons may participate in cycle skipping networks (Supplementary Fig. 5).

Directional segregation of head direction cell assemblies
Is there a difference between the directional tuning curves of cell pairs that are synchronous (assembly partners) and anti-synchronous (not assembly partners)? Notably, the cells of anti-synchronous pairs rarely shared similar head direction tuning preferences, whereas the head direction tuning of synchronous pairs were often very similar (Fig. 4a). Only 3 of 41 anti-synchronous pairs had a tuning angle difference of less than 60 degrees, and none were less than 40 degrees (Fig. 4a). Between-neuron pairs with synchronous cross-correlations had significantly smaller angular distance between their preferred head direction angles (synchronous, n = 58 neuron pairs, median ± median absolute deviation, 35.5 ± 42.4 degrees; anti-synchronous, n = 41, 103.9 ± 27.8 degrees; P < 0.001 unpaired Wilcoxon rank sum test; Fig. 4a). These results capture a temporal organization in the head direction network. When a rat faces a direction in the shared region of overlapping tuning curves of anti-synchronous head direction cell populations, both assemblies are coactive when considered on longer timescales, but their spiking is separated at shorter timescales on alternating theta cycles (Fig. 3).

We developed a statistical model to estimate the dependence of theta cycle synchrony on the similarity of preferred head direction angles (Fig. 4a,b and Supplementary Modeling). In contrast with the data (Fig. 4a,c,d), the statistical model generated a broad distribution of head direction preference angle differences that was common for both synchronous and anti-synchronous pairs (Fig. 4a,b). These results illustrate the independence of theta cycle timing from the timing governed by head direction tuning (that is, cells highly coactive during matching head direction are not required to be coactive during theta cycles and vice versa, although this was observed in the data). These results demonstrate that at least two simultaneously active assemblies of head direction cells (or information streams) are separated in time by the theta cycle. The fixed synchronous or anti-synchronous relationships between neuron pairs argue against underlying intrinsic cellular mechanisms in favor of the network-level temporal organization of the cortical head direction signal.
Figure 7 Stronger input can induce theta cycle skipping. (a) With weaker theta rhythmic input, excitatory cells spike on each cycle, but there is less driving force on inhibitory cells. (b) With stronger input, the theta rhythmic input drives multiple spikes on a single theta cycle, as in the experimental data. The activation of multiple spikes causes activation of coupled inhibitory interneurons. The influence of inhibitory interneurons on slow GABA-B conductances can prevent generation of spikes on the next theta cycle, resulting in cycle skipping, in which bursts of spikes appear on alternating theta cycles. (c) A simple network with feedback inhibition and excitatory synapses between stellate cells and regular spiking cells can show anti-synchronous theta cycle skipping. A hyperpolarizing step function at start caused rebound spiking of five stellate cells. Excitatory connections between stellate and pyramidal cells caused bursts of spikes. The excitatory input activates spiking in the inhibitory neurons that hyperpolarizes the other population of stellate cells, causing rebound spiking. The interaction of feedback inhibition with properties of stellate cells causes emergence of stable anti-synchronous theta cycle skipping in simulations.

Theta cycle skipping is sensitive to septal inactivation

What downstream information is provided by head direction cell assemblies that are offset by at least 40 degrees of head direction and are segregated in time by alternating theta cycles? Versions of oscillatory interference and attractor network models of grid cells use theta rhythmic head direction cells as input to generate grid cells with both a rate code and phase code for position. Previously, we found that grid cell spatial periodicity was eliminated by pharmacological inactivation of the medial septum that reduced the power of theta oscillations and reduced the total number of theta rhythmic cells in the medial entorhinal cortex. We re-examined the subset of data for which inactivation was performed, noting that all cycle skipping in head direction cells and conjunctive grid—by—head direction cells was eliminated (before muscimol infusion: n = 20, theta skipping index = 0.26 ± 0.02; immediately after muscimol infusion: n = 19, theta skipping index = 0.11 ± 0.02; 3 h after muscimol infusion: n = 19, theta skipping index = 0.25 ± 0.04; 24 h after muscimol infusion: n = 19, theta skipping index = 0.23 ± 0.04; P < 0.001, Wilcoxon paired signed rank test; Fig. 5a–c). Although theta cycle skipping dynamics were always lost, theta rhythmicity was still apparent in some cells (Fig. 5a). The number of theta skipping cross-correlations was greatly reduced during the infusion (pre, 23 theta skipping index cross-correlations > 0.2; infusion, 4; 3 h, 26; 24 h, 22; Fig. 5b,d), and TScorr dropped in theta skipping cross-correlations that retained rhythmicity during the infusion (pre, n = 23, TScorr = 0.35 ± 0.03; infusion, n = 4, TScorr = 0.19 ± 0.02; 3 h, n = 19, TScorr = 0.35 ± 0.04; 24 h, n = 22, TScorr = 0.29 ± 0.04; P = 0.06, Wilcoxon signed rank test). The gridness signal was also lost in theta skipping (and non—theta skipping) neurons during the infusion (pre, n = 32 cells, gridness score = 0.73 ± 0.05; infusion, n = 32, −0.07 ± 0.03; 3 h, n = 29, 0.44 ± 0.08; 24 h, n = 23, 0.63 ± 0.07; P < 0.001, Wilcoxon paired signed rank test; Fig. 5e), whereas head direction tuning was unaffected by the infusion (pre, n = 36 cells, Rm = 0.41 ± 0.04; infusion, n = 35, Rm = 0.43 ± 0.04; 3 h, n = 29, Rm = 0.41 ± 0.04; 24 h, n = 21, Rm = 0.40 ± 0.05; P > 0.05, Wilcoxon paired signed rank test; Fig. 5f).

These data suggest that the cycle skipping head direction code could be useful in various theories of the generation of spatial periodicity of grid cells. It remains to be determined why theta cycle skipping, which predominantly appears to be a feature of the head direction system, is sensitive to septal inactivation, whereas head direction tuning is not. Septal inactivation may disrupt lateral inhibition between theta cycle skipping head direction cells, as a large percentage of septal projections innervate medial entorhinal (MEC) interneurons (personal communication, M. Witter). This inhibition may normally contribute as a network mechanism for theta cycle skipping and the generation of separate anti-synchronous populations of theta cycle skipping cells.

To further support the network mechanism hypothesis, we performed in vitro whole-cell patch-clamp recordings from synaptically isolated pyramidal neurons in layer V of MEC and found that, in contrast with in vivo conditions (Fig. 2), increased drive to an isolated neuron reduced theta cycle skipping (Fig. 6). We then built a simple network spiking model that uses lateral feedback inhibition between a population of stellate cells or regular spiking cells and a population of inhibitory interneurons. Increased drive to this network results in increased theta cycle skipping (Fig. 7 and Supplementary Modeling).

DISCUSSION

The function of theta oscillations in the neural coding of memory is debated. Beyond the data on phase precession in place cells and grid cells, there has been limited evidence to indicate that theta oscillations coordinate neural representations. We found that head direction cells are segregated in time by alternating theta cycles according to their directional preference, indicating that theta oscillations facilitate the segregation of information.

Theta cycle skipping has been reported, but the link to the head direction system has not been emphasized. Theta cycle skipping was first described in the medial entorhinal cortex and was more recently quantified. However, these studies did not report the head
direction specificity of these cells. Theta cycle skipping was readily visible in a study that quantified theta modulation of head direction cells in the presubiculum, parasubiculum and medial entorhinal cortex. It remains unknown whether head direction cells in the retrosplenial cortex also exhibit theta cycle skipping. Notably, theta cycle skipping is also visible in a class of medial septal neurons that are only theta rhythmic when the animal runs in a particular direction. In our study, inactivation of medial septum eliminated all theta cycle skipping in head direction cells despite leaving many cells with normal theta rhythmicity. These data suggest the medial septum may be involved in the theta cycle skipping code in cortical head direction cells.

Segregation of cell assemblies by theta cycles
We found that pairs of theta cycle skipping neurons, sensitive to head direction, had a fixed synchronous or anti-synchronous cycle relationship throughout the duration of a recording session, suggesting that assembly segregation occurs on different theta cycles. This temporal organization was maintained through hundreds of trials involving the rat passing its head through the cell pair’s preferred head directions (Figs. 3 and 4c,d), and was even maintained in recordings separated by 6 d (Fig. 3d).

Anti-synchronous theta skipping pairs rarely contained preferred head directions within 60 degrees, whereas many synchronous pairs had preferred directions in this range (Fig. 4a). Could this reflect a mechanism that promotes the 60 degree offsets in grid cell firing field orientation? Oscillatory interference models predict a specific organization of head direction input at 60 or 120 degree intervals. This prediction has lacked experimental support, as several studies have reported an even distribution of preferred direction in populations of head direction cells; however, these studies did not account for theta cycle skipping relationships between neurons. We re-approached this topic, taking advantage of theta cycle skipping rhythms to reveal orchestration of assemblies, rather than focusing on the distribution of preferred head direction across individual neurons, and our results support selective functional coupling between subpopulations of head direction cells with characteristic tuning separations. We found that some head direction cell populations were segregated by a theta cycle and by about 60 degrees or more of head direction. These results suggest a complex organization that may be consistent with models of grid cells that require organized head direction cell inputs. Future modeling can capitalize on this temporal and directional organization of the head direction network to simulate the 60 degree angles between the dominant orientations of grid cell firing fields. Separation on different cycles may facilitate distinct shifts in the dimensions separated by 60 degrees (Supplementary Fig. 6), and the combination of two wider peaks from anti-synchronous theta cycle skipping cells may allow maintenance of attractor dynamics during the inactive troughs of the theta rhythm.

Do synchronous populations of cycle skipping head direction cells form anatomically distinct modules? This is difficult to answer, as the probability of recording multiple head direction cells that display cycle skipping with overlapping directional preferences at the same recording location is low. However, in one case, three such neurons were isolated on a single tetrode, making both synchronous and anti-synchronous pairs (Fig. 3e). These data suggest that segregated head direction cell assemblies are not anatomically distinct in a strict sense, but the possibility exists that these recordings were obtained on the border between modules.

To understand the functionality of the theta cycle skipping head direction network, we focused in part on a data set in which medial septum inactivation disrupted the grid cell spatial periodicity, but left head direction tuning intact. Many head direction cells and conjunctive grid–by–head direction cells from this data set displayed significant theta cycle skipping before septal inactivation (Fig. 5a,c). Muscimol infusion into the medial septal dissociated theta cycle skipping from head direction tuning (Fig. 5a,c,d,f), suggesting that the head direction signal itself does not require theta cycle skipping, and simultaneously disrupted the spatial signal in grid cells (Fig. 5a,e). These results suggest that the temporal precision of the head direction cell inputs may be important for grid cell function, although the disappearance of the two phenomena may be independent of each other. The data also point to the medial septum as a driving force of the theta cycle skipping phenomenon.

The entorhinal cortex provides the majority of cortical information projecting to the hippocampus. What benefit could theta skipping head direction cells and conjunctive grid–by–head direction cells offer the hippocampus? A previous study found that CA1 place cell assemblies are organized into 25-ms windows that appear to skip theta cycles. These assemblies of neurons in region CA1 may be influenced by assemblies of cells in layer III of MEC. A recent study on the time course of hippocampal content noted that instantaneous transitions between spatial contexts caused spatial representations in the hippocampus to ‘flicker’, or alternate, between theta cycles. In combination, these data support a view that the theta cycle is a unit of hippocampal content; however, our data do not preclude additional mechanisms to support hippocampal content at faster timescales, such as gamma cycles nested in the theta cycle.

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

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AUTHOR CONTRIBUTIONS
M.P.B. and M.E.H. designed the in vivo experiments. M.P.B. collected the in vivo data. M.P.B., A.R.B. and N.W.S. designed, and A.R.B. implemented, the in vivo analyses. N.W.S. and M.E.H. designed the in vitro experiments. N.W.S. collected and analyzed the in vitro data. M.E.H. created the network simulations and A.R.B. developed the Poisson model. M.P.B., A.R.B., N.W.S. and M.E.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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In vivo neural recordings. Rats were recorded from daily in the open field to search for conjunctive grid–by–head direction cells and head direction cells and head direction cells. Once theta oscillations and theta rhythmic neurons were prevalent, tetrodes were turned a maximum of ~32 μm per second, and experiments never took place on days in which any tetrode was moved. Data collected during inactivation of the medial septum were presented previously, but the current data set includes a much larger number of isolated cells from non-inactivation recordings. Neural signals were pre-amplified by unity-gain operational amplifiers on the headstage (Neuralynx) and were then amplified (5,000–20,000×) and band-pass filtered (0.3–6 kHz, Neuralynx). When a signal crossed threshold, all four channels of the tetrode were digitized at 32 kHz and recorded. Local field potentials obtained from the MEC were referenced to the rat ground or to a cortical tetrode. A ceiling-mounted video camera (30-Hz sampling rate) tracked the position of two LEDs, one red and one green, mounted on the recording headstage. Position of the rats was defined as the centroid of the two LEDs and a reference defined by camera orientation. Up to five lost samples resulting from occlusion of tracking LEDs or reflections in the environment were replaced by a linear interpolation for both position and directional data. Running velocity was calculated from the derivative of the Kalman-filtered position. Methods for cluster cutting and histological analysis were described previously. Although it was difficult to reconstruct the exact location of each recording, as many tetrodes passed through layer I and out of the cortex, all tetrodes passed through the medial entorhinal cortex or parubiculum. Previous work on the location of head direction cells in these structures suggests that the cells analyzed here were recorded in layers III and V (refs. 29,31,33).

Septal inactivation. Muscimol, a GABA_A agonist, was diluted in phosphate-buffered saline. Prior to an infusion, the dummy cannula was removed and replaced with a primed injector cannula (33 gauge) that extended 1 mm past the guide cannula into the medial septum. A microinfusion pump (Harvard Apparatus) infused 0.50 µl of diluted muscimol at 0.125 µl min⁻¹. The injector cannula remained connected for 2 min after the infusion to allow the drug to perfuse through the neural tissue. The experimenter was not blind to whether an infusion of muscimol had occurred. Theta oscillations, gridness and theta cycle skipping were reduced by all of the septal muscimol infusions. We conducted 20-min pre-infusion baseline recordings before each infusion. Post-infusion recordings lasting up to 60 min were started 15 min after the completion of the infusion. Recovery recordings were conducted 3–6 h after the infusion to test whether theta oscillations and grid cells had returned and 24 h post-infusion recordings were also attempted when neurons were held across days. Waveform profiles across the four electrodes were compared across recordings sessions to confirm stability of each neuron across recording sessions.

Single-unit classification. We categorized some neurons as theta cycle skipping, head direction or conjunctive grid–by–head direction cells. Only neurons with more than 100 spikes in a given session were included in the study. Theta cycle skipping neurons were those cells with a satisfactory fit to the model equation (equation (3)) and in which the theta skipping index was greater than 0.1. A satisfactory fit was described as those in which the correlation coefficient, was greater than 0.7. The autocorrelation theta power, defined as the time-averaged square amplitude of the oscillatory, the first term of the equation divided by the time-averaged squared amplitude of the autocorrelation signal itself, was required to be greater than 0.01 to ensure an oscillatory component. Head direction cells were defined as those neurons with an R_m > 0.2 (ref. 42). Neurons with a grid score greater than zero were considered grid cells and those grid cells that were also classified as head direction cells are labeled conjunctive grid–by–head direction cells.

We took a conservative approach to ensure that all reported head direction cells were unique across sessions and days (Supplementary Fig. 4). Specifically, we ensured that no two head direction cells recorded from the same tetrode had similar angles of preferred head direction. To accomplish this, we clustered (‘linkage’ in MATLAB R2010b, MathWorks) head direction cells on any given tetrode by the angular distance between their preferred directional heading. A dendrogram represents the output of the clustering algorithm for one tetrode in one rat over many recording sessions (Supplementary Fig. 4). Clusters were cut at 30 degrees angular distance. Clusters labeled C1–6 show head direction neurons that were flagged as potential repeat cells (Supplementary Fig. 4). From each cluster, one neuron was automatically selected by maximizing Watson’s U² test statistic, which rewards high numbers of spikes and clear head direction tuning (Supplementary Fig. 4). The other units in the cluster were not used to take the strictest approach to ensure that no cell was included twice. Of 435 head direction cells, 157 unique cells were identified, and 278 cells representing their repeated recordings were not included in the analysis. Of the 278 cells not included in the analysis, 62 were theta cycle skipping neurons.

Gridness and directionality. Firing rate maps were constructed by calculating the occupancy normalized spike count for 3-cm x 3-cm bins of position data. Data were smoothed by a two-dimensional convolution with a pseudo-Gaussian kernel with a s.d. of one pixel (3 cm). We measured the gridness of neurons using an analysis that accounts for elliptical distortions in assumed hexagonal firing patterns of grid cells. Occupancy normalized polar plots of firing rate by head direction were generated using 6-degree directional bins. To quantify the degree of head direction selectivity, we calculated the mean resultant, R_m, of the directional firing rate map.

\[
R_m = \frac{\sum_{i=1}^{n} \left( \sum_{j=1}^{n} F_j \cos(\theta_j) + \sum_{j=1}^{n} F_j \sin(\theta_j) \right) \cos(\bar{\theta})}{\sum_{i=1}^{n} F_i} \tag{1}
\]

where \( \bar{\theta} \) represents the preferred firing direction of the cell and is calculated by

\[
\bar{\theta} = \arctan \left( \frac{\sum_{i=1}^{n} F_i \sin(\theta_i)}{\sum_{i=1}^{n} F_i \cos(\theta_i)} \right) \tag{2}
\]

and \( F_i \) and \( \theta_i \) are the firing rate and heading direction for bin \( i \).

Theta cycle skipping index. To measure theta cycle skipping in spike trains, we used the spike time autocorrelogram. The spike time autocorrelogram was estimated by collecting all nonzero lags between spikes within 400 ms of one
another, and generating a histogram of the lags with 10-ms-wide bins. The resulting autocorrelation signals were divided by the number of spikes in the train to yield probability of synchronous firing at a given lag. Each autocorrelation was fit (‘fit’ in MATLAB R2010b) with an equation adapted from ref. 41, to include a second interfering oscillation:

$$y(x) = a_1 \cos(ax + \phi) + a_2 \cos(0.5ax + \phi) + b \times \exp\left[-\frac{|x|}{\tau_1}\right] + c \times \exp\left(-\frac{x^2}{\tau_2^2}\right)$$

where $x$ is the autocorrelation lag, $a_1 = [0, m]$, $a_2 = [0, m]$, $b = [0, n]$, $c = [-m, m]$, $\phi = [0.3, 18\pi]$, $\tau_1 = [0, 0.05]$ and $\tau_2 = [0, 0.05]$ are the fit parameters, and $m$ is the maximum value of the autocorrelation. A subset of the parameters was used to calculate the theta cycle skipping index, specifically defined as the difference between the first and second peaks in the autocorrelation, normalized by the larger of the two:

$$TS = \frac{P_2 - P_1}{\max(P_1, P_2)}$$

where $P_1$ and $P_2$ are the model values at one and two full cycles ($x = 2\pi/\omega$ and $x = 4\pi/\omega$) to the right of the center peak. The theta cycle skipping index is bound between $-1$ and $1$, and higher values indicate more theta cycle skipping.

A similar equation (equation (5)) was used to model cross-correlations, which did not make all of the same assumptions as the autocorrelation case. Namely, we did not assume that the center peak was the peak of the beat oscillation or that the signal was symmetric about zero lag, nor did we expect the same sharp center peak that was common in the autocorrelation.

$$y(x) = a_1 \cos(ax + \phi) + a_2 \cos(0.5ax + \phi) + b \times \exp\left[-\frac{|x| + \omega_0 ax}{\tau_1}\right]$$

To address these issues, we introduced a phase offset parameter, $\phi$, allowed $a_2$ to drop below zero, and eliminated the sharp center peak term (set $c = 0$). The intervals for the parameters in equation (3) were $a_1 = [0, m]$, $a_2 = [-m, m]$, $b = [0, n]$, $\omega = [10\pi, 18\pi]$, $\phi = [-\pi, \pi]$ and $\tau_1 = [0, 5]$. The average phase offset was 24.9 degrees of the theta frequency, corresponding to an offset of about 8 ms.

Theta skipping in cross-correlations was defined using the fit equation and parameters from equation (5) as

$$TS_{xcorr} = \frac{P_{0.2} - P_{0.3}}{\max(P_1, P_{0.2})}$$

where $P_{0.2}$ is the mean of the model values at $x = 0$ and $x = 4\pi/\omega$ corresponding to the center and second side peak, and $P_{0.3}$ is the model values at $x = 2\pi/\omega$, corresponding to the first side peak. The theta skipping index is bound between $-1$ and $1$, with negative values indicating anti-synchrony, positive values indicating synchrony and increasing magnitude indicating more theta cycle skipping.

LFP channels were selected to maximize the ratio of power of the theta-filtered (6–10 Hz) LFP signal to the power of the delta-filtered (2–4 Hz) LFP signal to ensure accurate theta phase values. Spike phase was determined as the instantaneous phase of the theta band-pass filtered LFP signal was estimated by the phase angle of the Hilbert transform.

A control analysis was designed to include only spikes during right or left turns to test the null hypothesis that peaks in the cross-correlation were the result of repeated turns between the preferred head directions of the pair of neurons. This would be verified if the corresponding cross-correlations lost their symmetry as a result of repeated and asymmetric activation of head direction cells. However, the symmetry evident in the raw cross-correlations for all spikes was maintained in the controls (Supplementary Fig. 5a), as summarized by the cumulative distribution function of Pearson’s correlation coefficient between the negative lags cross-correlation signal and the positive lag values (Supplementary Fig. 5b).

For both synchronous and anti-synchronous pairs, symmetry in the cross-correlations was maintained when controlling for behavior, indicating that it does not result from sequential movements of the head through different preferred angles of head direction cells (all, Pearson’s $R = 0.42 \pm 0.02$; control-right, 0.37 ± 0.02; control-left, 0.35 ± 0.02).

**In vitro experiments.** Male and female, 17–24-d-old Long Evans rats (Charles River Laboratories) were deeply anesthetized with Isoflurane (Abbott Laboratories) and decapitated. Brains were rapidly removed under ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 1.8 mM MgSO4, 10 mM dextrose, 26 mM NaHCO3, 1.09 mM Na2PO4, 1.6 mM CaCl2. Saturation of ACSF with 95% O2/5% CO2 was maintained throughout each experimental session. Brain slices (400 μm) were prepared in ice-cold ACSF with a Leica VT 1000S vibratome (Leica Microsystems) and incubated for 25 min at 31 °C and at 20–23 °C for 35 min.

Layer V medial entorhinal neurons were visualized with an Olympus BX51WI upright microscope with a 40× water-immersion objective (Olympus America). Experiments were performed at physiological temperature of 37 °C with continuous gravity-driven exchange of ACSF containing 2 mM kynurenic acid and 100 μM picrotoxin (Sigma-Aldrich) to block spontaneous fast glutamatergic and GABAergic synaptic transmission. Patch recording pipettes (3.5–7 MΩ) were pulled from borosilicate glass capillary tubes with a Sutter Instruments model P-87 micropipette puller (Sutter Instruments) and filled with intracellular fluid containing 120 mM potassium gluconate, 10 mM HEPES, 0.2 mM EGTA, 20 mM KCl, 2 mM MgCl2, 7 mM Phosphocreatine di(tris) salt, 4 mM Na2ATP, 0.3 mM TrisGTP (pH adjusted to 7.3 with KOH) and 0.1% biocytin (vol/vol) for staining and morphological identification of cells. Pipette-cell membrane seals of >1 GΩ were established with slight, initial negative pressure while holding the membrane at ~70 mV. Access resistance during recordings was maintained below 20 MΩ. Voltage was amplified with a Multiclamp 700B (Molecular Devices), capacitance neutralization and bridge balancing were applied, and data were digitized with a Digidata 1320 (10 kHz) and recorded with pClamp 10 software (Axon Instruments). Cell health was deemed suitable for data collection if the resting membrane potential was ~60 mV, input resistance was >125 MΩ, spike height was >60 mV and spike amplitudes were >15 mV. After recording, slices were placed in 4% paraformaldehyde (Sigma-Aldrich) and staining with diaminobenzidine (Vector Laboratories) and decapitated. Brains were rapidly removed under ice-cold PBS. Saturation of ACSF was 95% O2/5% CO2 was maintained throughout each experimental session. Brain slices (400 μm) were prepared in ice-cold ACSF with a Leica VT 1000S vibratome (Leica Microsystems) and incubated for 25 min at 31 °C and at 20–23 °C for 35 min.

Stimuli delivered in current-clamp mode were 8-Hz sinewaves (80 pA peak to peak, with 0–250-pA DC current). Spiking probability was calculated as the proportion of stimulus cycles with spikes and plotted as a function of DC stimulus level. The in vitro theta cycle skipping metric was calculated using the area under the autocorrelation of spiking during stimulus delivery. The difference between the area in 50-ms windows centered on 125 ms and 250-ms lags was normalized by the area under the autocorrelation at 250 ms, giving a maximum measure of 1 for perfect theta cycle skipping. This conservative measure of theta skipping is a necessary modification of our in vivo metric, as spike timing in vitro in response to sinewave current injections is far more precise than observed in vivo.

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