Evidence for a subcircuit in medial entorhinal cortex representing elapsed time during immobility

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The medial entorhinal cortex (MEC) is known to contain spatial encoding neurons that likely contribute to encoding spatial aspects of episodic memories. However, little is known about the role MEC plays in encoding temporal aspects of episodic memories, particularly during immobility. Here using a virtual ‘Door Stop’ task for mice, we show that MEC contains a representation of elapsed time during immobility, with individual time-encoding neurons activated at a specific moment during the immobile interval. This representation consisted of a sequential activation of time-encoding neurons and displayed variations in progression speed that correlated with variations in mouse timing behavior. Time- and space-encoding neurons were preferentially active during immobile and locomotion periods, respectively, were anatomically clustered with respect to each other, and preferentially encoded the same variable across tasks or environments. These results suggest the existence of largely non-overlapping subcircuits in MEC encoding time during immobility or space during locomotion.

Over the past 50 years, research from humans and animal models have implicated the medial temporal lobe, which includes the hippocampus and MEC, in the formation of personal memories of events that occur at specific places and involve specific time intervals. While a vast amount of research has uncovered cellular substrates in the hippocampus and MEC that likely make up the spatial representation required for these episodic memories, our understanding of the temporal representation is substantially less advanced and has focused mostly on the hippocampus. Time-related neurons were first demonstrated in the hippocampus using studies in which rodents were moving to some degree, either in a running wheel, on a treadmill, or in a small box. Notably, one study found hippocampal time-related activity during immobility. These so-called hippocampal ‘time cells’ fire briefly and consistently at specific times during the task, such that behavioral time periods are tiled by a sequence of brief neuronal activations. Strikingly, specialized circuitry representing spatial information during immobility has also been demonstrated in the hippocampus. This suggests that separate circuitry within the medial temporal lobe might be used to encode behaviorally relevant variables between mobile and immobile periods, though it is unclear from these studies whether the representation of elapsed time maps onto a particular circuit(s).

In MEC, one study found that MEC grid cells can provide timing-related information during treadmill running, and a separate study found MEC neurons that were more active at low running speeds rather than high speeds during locomotion. Inactivation of MEC during such mobile periods was found to produce deficits in encoding memories across trace periods, produce deficits in a temporal memory task, and cause instability in downstream hippocampal time cells. These studies suggest that a code for elapsed time may exist in MEC during locomotion, but it is currently unknown whether the neural circuitry in MEC forms a representation of elapsed time during immobility, when sensory cues may not change in a temporally informative manner. Furthermore, if such a representation exists in MEC, it is unknown how the neural circuitry might be organized to generate it.

Results
To explore these ideas, we used our previously developed functional two-photon imaging methods to optically record from populations of layer II MEC neurons (Fig. 1a and Supplementary Fig. 1) during mouse navigation in a novel virtual Door Stop task. The Door Stop task combines both a locomotion-dependent virtual navigation phase and an explicit instrumental timing phase that was separated in time and location from reward delivery (Fig. 1b and Supplementary Fig. 2a). Mice were trained to run down a linear track to a specific location where they encountered an invisible door, which they could not run past, though they could still run on the treadmill. At the door location, the mice were required to stop and wait for at least 6 s (an auditory click signaled the start of the 6-s interval once the treadmill velocity fell below a threshold; see Methods); if the mice began running on the treadmill before the expiration of the 6 s interval, the mice could not progress past the closed door and the trial would start over (signaled by another click). After the 6-s interval, the door would open and the mice could run down the remaining length of the track to the reward zone. After 6–8 weeks of training, mice ran to the invisible door and stopped on their first attempt for the full 6 s wait period on 55.1% of trials (Fig. 1c), referred to as ‘correct trials’. To easily compare neural activity during immobile timing periods and neural activity during locomotion periods, we excluded a transition zone between these periods and excluded the reward zone when behavior was more ambiguous (Fig. 1c, Supplementary Fig. 2a, and see Methods). During the wait periods, mice mostly sat immobile with essentially 0 velocity with small jerky movements occurring during 12.9% of the wait period to maintain balance on the treadmill (velocity over wait periods = 0.33 ± 1.00 cm/s (mean ± s.d.); Fig. 1d,e). All of the data presented in Figs. 2–4 using the (invisible door) Door Stop task come only from these correct trials (see Supplementary Fig. 2b–f for velocity on all trials). Since the mice could not see the invisible door opening at the end of the 6-s interval, this Door Stop task therefore required an internal temporal representation for efficient completion.
We first explored the possibility that MEC contains neurons active preferentially during immobility. We optically recorded from populations of layer II MEC neurons labeled with GCaMP6f (Fig. 2 and Supplementary Fig. 3) during the Door Stop task (11 imaging sessions, i.e., fields of view (FOV), across 7 mice, FOV 414 ± 50 by 328 ± 40 μm (mean ± s.d.), depth below surface = 89 ± 26 μm). Across the population (156 ± 54 (mean ± s.d.) active neurons per FOV for a total of 1,497 active neurons; see Methods), active neurons exhibited calcium transients with different selectivities for running and immobile periods. This is consistent with previous reports of MEC neurons displaying positive and negative correlations with running speed15 (Supplementary Fig. 3f). However, in addition to neurons that were negatively tuned to speed, we also observed neurons that were essentially silent during running periods and active selectively during immobility (when running speed was essentially 0; Supplementary Fig. 2 and see Methods). Therefore, to quantify this run–rest selectivity across the population, we developed a ‘run–rest index’ (RRI) that measures the run-versus-rest selectivity of a neuron, such that if all significant transients ($P < 0.01$; see Methods) occurred during resting, $\text{RRI} = -1$, and if all occurred during running, $\text{RRI} = +1$ (see Methods). The distribution of RRI values across the active MEC population appeared bimodal, with neurons active preferentially during rest (immobility) periods or preferentially during run periods (Fig. 2a,b; across all active neurons $\text{RRI} = -0.24 ± 0.71$, mean ± s.d.), though neurons with RRI values spanning the full range were observed. Thus, during the virtual Door Stop task, MEC contains subsets of run-selective and (previously unknown) rest- or immobility-selective neurons.

We then sought to determine whether any of the rest-specific neuronal activity might encode elapsed time during the immobile timing phase of the Door Stop task (11 FOVs in 7 mice). We identified all timing interval trials during individual sessions and, for all active neurons, plotted the change in fluorescence (calcium transients) as a function of time after stopping. Across the different timing intervals, many neurons displayed calcium transients that occurred regularly at a specific time delay from the start of immobility (Fig. 2c), with the mean activity over all trials forming significant timing fields. Thus, the population-level activity of active neurons ($P < 0.05$ for bootstrap test, 11 of 11 FOVs contained cells...
Fig. 2 | Functionally and anatomically clustered populations of neurons in MEC encode space during locomotion and elapsed time during immobile intervals of the Door Stop task. a, ∆F/F traces of significant transients (green traces; P < 0.01; see Methods) from individual example rest-selective cells (left) and run-selective cells (right) during running and resting periods (black traces) in Door Stop task. b, Histogram of RRI for all active cells across all FOVs in all mice during Door Stop task; transition periods and reward zone excluded. c, Top: mean ∆F/F vs. time across all correct trials of a single session for 6 individual neurons from the same FOV during the 6-s Door Stop wait interval. Purple dashed lines and arrows indicate transition period. Bottom: ∆F/F vs. time for each correct trial. Scale bars indicate 100% ∆F/F. d, Mean ∆F/F vs. time across all correct trials in a single session for all time-encoding cells (each row represents an individual neuron mean ∆F/F value) in a single FOV during the 6-s Door Stop wait interval. Mean ∆F/F is normalized to peak for each neuron (each row). e, Histogram of RRI for all time-encoding cells (red) and all space-encoding cells (blue) across all FOVs in all mice during Door Stop task; transition periods and reward zone excluded. f, MEC FOVs of GCaMP6f-labeled populations (top) colored red or blue to indicate cells encoding time or space, respectively (bottom). g, Mean pairwise distance (left) or fold-change (right) between neurons in various groups. All space- or time-encoding cells from all mice in Door Stop task; transition periods and reward zone excluded. MEC FOVs of GCaMP6f-labeled populations (top) colored red or blue to indicate cells encoding time or space, respectively (bottom).
with timing fields: 22.5 ± 3.4 (mean ± s.e.m.) cells had timing fields in each FOV; range of number of cells in each FOV: 6–40, wait times between 6–9 s included). In individual FOVs during single sessions, timing fields of different neurons across the population tiled the full timing interval (Fig. 2d; for similar results across all FOVs, see Supplementary Fig. 3c). Therefore, during periods of immobility in a virtual Door Stop task, a subset of neurons in MEC form a representation of elapsed time through their sequential activation across the full wait interval.

During the locomotion phase of the task, we found many neurons with significant spatial fields (17.9 ± 3.0% (mean ± s.e.m.) of all active neurons; \( P < 0.05 \) for bootstrap test; 22.2 ± 11.9 (mean ± s.e.m.) cells had spatial fields in each FOV; range in each FOV: 9–45; Supplementary Fig. 3d,e); this population likely contained many of the navigation-encoding cell types previously described (grid cells, border cells, velocity cells, etc.)22,23,24. Across all 11 FOVs, the majority of space- or time-encoding cells had either spatial or timing fields (92.1 ± 1.8% (mean ± s.e.m.) were time- or space-encoding cells only), but not both (7.9 ± 1.8% (mean ± s.e.m.) had both timing and spatial fields; 3.1 ± 0.7% (mean ± s.e.m.) of all active cells; \( t_w = 7.9, P < 0.00001, \) Student's paired t test). While the time-encoding cells were most active during periods of rest (\( RRI = 0.86 ± 0.02, \) median ± s.e.m.), the space-encoding cells were most active during periods of locomotion (\( RRI = 0.69 ± 0.03, \) median ± s.e.m.); with little overlap in their RRI distributions (Wilcoxon rank-sum \( z \)-statistic = 17.3630; \( P < 0.00001; \) Fig. 2e). Notably, similar results suggesting functional bimodality within the MEC population were obtained using information-theoretic metrics (Supplementary Fig. 4). Thus, in a particular environment, the subset of neurons encoding elapsed time during immobility was largely non-overlapping with a separate subset of neurons encoding space during locomotion.

We then explored the anatomic organization of time- and space-encoding cells in MEC during the Door Stop task. Visual inspection of the anatomic location of the time- and space-encoding cells revealed that cells encoding similar information were often spatially clustered within individual imaging fields (Fig. 2f). Across all 11 FOVs, time-encoding cells were significantly clustered together in MEC compared to space encoding cells and compared to all active neurons (distance between time-encoding cells: 115.0 ± 5.9 \( \mu m \) (mean ± s.e.m.)); distance between space-encoding cells: 136.1 ± 7.4 \( \mu m \), distance between all neurons: 142.8 ± 5.6 \( \mu m \), distance between time- and space-encoding cells: 134.2 ± 8.0 \( \mu m \); repeated-measures ANOVA, \( F_{(3,6)} = 11.8, P < 0.0001; \) distance between time-encoding cells versus distance between space-encoding cells, \( P < 0.001, \) Tukey's post hoc test with Bonferroni correction; distance between time-encoding cells versus distance between time- and space-encoding cells, \( P < 0.01, \) Tukey's post hoc test with Bonferroni correction), resulting in significant 24% and 17% differences in neuron–neuron distances between all neurons versus between time-encoding cells and between time- and space-encoding cells versus between time-encoding cells, respectively (Fig. 2g). Together, the above results demonstrate the existence of different subsets of neurons in MEC during a navigation task: the canonical space-encoding subset active during locomotion and a novel time-encoding subset active during immobile timing intervals.

We observed that during correct trials in the Door Stop task, mice sometimes waited close to the ideal 6 s and sometimes waited longer than 6 s (Fig. 1c). Based on this observation, we next asked whether the temporal representation in MEC reflected this difference in timing behavior, as might be expected for such a representation, and, if so, whether the sequential activation of time-encoding cells advanced more slowly or whether additional time-encoding cells were added to encode the additional wait time. For 4 sessions
13 imaging fields; (gray) environments or tasks. Mean for each group indicated by solid black bars ($n = 3$ mice), The fraction of cells that encoded the same variable (orange) or switched variables (blue) within the population of cells encoding a variable in both environments (or tasks). Mean for each group indicated by solid black bars; s.d. bounds for randomly shuffled distributions for each type of environment or task switch shown by dashed lines (across all conditions: $n = 13$ imaging fields; $t_{(13)} = 13.8$, ***$P < 0.0001$, two-sided Student’s paired $t$-test).

Fig. 4 | Subsets of neurons encoding time or space in one track (or task) are more likely than chance to encode the same variable in a different track (or task). a. Environment switches in the Door Stop task with invisible door; Door Stop task with visible door, linear track task, and task-switch from classical trace-conditioning to virtual linear track task (from left to right). CS, conditioned stimulus. b. The fraction of cells that encoded the same variable (orange) or switched variables (blue) within the population of cells encoding a variable in both environments (or tasks). Mean for each group indicated by solid black bars; s.d. bounds for randomly shuffled distributions for each type of environment or task switch shown by dashed lines (across all conditions: $n = 13$ imaging fields; $t_{(13)} = 13.8$, ***$P < 0.0001$, two-sided Student’s paired $t$-test). c. The fraction of cells that encoded a variable in only one (black) or across both (gray) environments or tasks. Mean for each group indicated by solid black bars ($n = 13$ imaging fields; $P < 0.001$ for shuffle test for each track or task-switch experiment; $P < 0.001$ for paired signed-rank test for pooled data across all track or task-switch experiments).

The subsets of MEC neurons encoding space and time together formed a sequence of neuronal activations that encoded the full spatiotemporal extent of each trial of the task (from track start to end in the Door Stop task). These subsets could be recruited at random from the pool of all MEC neurons, suggesting that MEC contains a single general, flexible circuit designed to generate sequences independent of the behavioral variable being encoded. Alternatively, the subsets could be recruited from largely non-overlapping pools of MEC neurons: one pool largely encoding time and a different pool largely encoding space. To distinguish between these possibilities, we asked whether the subsets encoding time or space in one track (or task) were more likely than chance to encode the same variable in a different track (or task), suggesting largely non-overlapping pools, or were more likely to randomly switch their encoding between variables, suggesting a single large pool. We imaged the same population of MEC neurons and compared their space- and time-encoding properties across the following experiments in which mice navigated across different (familiar) tracks or performed different (familiar) tasks: (i) mice were switched between two (invisible door) Door Stop task tracks substantially different in visual appearance (Fig. 4a–c; $n = 3$ mice), (ii) mice were switched between two (visible door) Door Stop task tracks substantially different in visual appearance and door wait-times (Fig. 4a–c and Supplementary Fig. 6a,b; $n = 3$ mice), (iii) mice were switched between two different linear tracks (no Door Stop, time-encoding during voluntary rest periods, Fig. 4a–c, Supplementary Fig. 6c–f, and see Methods; $n = 3$ mice), and (iv) mice were switched between an immobile classical trace-
conditioning task and the linear-track navigation task (Fig. 4a–c and Supplementary Fig. 6g–j; n = 3 mice). The tracks in environment-switch experiments i–iii were sufficiently different in visual appearance (Fig. 4a) to cause global remapping across populations of space-encoding cells in MEC (mean spatial activity pattern correlations across environments not significantly different (P > 0.01 for shuffle test) compared to chance in 7 of 9 mice, Pearson’s correlation r = 0.12 ± 0.05 (mean ± s.e.m.); n = 9 mice; Supplementary Table 1) and in place cells in the hippocampus. Many cells with timing or spatial fields in one track (or task) did not have significant timing or spatial fields, respectively, in the other track (or task), becoming largely inactive (77.3 ± 0.02% (mean ± s.e.m.); Fig. 4c and Supplementary Fig. 6b). This observation led to a refinement of the above two different possibilities: either two largely non-overlapping pools of MEC neurons exist, from which some fraction of cells can be recruited to encode time (from the time-encoding pool) or space (from the space-encoding pool) for any given environment and context, or a single large pool of MEC neurons exists, from which some fraction of cells can be recruited to encode time or space for any given environment and context. Notably, we found that of the 22.7% of cells with timing or spatial fields in both tracks (or tasks), 83.7 ± 2.7% (mean ± s.e.m.) encoded the same variable in both while 16.3 ± 2.7% (mean ± s.e.m.) switched their encoding (Fig. 4b and Supplementary Table 2; t1,= 13.8, P < 0.0001, Student’s paired t test), fractions highly unlikely to have arisen from chance (P < 0.001 for shuffle test for groups of timing cells and spatial cells in each of the 4 track or task switches). We also found that cells with significant timing or spatial fields (P < 0.05 from bootstrapping; see Methods) present across multiple days were much more likely to encode the same variable rather than switch (Supplementary Fig. 6k–m). Additionally, RRI values of the active cells in both tracks (experiments i–iii) were similar across tracks (RRI differences for each cell between tracks = 0.30 ± 0.031 (mean ± s.e.m.); Supplementary Fig. 6n), and this difference was unlikely to have arisen from chance (shuffled RRI differences for each cell between tracks = 0.864 ± 0.002 (mean ± s.e.m.; P < 0.001 for shuffle test). Together, the above results suggest the possible existence of largely non-overlapping pools of MEC neurons, one that preferentially encodes time during animal immobility and another encoding space during animal locomotion.

Next, we asked whether time- and space-encoding neurons within MEC were capable of forming temporal or spatial representations from the first moments of new spatiotemporal experiences, as might be expected if the representations arise from largely non-overlapping subcircuits that specialize in encoding space or time. Alternatively, it is possible that the representations developed only after learning, as might be expected for nonspecialized, flexible circuitry. To answer this question, we used the virtual linear-track task (no Door Stop), during which we observed a similar MEC circuitry. To answer this question, we used the virtual linear-track task (no Door Stop), during which we observed a similar MEC circuitry. To answer this question, we used the virtual linear-track task (no Door Stop), during which we observed a similar MEC circuitry. To answer this question, we used the virtual linear-track task (no Door Stop), during which we observed a similar MEC circuitry. To answer this question, we used the virtual linear-track task (no Door Stop), during which we observed a similar MEC circuitry.
track (Supplementary Fig. 6c–f). Mice were trained to familiarity in one linear track (familiar) and then switched to a second virtual linear track (novel), which was sufficiently different in visual appearance (Fig. 5a) to cause global remapping across populations of space-encoding cells in MEC (spatial activity pattern correlations across environments not significantly different (P > 0.01 from shuffle test) compared to chance in 3 of 3 mice, Pearson's correlation ρ = −0.07 ± 0.03 (mean ± s.e.m.), n = 3 mice; Supplementary Table 1) and in place cells in the hippocampus36. From the first moments of exploring the novel track, mice displayed periods of resting and running, similar to their observed behavior during navigation in a familiar linear track (Supplementary Fig. 6d). Across all rest periods from single sessions in the novel track, we observed time-encoding cells (Fig. 5b) with properties similar to those described above (Fig. 2 and Supplementary Fig. 6). Notably, time-encoding cells very often were active in their respective timing fields during the very first rest period in the novel track (Fig. 5b; time to first rest after transition from familiar to novel: 35.8 ± 14.1 s (mean ± s.d.); 56% active on rest 1, 88% active by rest 3; Fig. 5c,d), resulting in correlation values across all time-encoding cells (between the calcium transients during each rest period and the mean timing field over all periods for each cell) that did not depend on exposure time in the novel track (Fig. 5c). Furthermore, the fraction of trials during which a transient occurred within a cell's timing field did not change between the first half and second half of the session (Fig. 5c). Similar results were also observed for the space-encoding subsets (Supplementary Fig. 7a–e). Thus, the temporal (or spatial) representation formed by subsets of time- or space-encoding cells in MEC is present from the first moments of new experiences.

**Discussion**

Altogether, our results establish the existence of a representation of elapsed time in MEC during immobility and further suggest the possible existence of largely non-overlapping functional subcircuits in MEC that encode either time during animal immobility or space during animal locomotion. This later notion may differentiate MEC time-encoding cells from previously described hippocampal time cells and from time encoding neurons in other brain regions. The following data presented above are consistent with this notion. First, MEC time-encoding cells were present from the first moments of exposure to novel environments, suggesting that learning was not required for their formation; time cells in the hippocampus12,14,15 and other time-encoding cells in other brain regions33–35 have only been described in well-trained animals, and thus it is not clear whether or not learning is required for their formation. We note that time cells in the hippocampus and MEC track time via the evolution of a neuronal ensemble over time; this differs from timing activity described in other brain regions, which is more often characterized by individual neuron firing patterns that ramp up or down in time after a cue32,34. Second, the subsets of neurons encoding time or space in one track (or task) were more likely than chance to encode the same variable in a different track (or task). This suggests that neurons encoding time or space during any given experience may be recruited from largely non-overlapping pools of neurons that may arise from specialized circuitry. Third, subsets of MEC time-encoding cells formed temporal representations during different behavioral tasks, one with an explicit timing requirement during immobile periods of a navigation task (Door Stop), another with no explicit requirements during periods when the mice chose to rest during navigation (linear track), and yet another in a non-navigation, immobile task with an explicit timing period defined (classic trace conditioning). This suggests that MEC contains circuitry that can be used to represent elapsed time given different tasks or contexts. Fourth, time-encoding and space-encoding cells were anatomically clustered, possibly suggesting that the separate circuits may have developed from separate precursor populations35 or developed to minimize wiring distance between their respective internal components36.

The neural basis for generating a temporal representation during immobility in MEC is thus far unknown, but previous research on the generation of grid cell firing patterns in this same brain region may provide insights, as separate similarly organized circuits could be used to form a temporal representation. For example, circuit-connectivity patterns capable of generating bumps of activity in continuous attractor networks have been described in MEC37,38. These networks could possibly generate sequential firing with appropriate input to move the bumps in one dimension for timing cell networks, rather than in two dimensions for grid cell networks, or could be slightly rewired to form a one-dimensional ring attractor for sequential timing cell activation. Additionally, beyond MEC, several general cortical or subcortical neural timing mechanisms have been proposed39–41. Our results here demonstrate that time-encoding cells can exhibit fully formed timing fields from the first moment of exposure in a novel environment, suggesting that the mechanisms necessary for encoding elapsed time in MEC do not require learning. However, given our current data, it is not possible to determine whether the temporal encoding circuit arose through a developmental program or whether it could be formed through previous learning in related contexts.

The MEC timing representation described here could be used for online perception of elapsed time or could contribute to encoding temporal aspects of episodic memories, or both. While further research is required to discriminate between these possibilities, some insights can be drawn again from studies of grid cells. For example, grid cells appear to be important components of an online path-integration system used during navigation42 and for constructing context-dependent spatial memories downstream in the hippocampus43–45. Therefore, it is possible that MEC timing circuitry is important both for the online perception used for interval timing46 and for supporting the formation of memories of events, occurring over time and including temporal intervals, in the hippocampus47. For the latter case, inactivation of ‘island’ cells in MEC have demonstrated deficits in encoding memories across trace periods48, and distinct populations of neurons in the CA2 area of the hippocampus become active during periods of immobility49. Since strong and direct synaptic projections exist from superficial MEC to CA232, it is possible that distinct subnetworks within the hippocampus and MEC are recruited to encode elapsed time during immobility.

Similarly to recent reports examining grid cells and spatially selective nongrid cells50, time-encoding cells in MEC display a range of environment or context dependent selectivity, with the majority of cells being selective for only one of two environments or contexts. Results presented here suggest that time-encoding and space-encoding cells are predisposed to encode either time during immobility or space during locomotion, respectively, and may arise from subcircuits that specialize in encoding these behavioral variables. However, several additional points should be considered for a rigorous interpretation of these findings. First, the majority of time- and space-encoding neurons (~77%) were active in only one of the two contexts during the environment (or task) switch experiments. This suggests that most of these cells are likely not part of ‘hard-wired’ neural circuits, whereby the exact set of neurons are recruited to encode the same behavioral variable across all contexts. Second, although a large majority (~84%) of the neurons active in both contexts encoded the same behavioral variable (time or space), it is possible that this predisposition is caused by the animal encoding the contexts in a similar way (i.e., familiarity), rather than through specialized subcircuits. This possible explanation seems unlikely based upon the following observations indicating that global remapping took place across the context switches: (i) of the neurons in MEC that encoded time or space in at least one context, 77% encoded only one of the two contexts in the environment- or task-switch
experiments (~23% encoded both), and (ii) space-encoding cells displayed signal correlations across environments not significantly (P > 0.01 from shuffle test) different compared to chance in 7 of 9 mice (Supplementary Table 1). Third, of the neurons in MEC that encoded both contexts, a subset (~16%) switched from encoding time to encoding space (or vice versa) across environments or tasks. If specialized subcircuits do indeed exist in MEC for encoding time during immobility and space during locomotion, then how might these subcircuits be organized to account for such switching across contexts? One possibility is that given enough context switches, all MEC neurons could be eventually recruited to encode either time or space, which would indicate that any predisposition of subcircuits to encode time or space is short-lived and continually evolving due to experience and plasticity. Alternatively, it is possible that within the time- and space-encoding subsets of MEC neurons, distinct subpopulations exist that reliably encode time or space across contexts (the ~20% of time- or space-encoding neurons here that encoded the same variable across contexts), while the rest of the population may be less tuned for one behavioral variable or the other. Consistent with this idea, a recent study showed that the most spatially selective grid cells encode space across environments and exhibit a coherent population phase shift with respect to their firing fields, while spatially selective nongrid cells in MEC randomly turn off or turn on across environments, and, as a population, exhibit global remapping. Here we found that in 3 of 9 mice, time-encoding cells did exhibit temporal activity pattern correlations across environments (or tasks) that were above chance (Pearson’s correlation $r = 0.33 \pm 0.11$ (mean $\pm$ s.e.m.), n = 9 mice; Supplementary Table 3), though population coherence was not analyzed.

Finally, correlation between animal wait time and speed of sequence progression (Fig. 3 and Supplementary Fig. 5) did not indicate how the stretching between long- and short-wait trial neural sequences was read out by downstream brain structures, nor did it reveal much about the animal’s temporal perception during long- versus short-wait trials. It is not possible to say whether the stretching was or was not a context-invariant neural representation of the time interval. For example, it is possible that the animal perceived the same amount of time during the short and long trials (egocentric timing perspective, no context difference). It is also possible that the animal did indeed perceive a difference in the amount of elapsed time (allocentric timing perspective, context difference). Our results only demonstrate a correlation between the wait-time behavior of the animal and the speed of the neural sequence progression, suggesting that this temporally structured neural activity in MEC may play a role in the animal’s estimation of elapsed time.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-018-0252-8.

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Author contributions

J.G.H. designed and performed experiments, conducted analyses, and wrote the manuscript. D.A.D. designed experiments, conducted analyses, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Methods

Surgery and behavior. All experiments were approved and conducted in accordance with the Northwestern University Animal Care and Use Committee. Methods for MEC virus injection and microprism implant have been described previously (25, 26). Briefly, 12–15 C57BL6 mice (P0–P4) were anesthetized with isoflurane (1–2%). For virus injection, a small (~0.5 mm) craniotomy was made over the dorsal surface of the cortex (above MEC) and cerebellum with corners positioned ~2.1 mm lateral of bregma, ~7.75–8 mm caudal of bregma (~3.25–3.5 mm caudal of the transverse sinus); and (iv) ~4.5 mm lateral of bregma, ~7.75–8 mm caudal of bregma. After the craniotomy and the inner edge of the metal ring was covered with opaque cement (Metabond, Parkell, made opaque by adding 0.5 g of carbon powder, Norland), and this assembly was then positioned by aligning the front face of the microprism parallel to the caudal surface of the MEC and aligning the top surface of the microprism perpendicular to the (eventual) axis of excitation light propagation. A thin layer of Kwik-Sil was applied to the caudal MEC surface before microprism implantation to fill the void between the brain and the front surface of the microprism. The microprism and mount were rigidly held in place and the craniotomy was sealed by application of a thin layer of Metabond to all exposed sides of the microprism (except the top surface of the prism) and mount and onto any exposed skull or brain. Subsequently, a titanium headplate (9.5 mm side length and reflective enhanced aluminum coating on the hypotenuse, Tower Optical) was mounted on a custom stainless-steel mount (using UV-curable adhesive, Norland), and this assembly was then positioned by aligning the front face of the microprism parallel to the caudal surface of the MEC and aligning the top surface of the microprism perpendicular to the (eventual) axis of excitation light propagation. A thin layer of Kwik-Sil was applied to the caudal MEC surface before microprism implantation to fill the void between the brain and the front surface of the microprism. The microprism and mount were rigidly held in place and the craniotomy was sealed by application of a thin layer of Metabond to all exposed sides of the microprism (except the top surface of the prism) and mount and onto any exposed skull or brain. Subsequently, a titanium headplate (9.5 mm × 38 mm) was attached to the dorsal surface of the skull, centered upon and aligned parallel to the top face of the microprism. The headplate was used to head restrain the mouse as described previously (24). A titanium ring (27-mm outer diameter and 12.5-mm inner diameter, with a 3-mm high edge) was then attached to the top surface of the headplate, centered upon and aligned parallel to the microprism, around the area between the craniotomy and the inner edge of the metal ring was covered with opaque dental cement (Metabond, Parkell, made opaque by adding 0.5 g of carbon powder, Sigma Aldrich). The metal ring and opaque Metabond, combined with the loose-fitting black rubber tube and tight fitting metal rings described previously (25), were secured to the block away from the virtual reality screen. After the surgery, the mice recovered in their home cages for ~2–3 d and were regularly exposed to a linear track (no Door Stop) for ~1 h per day until they ran ~2 laps/min. Once mice reached this criterion, they were transitioned to the ‘visible door’ version of the Door Stop task. In this task, mice ran 1.5 m down a linear track on the cylindrical treadmill to a visible door. At the door, the mice were required to stop (locomotion velocity below threshold of either 5.2 cm/s for 6 of 7 mice and 6.9 cm/s for 1 of 7 mice) within 10 cm of the door location. An instrumental cue in the form of an auditory click was presented to instruct the mice that the Door Stop had begun. Once only the mice had stopped for a given interval did the door open, at which point they could run forward through the open door another 1.5 m to the black environment zone to gain a small water reward (4 μL). Because the treadmill was not fixed in place during the timing interval, the mice could begin running on the treadmill before the interval was complete. In such cases, the door did not open and the mice could not progress forward along the virtual track; once the mice stopped again, the interval started over with another auditory click sound. At the beginning of training on the visible door task, the timing interval was set to 2 s, and it was gradually increased over weeks of training to 6 s, as each mouse gained >1 reward/min averaged over an entire 1 h training session. During this training period on the visible-door Door Stop task, the virus injection and MEC window implant surgeries described above were performed, with multiple days for recovery (without training) following each.

Once the mice were able to obtain ~1 reward/min on the 6-s interval visible-door Door Stop task, the door was made invisible. This task was identical to the visible-door version of the task, except that the door was made completely invisible. Mice were therefore not able to see when the door was present or not, but when the door was present, it would block the forward progress of the mice down the track. Further, since the mice could not see the invisible door opening at the end of the 6-s interval, this Door Stop task therefore required an internal temporal representation for efficient completion. If mice did not reach the invisible door after switching from the visible door task, the timing interval was reduced to ~4–5 s until performance improved, at which point it was again increased to 6 s. Once mice were able to obtain ~1 reward/min on the 6-s interval invisible-door Door Stop task (referred to in the text as the Door Stop task, ~6–8 weeks of total training time), imaging experiments began.

During wait periods at the invisible door, jerky movements (for the animal to maintain balance on the treadmill) occurred intermittently (0.61 ± 0.25 Hz, mean ± s.d.), aperiodically (randomly), and for short durations (lasting 0.23 ± 0.23 s, mean ± s.d.), and occurred during 12.9 ± 6.6% of the rest periods (mean ± s.d.), with a mean amplitude of 1.1 ± 1.5 cm/sec (mean ± s.d.; see Supplementary Fig. 2b–f).

Linear track environment switch (Fig. 4) and novel environment exposure (Fig. 5, Supplementary Fig. 7, and Supplementary Tables 1–3). Before exposure to the novel linear track (Fig. 5 and Supplementary Fig. 7), mice ran on a different (familiar) linear track for 2–8 weeks. When transitioning to the novel track, mice either ran on the familiar environment test and were switched to the novel track during the same recording session (3 transitions) or were exposed to the familiar environment on one day and switched to the novel at the beginning of the session on the next day (2 transitions). Once the second environment became familiar, the same FOV of MEC neurons were imaged across the two familiar environments (Fig. 4) in 3 mice.

Visible and invisible Door Stop task environment switch (Fig. 4, Supplementary Fig. 6a–k, and Supplementary Tables 1–3). Three mice were trained on the Door Stop task with a visible door (described above) using two visually different environments: one had an 8-s wait interval and the other had a 6-s interval wait.
Once mice were able to obtain ~1–2 rewards per min over a 1-h training session (averaged over trials in both environments) imaging experiments commenced. The same FOV was imaged as mice navigated one familiar Door Stop environment for the first half of the imaging session and the second familiar Door Stop environment for the second half of the imaging session. The same procedure was repeated for 3 mice trained and imaged in the invisible-door Door Stop task-switch experiment, except in this case both environments had the same 6-s wait interval.

**Classical trace conditioning task and switch to virtual linear navigation task** (Fig. 4 and Supplementary Fig. 6g-j). Three mice were trained in an immobile, headfixed classical trace-conditioning task. Mice were headfixed in the dark on top of a cylindrical treadmill that was held in place (i.e., mice could not move the treadmill). Mice were presented with a conditioned stimulus (CS) in the form of an auditory click, followed by a fixed trace period, and subsequently an unconditioned stimulus in the form of a water reward (2μl). All visual stimuli were turned off, and a 10-s baseline combined with a random time interval drawn from a uniform distribution from 0 to 20 s. Early in training, the fixed trace period was set at 1 s, and as mice began to display predictive licking during the 5.5 s trace interval. Once mice displayed predictive licking during the 5.5 s trace period, MEC imaging experiments began. Prior to this classical conditioning task, mice had been trained to run on the (no door) virtual linear track navigation task described above. Mice were imaged during the classical conditioning task, and, during the same imaging session, the 3-m virtual linear track was presented on previous days dark screens around the headfixed mouse and the treadmill was freed to allow for the mice to run and navigate along the track for water rewards at the end of the track. During navigation on the linear track, the same FOV was imaged as during the classical conditioning task.

**Data analysis.** Imaging data was analyzed on a Dell Power Edge 720 Server using Imaging (Version 1.47) and custom software written in Matlab (2017a/b). No statistical methods were used to predetermine sample sizes. Sample sizes were based on reliably measuring experimental parameters while remaining in compliance with ethical guidelines to minimize the number of animals used. Spearman’s rank tests, t tests, repeated measures ANOVA, Tukey’s post hoc test with Bonferroni correction, bootstrap tests, shuffle tests, and Wilcoxon rank-sum tests were used to test for statistical significance when appropriate, and all statistical tests were two-sided unless stated otherwise. For tests assuming normality, data distributions were assumed to be normal, but this was not formally tested. Data collection and analysis were not performed blind to the conditions of the experiments. Data collection was not randomized and different experimental groups were not used or defined. All data in the text and figures are labeled as either mean ± s.d. or mean ± s.e.m. See the Nature Research Reporting Summary for more information.

**Image processing, ROI selection, and transient analysis.** Motion correction was performed using whole-frame cross-correlation, as described previously, and the motion-corrected time-series was used for all subsequent analysis. Regions of interest (ROIs) were defined using Cell Sort (threshold = 1.5–3; area limits = 150–4,000 pixels; smoothing width = 0.75–1.0; mu = 0.5; principal components = 150) as described previously, but with the following modification. The motion corrected time-series first was divided spatially into subregions of ~160 × 200 μm (for example, a time-series of a 320 × 400-μm field would be broken up into 4 time-series, each one containing a 160 × 200-μm portion of the original field). Cell Sort was then applied to each subvideo independently, and all ROIs generated by Cell Sort were visually inspected to select for neuronal somata. The ROIs for the original field were then reconstructed using the ROIs defined on the original field. ROIs at borders were determined to be from the same cell split into different subfields, the ROIs were merged and the ∆F/Fl traces were averaged to generate a single cell and single ∆F/Fl trace. Cells were determined to be the same cell if at least 50% of each ROI’s pixels along the border were shared between ROIs and the Pearson’s correlation between the two ROIs ∆F/Fl traces was >0.7. To avoid double labeling of ROIs in a single subfield, if two ROIs were separated by less than 50 μm and the Pearson’s correlation between the two cells significant transient-only traces was greater than 0.8, then only one of the ROIs was retained. ROIs that appeared to be dendrites were also removed from each dataset. ∆F/Fl versus time traces from each ROI were then processed as previously described. Briefly, slow changes in the fluorescence traces were removed by examining the distribution of fluorescence in a 10–2 s interval centered on each sample in the trace and normalized by the eighth percentile value. We then analyzed these baseline-corrected somatic fluorescence traces for the ratio of positive- to negative-going transients of various amplitudes and durations, as described previously. We used this analysis to identify significant transients with <1% false-positive error rates and generated the significant transient-only traces that were used for all analysis in this research.

Defining immobile timing and locomotion dependent behavioral epochs in the **Door Stop task.** The immobile timing phase of the (visible and invisible) Door Stop tasks was defined as the middle 75% of the timing period (Fig. 1e and Supplementary Fig. 2a). The first 12.5% (0.75 s) and last 12.5% (0.75 s) of the 6-s wait period were considered part of a transition period between locomotion and immobility and were removed from the analysis. The locomotion-dependent navigation phase of the Door Stop task was defined as the middle 75% of the 3 m track; 12.5% of the track (3.75 cm) before and after the door and 8.3% of the track (25 cm) before and after (beginning of track) the reward zone were considered part of a transition area between locomotion and immobility or a transition area where mice slowed down and stopped to consume the reward, respectively, and were removed from the analysis. The transition periods were purposely chosen to be large so as to include only clearly defined behavioral periods. These steps thus allowed us to exclude ambiguous behavioral periods and more clearly identify the small number of neurons with separate/isolated fields in both the immobile timing period and the locomotion-dependent navigation period, rather than cells with single fields straddling the transition regions.

**Defining active cells.** All active cells included in this study were defined as cells identified using Cell Sort segmentation software (see above) that had a mean ∆F/Fl > 0.7% in at least one of the two defined behavioral epochs (the immobile timing phase at the door location or the locomotion-dependent navigation phase along the track).

**Defining temporal encoding and spatial encoding cells in the Door Stop, classical trace conditioning, and linear track tasks.** Spatial fields were defined as described previously, with minor changes. First, running periods were defined when mice were running at least 5.2 cm/s. The significant spatial fields were then defined as contiguous fields of this run period and maintained at least 5 cm/s for at least 25 cm of movement along the track (excluding transition zones). Note that these are the running periods used for calculating the run–rest index (see below). For each cell, the mean somatic ∆F/Fl was calculated as a function of virtual track position for 35 (Door Stop) position bins and 50 (linear track) position bins (5 cm per bin). Potential spatial fields were first identified as contiguous periods of this plot in which the mean in-field ∆F/Fl was greater than 20% of the difference between the peak somatic ∆F/Fl value (for all bins) and the baseline value (mean of the lowest 25% somatic ∆F/Fl values). These potential spatial field regions then had to satisfy the following criteria: (i) The field must be >15 cm in width; (ii) the field must have one value of at least 6% mean ∆F/Fl; (iii) the mean in-field ∆F/Fl value must be >2× the mean out-of-field ∆F/Fl value; and (iv) significant calcium transients must be present >33.3% of the time the mouse spent in the spatial field and each cell must have >4 transients that occurred in the field. Potential spatial field regions that met these criteria were then defined as spatial fields if their P value from bootstrapping was <0.05, as described previously, and their mean widths were <125 cm. The ∆F/Fl time-series for each cell was broken into periods of individual transients and intertransient intervals. The sequence of these events was then randomly shuffled to produce a random ∆F/Fl time-series from which mean shuffled ∆F/Fl versus position plots were generated and subjected to the same criteria outlined above. This process was repeated 1,000 times and the P value was defined as the ratio of the number of times out of 1,000 that the random shuffled trace generated a spatial field that met the above criteria.

Significant timing fields were defined using a similar method (to spatial fields) applied to calcium transients during the wait periods of either the Door Stop or linear track tasks. For each recording session in the Door Stop task, wait trials were in 1-s increments, the door location was maintained and maintained velocity below a threshold (5.2 or 6.9 cm/s) for the entire 6-s wait period and then began running velocity >5.2 or 6.9 cm/s) within 3 s from the door opening. Wait trials in which the mouse stopped at the door and began running before the end of the 6-s wait period or trials in which the animal waiting longer than 9 s were not included (except for error, short-, and long-wait trial data included in Fig. 3 (6 s ≤ wait ≤ 9.3 s) and Supplementary Fig. 5a,b (4.5 s ≤ wait ≤ 8.5 s)). For each recording session in the linear track task, rest periods were included if the mouse went below 2.5 cm/s and subsequently maintained velocity below 5 cm/s for at least 9 s and not longer than 30 s. To measure the percentage of stopping periods at different locations along the track, the linear track was divided into four equal spatial segments. For each such segment, the number of stops was divided by the total number of stops. Region 1 (start/reward location) contained the first 1/8 and last 1/8 of the track; regions 2 to 4 were spaced from 1/8 to 3/8 of the track, 3/8 to 5/8 of the track, and 5/8 to 7/8 of the track, respectively. Since each mouse nearly always stopped at region 1 (77.9 ± 19.4% for mice ≥ 8 s) and the P value and the baseline value (mean of the lowest 25% ∆F/Fl bins). These potential timing field regions then were required to satisfy the following criteria: (i) the field must persist for >0.5 s; (ii) the field must have one value of at least 6% mean ∆F/Fl; (iii) the mean in-field ∆F/Fl value must be >2× the mean out-of-field ∆F/Fl value; and (iv) significant calcium transients must
be present in the field during >33.3% of the trials. Potential timing field regions that met these criteria were then defined as timing fields if their $P$ value from bootstrapping (see above) was <0.05, and their mean duration <5 s. Time-encoding cells in the classical conditioned fear paradigm were defined as outlined above, but during the 5.5-s trace period of the task.

Time-encoding cells were active almost exclusively during periods of immobility (Fig. 2e), which is different from previous reports of negative-velocity tuning in MEC neurons (see Hinman et al)\(^{16}\). In contrast to the time encoding cells shown here, the negatively tuned velocity cells presented by Hinman et al\(^{16}\) were active during locomotion, but with firing rates that were negatively correlated with the animal’s velocity. Most temporal coding cells were active during periods of immobility (mean velocity $\leq 0.33 \pm 1.00$ cm/s (mean $\pm$ s.d.) for the wait periods in the Door Stop task) and much less active during locomotion, even at low velocities (Fig. 2e and Supplementary Fig. 3f).

In the (invisible door) Door Stop task, cells that had significant fields in both the temporal and spatial epochs made up 3.1 $\pm$ 0.7% (mean $\pm$ s.e.m.) of all active cells and 7.9 $\pm$ 1.8% (mean $\pm$ s.e.m.) of all cells with at least one significant field in the temporal or spatial phase of the Door Stop task. Data presented in Fig. 2e,f,g are shown for exclusively timing-encoding selective or exclusively spatial-selective cells.

For the remaining cells, we used the pairwise distance between neurons as the Euclidean distance between the centroids of each neuron’s ROI. A repeated-measures ANOVA (with Tukey’s post hoc test with Bonferroni correction) across 11 unique (non-overlapping) imaging fields in 7 mice was used in order to compare the intercell and intracellular distances between time-encoding cells, space-encoding cells, and all cells.

To measure the speed of sequence progression (Fig. 3 and Supplementary Fig. 5), we included only temporally selective cells with transients on at least 5 trials in each of the long- and short-wait trials epochs. The center of mass (COM) of each timing cell’s mean $\Delta F/F$ versus time trace was calculated as previously described\(^{17}\).

For data presented in Fig. 3b, cells were ordered according to each cell’s mean COM across all short-wait trials (earliest mean COM at top, latest at bottom). All cells in one FOV from a single imaging session, except for Fig. 3b bottom, which was sorted across all cells from all fields. The same indices used to sort the cells on short-wait trials were then applied to sort cells on the long-wait trials (Fig. 3b). The trial-by-trial measure of the speed of sequence progression (unit sequence progression/s) was computed for each trial as the slope of the linear fit of the sequence-sorted COMs for all time-encoding cells active on that trial (Fig. 3bc and Supplementary Fig. 5a,b). For data presented in Fig. 3c and Supplementary Fig. 5a,b, cells were ordered according to each cell’s mean COM across all correct (6- to 9.5-s) trials (earliest mean COM at top, latest at bottom) for all cells in one FOV from a single imaging session. To compare linear fits across FOVs (with different numbers of cells in each sequence), we normalized the position of each cell in the sorted sequence such that the 4th cell in the ordered sequence had a position of $k/N$, where $N$ is the total number of cells in the sequence. Therefore, for each trial, we computed the COM for each cell’s $\Delta F/F$ versus time trace on that trial, and left the ordering of the COMs according to each cell’s mean COM across all correct trials. A linear fit was then computed for the normalized cell sequence of COMs on each trial (example fits for error, short-, and long-wait trials displayed as overlaid lines in Fig. 3b and Supplementary Fig. 5a). For each fit the intercept was fixed at (0,0), and cells that did not have transients on a given trial were not included in the fit.

To compare observed fractions of time- and space-encoding cells that either encoded the same variable or switched codes between environments (or tasks) to the fraction expected by chance (Fig. 4b), we performed a shuffle test as follows. For each environment-switch (or task-switch) experiment (visible door, invisible door, linear track, and classical conditioning to linear track) we started by identifying the number of cells with timing or spatial fields in the first environment/task (e.g., 50 total cells with fields, 30 timing and 20 spatial cells). We then found the number of these cells in the real data that encoded a variable (time or space) or did not encode a variable in the second environment/task (e.g., 10 cells encoding a variable across both environments, 40 cells only encoding in the first environment, for the 50-cell example above) and used these numbers to estimate variable-encoding probability across the switch (20% chance for a cell encoding a variable in the first environment to encode a variable in the second environment, 80% chance to encode a variable only in the first environment). Across all of our datasets, we found that 18% of active cells were time-encoding cells and 18% were space-encoding cells, implying that if a cell was randomly assigned to encode time or space in the second environment, it would have a 50% chance to be either. These probabilities were then used to generate a surrogate dataset by randomly assigning each cell that had a timing or spatial field in the first environment (the 50 cells in our example) an identity in the second environment (80% chance to encode no variable, 10% chance to encode time, and 10% chance to encode space). Among the subset of cells in the surrogate dataset that encoded a variable in both environments, we calculated the fraction of cells that encoded the same variable as switched vs not encoding. This process was repeated 1,000 times, and a $P$ value was defined as the ratio of the number of times out of 1,000 that the randomly shuffled fraction of cells that kept the same identity was at least as large as the observed fraction that kept the same identity in the real data. The s.d. of this randomly shuffled fraction distribution, which is centered on 50%, is shown in Fig. 4b (dashed line).

Measuring temporal and spatial information for active cells in the (invisible door) Door Stop task (Supplementary Fig. 4). In order to measure temporal information during wait periods and spatial information during locomotion periods for each cell, we used information rate as defined in Skaggs et al. and recently applied to GCaMP6f \([Ca^{2+}]i\) imaging data\(^{18,19}\). The only threshold used in this analysis was a minimum-activity threshold that required neurons to have a mean $\Delta F/F > 0.7$% during either locomotion periods along the track or during immobile periods waiting at the door, which equates roughly to at least 5 calcium transients during one of these behavioral periods (the transition periods mentioned above were also excluded from analysis. Because the different parameters (spatial location versus wait time) occurred during different behavioral periods, we applied the below-described information-significance test separately to spatial (during locomotion periods) and temporal (during immobile periods) information for each cell.

During rest/wait (or running) periods defined above, the temporal (or spatial) information rate was computed for each cell as:

$$\sum_{i=1}^{N} p_i \log_2 \left( \frac{1}{p_i} \right)$$

Where $\lambda$ is the mean $\Delta F/F$ versus time across all wait trials (or versus position across all traversals across the track) excluding transition periods (or zones), $\lambda$, is the mean $\Delta F/F$ at the $i$th wait time (or $i$th position along the track) and $p_i$ is the occupancy probability at the $i$th bin. The number of correct wait trials was less than the number of track traversals; therefore, to compare the temporal and spatial information measures attributed to each cell directly, a random subset of the traversals along the track was included, such that this number was equal to the number of correct wait trials for each recording session. Cells with significant temporal (or spatial) information were generated by using a shuffle procedure. The $\Delta F/F$ time-series during the rest/wait (or running) periods for each cell was broken into periods of individual transients and intertransient intervals. The sequence of these events were then randomly shuffled to produce a random $\Delta F/F$ time-series from which surrogate $\lambda$ and $\lambda$, values were computed for the temporal (or spatial) period and these parameters were used to compute a surrogate temporal (or spatial) information rate. This process was repeated 1,000 times and a shuffled $P$ value was defined as the ratio of the number of times out of 1,000 that the surrogate temporal (or spatial) information measure was greater than the temporal (or spatial) information rate measured in the real data. Cells were determined as having significant temporal (or spatial) information if they had a $P < 0.05$

The random shuffle distributions shown in Supplementary Fig. 4c were generated by random shuffling. For all cells carrying significant spatial or temporal information, the spatial information values were randomly shuffled with respect to the temporal information values across the population. A new histogram was generated for each random shuffle. $k$-means clustering (Supplementary Fig. 4e) was performed using the kmeans Matlab function with two clusters and Euclidian distance as the clustering parameter.

Defining the run–rest index. Running and resting periods included in the run–rest index were defined using the same behavioral criteria used for defining spatial cells and temporal cells in the Door Stop task and linear track task (see above). The run-rest index for the 4th cell was computed as follows:

$$RRI_{ki} = \frac{\sum_{i \in \text{real}(k \in \text{rest})} \Delta F \log \left( \frac{1}{p_i} \right) - \sum_{i \in \text{real}(k \in \text{run})} \Delta F \log \left( \frac{1}{p_i} \right)}{\sum_{i \in \text{real}(k \in \text{run})} \Delta F}$$

where $\Delta F/F_{ki}$ is the calcium transient $\Delta F/F$ value for the $k$th cell at the $i$th frame, $R_{ki}$ is the set of all frames that occur during running periods, and $R_{ki}$ is the set of all frames that occur during rest periods. $RRI$ was computed for all active cells (Fig. 2b) and all significant time- and space-encoding cells (Fig. 2a). For data shown in Supplementary Fig. 3b, RRI was computed using the same equation, but in this case, $R_{ki}$ is the set of frames that occurred during the long-wait periods at the Door Stop location (i.e., running on the treadmill, but with the door closed, so the mouse does not move in VR).

To generate a shuffled $P$ value to compare changes in $RRI$ of cells across environments, in the real data, active cells were included if they had RRI’s from either the top or bottom quartiles of the $RRI$ distribution in the first environment (i.e., run- or rest-selective cells). To generate the shuffled distribution, we randomly permuted the $RRI$ values of these cells measured in environment 2, and measured the $RRI$ difference across environments:

$$|RRI_{ki}^{(1)} - RRI_{ki}^{(2)}|$$

where $RRI_{ki}^{(1)}$ is the real RRI of the $k$th cell in environment 1 and $RRI_{ki}^{(2)}$ is the shuffled RRI of the $k$th cell in environment 2. We then computed the mean $RRI$ difference across all cells. This process was repeated 1,000 times and the $P$ value was defined as the ratio of the number of times out of 1,000 that the random shuffled mean $RRI$ difference was smaller than the real mean $RRI$ difference (Supplementary Fig. 6n).
Identifying space- and time-encoding cells across days. To identify with certainty the same individual time- or space-encoding cells from recording sessions across days (Supplementary Fig. 6k–m), we compared the neuronal morphology for each ROI across days in FOVs that could be visually matched across days. FOVs were visually matched across days if both FOVs had a similar vasculature pattern, similar spatial organization, and similar morphology of neuronal somata. Time- or space-encoding cells were then matched across days (FOVs) through visual inspection of their morphology (including both soma and dendrites) from frames averaged when the cells were active (Supplementary Fig. 6k,l). This criterion requiring matching dendritic morphology was conservative, but attempting to identify the same cells over days based only on the position of the somata often led to misidentification (based on dendritic morphologies).

Histology. The surface vasculature seen across the microprism was recorded using epifluorescence microscopy (Supplementary Fig. 1a). The location of each two-photon imaging field in MEC layer 2, with respect to the surface vasculature, was then recorded by comparing the surface vasculature pattern observed using two-photon microscopy above (caudal to) the imaging field to the previously recorded epifluorescence pattern. Once imaging experiments in each mouse were complete, the mouse was anesthetized with 1–2% isoflurane and the headplate and prism/mount were removed to expose the caudal surface of MEC. A pin coated with Alexa Fluor 594 was then inserted perpendicular to the caudal MEC surface at an identified region in the vascular pattern near to the two-photon imaging field locations. The tissue was sectioned in 50-µm sagittal slices using a freezing microtome. Free-floating slices were then incubated in 0.1 M PBS with 0.1% Triton-X for 10 min, washed three times with 0.1 M PBS, and incubated for 1 h in a 25:1 solution of 0.1 M PBS to 435/455 blue fluorescent Nissl stain (Invitrogen). MEC was identified based on lamina dissecans, the position of the post-rhinal border relative to the pin mark, and the circular shape of the dentate gyrus shown at the mediolateral position of the sagittal sections. Two-photon imaging fields were then confirmed to be located in the identified MEC regions based on known distances from the pin-mark sites (Supplementary Fig. 1a–c).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code and data availability statement. The data and custom code that support the findings of this study are available from the corresponding author upon reasonable request.

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Life sciences study design

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| Sample size | No statistical methods were used to predetermine sample sizes. Sample sizes were based on reliably measuring experimental parameters while remaining in compliance with ethical guidelines to minimize the number of animals used. Spearman’s Rank tests, T tests, repeated measures ANOVA, Tukey’s post-test with Bonferroni Correction, Bootstrap tests, Shuffle tests and Wilcoxon rank tests were used to test for statistical significance when appropriate and all statistical tests were two-sided unless stated otherwise. |
| Data exclusions | All inclusion/exclusion criteria were established independently and prior to analysis of effect sizes. For imaging experiments, mice were excluded from analysis if no GCAMP expression was observed or if motion artifact was too large. Only mice that were able to perform the virtual reality tasks detailed below (reward rate >1 reward/min) were included in this study because behavioral periods of poorly behaving mice were difficult to interpret. Selection for well defined behavioral periods was performed as follows to focus on reproducible and interpretable behavior periods across mice. Running periods were defined when mouse movement velocity along the virtual track first increased above 10 cm/sec and maintained at least 5 cm/sec for at least 25 cm of movement along the track (excluding transition zones). Wait trials were included if the mouse stopped at the door location and maintained velocity below threshold (5.2 or 6.9 cm/sec) for the entire 6 second wait period and then began running (velocity>5.2 or 6.9 cm/sec) within 3 seconds from the door opening. |
| Replication | Experimental results were based on neural activity patterns and mouse behavior during tasks. Multiple mice (n) were used for every experiment and observations of similar results across mice were used to infer replication. All attempts at replication were successful. |
| Randomization | Randomization was not required because this study did not allocate different mouse experimental groups. |
| Blinding | Blinding was not required because this study did not allocate different mouse experimental groups. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | n/a Involved in the study |
| --- | --- |
| ☒ Unique biological materials | |
| ☒ Antibodies | |
| ☒ Eukaryotic cell lines | |
| ☒ Palaeontology | |
| ☒ Animals and other organisms | |
| ☒ Human research participants | |

| Methods | n/a Involved in the study |
| --- | --- |
| ☒ ChIP-seq | |
| ☒ Flow cytometry | |
| ☒ MRI-based neuroimaging | |

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| Laboratory animals | male C57-BL6 mice (~p70). |
| Wild animals | This study did not involve wild animals. |
| Field-collected samples | This study did not involve samples collected from the field. |