Supplementary Methods

Subjects

Neuronal activity was recorded from 14 male Long-Evans rats (350-500 g at implantation and testing) housed individually in transparent Perspex cages (54 × 44 × 35 cm height) with water available ad libitum. Five of these rats were taken from a previous study[1] and were used only for the correlation of recording depth with grid spacing and field size in the square box. All rats were kept on a 12 hr light/dark schedule and tested in the dark phase.

Electrode implantation

Rats were anesthetized (Equithesin; 1 ml/250g) and 4 tetrodes of twisted 17 µm HM-L coated platinum-iridium wire (California Fine Wire Company, Grover Beach, CA) connected to a microdrive were implanted above the dorsocaudal medial entorhinal cortex (dMEC) 4.5 mm lateral to the midline[1]. The electrodes were inserted 0.2-0.3 mm anterior of the sinus at an angle of 6-10º in the sagittal plane with the tip pointing in the anterior direction. Six rats had double implants. In five of them, the second microdrive was implanted at the corresponding location in the contralateral dMEC. In the sixth rat, the second microdrive was implanted above the contralateral CA3 at AP 3.2 mm and ML 2.8 mm relative to bregma[2]. In the rats with only one microdrive, a separate pair of EEG electrodes consisting of twisted 70 µm Teflon-coated stainless-steel wire (Goodfellow, Cambridge, UK) was implanted close to the fissure of the contralateral dorsal hippocampus. A jeweller's screw fixed to the skull served as a ground electrode in all rats. The microdrives were secured to the skull using jewellers' screws and dental cement. Electrode tips were platinum-plated before surgery in order to reduce electrode impedances to 150-200 kΩ at 1 kHz.

Behavioural training and testing

All training was performed post-surgically. The rats were connected to the recording equipment (Axona Ltd., Herts, U.K.) via AC-coupled unity-gain operational amplifiers close to the rat's head, with counterbalanced hearing-aid wire allowing the animal to move freely within the available space[1]. Over the course of 5-10 days, the tetrodes were lowered in steps of ~50 µm or less while the rat rested on a towel in a large flower pot on a pedestal. Electrode turning stopped when multiple well-separated large-amplitude theta-modulated low-frequency neurons appeared at depths of about 2.0 mm or lower in the superficial layers of the dorsolateral band of entorhinal cortex[1].

Data collection started when signal amplitudes exceeded ~5 times the noise level (r.m.s. 20-30 µV) and the units were stable for more than 2h. Recorded signals were amplified 10 000 to 25 000 times and band-pass filtered between 0.8 and 6.7 kHz using a digital sinc filter with a 0.69 ms Hamming window. Triggered spikes were stored to disk at 48 kHz (50 samples per waveform, 8 bits/sample) with a 32 bit time stamp (clock rate at 96 kHz). EEG signals from the external electrode in the hippocampus were amplified 3000-5000 times, lowpass-filtered at 500 Hz (single pole), and stored at 4.8 kHz (16 bits/sample). EEG signals from tetrodes in the entorhinal cortex were amplified 8000-10000 times and filtered and stored as the hippocampal EEG. A tracker system (Axona Ltd.) was used to record the position of a red or infrared LED attached to the head stage at a rate of 50 samples per second. The tracked position was smoothed with a Kalman filter offline.

Square box. The rats were kept at 85-90 % of their free-feeding body weight during the training period. After each screening session, the animals were trained to collect scattered randomly thrown pieces of food in one or several recording boxes. During the screening

period, all 14 animals were trained for at least 10 min twice each day in a black square box (1.0 × 1.0 × 0.5 m height) with a white cue-card (20 × 30 cm) centred on one of the walls. The inter-trial interval was 15 min. Before and after each trial, the rat rested on a pedestal for 5-10 min. In the analysis of grid spacing and field size as a function of recording depth, 26 cells from 5 rats were added from a previous study[1], yielding a total of 57 cells from 11 rats.

Circular boxes. Six rats were also trained to run in two circular enclosures. These enclosures differed in diameter (2.0 m vs. 1.0 m) but were otherwise identical. The height was 0.5 m. Both boxes had black walls and a black floor. The boxes were centred at the same room location. A white cue-card (45 × 50 cm) was placed at corresponding angular locations in both circles. Trials were 20 min in the large circle (to obtain sufficient coverage) and 10 min in the small circle (sequence: large-small-large). Training and testing were performed with or without black curtains around the recording circle (4 and 2 rats, respectively). These data were merged because the presence of curtains had no detectable effects on the variables measured in this study. Three rats received separate trials in which the cue card in the small circle was rotated 90 degrees and back. Curtains were used on all rotation trials. Before and after each trial, the rat rested on a pedestal for 5-10 min.

Darkness trials. Four rats were tested in a black circular enclosure (1.0 m diameter) with room lights on or off (10 min on, 10 or 30 min off, 10 min on). These experiments were performed in a room with sound- and light-insulated walls and a similarly insulated door to prevent light from entering the room during darkness. The recording equipment and the computer were placed in another room. An infrared diode on the head stage and a camera sensitive to infrared light were used to track the animals' position in both light and dark trials. The rat was not taken

out of the recording enclosure between light and dark epochs. Before and after testing in the cylinder, the rat rested on a pedestal for 5-10 min.

Novel environment. Seven animals were tested on alternating trials in a familiar room and a novel room (10 min familiar, 30 min novel, 10 min familiar). The familiar room was the same room as the one used for cell screening. The rats had been trained in this room for a minimum of 10 days. A square box (1.0 × 1.0 × 0.5 m height) with a cue-card (20 × 30 cm) centered on one of the walls was used in both rooms. One animal was tested in the novel room with lights off (same room and same recording procedure as in the other darkness experiments). After 30 min in darkness, the light was turned on for 10 min followed by another 10 minutes of darkness. The rat was not taken out of the recording enclosure between trials.

Linear track. Three rats were trained to run back and fourth on a linear track (235 × 10 cm) with food rewards at the ends. The track was placed 50 cm above the floor. The rats were tested on alternating trials in light (5 min) and darkness (5 min). Recording procedures were the same as in the darkness experiments described above.

Spike sorting and cell classification

Spike sorting was performed offline using graphical cluster-cutting software (Axona Ltd.). Clustering was performed manually in two-dimensional projections of the multidimensional parameter space (consisting of waveform amplitudes), using autocorrelation and crosscorrelation functions as additional separation tools and separation criteria[1]. Putative excitatory cells were distinguished from putative interneurons by spike width, average rate and the occasional presence of bursts[1,3].

Analysis of place fields

To characterize firing fields, we used a spatial smoothing algorithm[1]. The average rate at any arbitrary position *x* was estimated as

$$\lambda(x) = \sum_{i=1}^{n} g(\tfrac{s_i - x}{h}) \Big/ \int_0^T g(\tfrac{y(t) - x}{h}) dt$$

where *g* is a smoothing kernel, *h* is a smoothing factor, *n* is the number of spikes, s_i the location of the *i*-th spike, *y*(*t*) the location of the rat at time *t*, and [0, T) the period of the recording. A Gaussian kernel was used for *g* and *h* = 3 cm. Positions more than 5 cm away from the tracked path were regarded as unvisited. The peak rate of firing in the box was estimated as the highest firing rate observed in any bin of the smoothed rate map. A firing field was estimated as a contiguous region of at least 100 bins (225 cm^2) where the firing rate was above 20% of the peak rate.

Firing patterns on different trials in the same box were compared with a spatial correlation procedure. Each map was smoothed and binned into matrices of 5 cm × 5 cm pixels, and the rates of firing in corresponding pixels of the two maps were correlated for each cell. Pixels visited less than 150 ms in either trial were excluded to avoid artifacts in the correlation measure.

Spatial autocorrelations and crosscorrelations

To determine whether the multiple fields of a cell in dMEC formed a regular structure, we calculated the spatial autocorrelation for the smoothed rate map of each cell as well as spatial crosscorrelations for pairs of cells recorded at the same time. Auto- and crosscorrelograms were estimated from the sample correlation coefficient to correct for edge effects and unvisited

locations. With $\lambda_1(x, y)$ and $\lambda_2(x, y)$ denoting the average rates of cell 1 and 2 at location (*x*,*y*), the crosscorrelation between these fields with spatial lags of τ_x and τ_y was estimated as

$$r(\tau_x, \tau_y) = \frac{n\sum \lambda_1(x,y)\lambda_2(x-\tau_x, y-\tau_y) - \sum \lambda_1(x,y)\sum \lambda_2(x-\tau_x, y-\tau_y)}{\sqrt{n\sum \lambda_1(x,y)^2 \left(\sum \lambda_1(x,y)\right)^2}\sqrt{n\sum \lambda_2(x-\tau_x, y-\tau_y)^2 \left(\sum \lambda_2(x-\tau_x, y-\tau_y)\right)^2}}$$

where the summation is over all *n* pixels in $\lambda_1(x, y)$ for which rate was estimated for both $\lambda_1(x, y)$ and $\lambda_2(x-\tau_x, y-\tau_y)$. The autocorrelation of $\lambda_1(x, y)$ was estimated similarly by substituting $\lambda_2(x-\tau_x, y-\tau_y)$ with $\lambda_1(x-\tau_x, y-\tau_y)$. Auto- or crosscorrelations were not estimated for lags of τ_x, τ_y where $n < 20$.

For each autocorrelogram, we compared distance and angle from the central peak of the autocorrelogram to each of the surrounding peaks. The angles relative to a fixed external reference (the orientation of the camera) were also determined.

Note that for both autocorrelograms and crosscorrelograms, the distance scale is half of that of the original maps, with points along the circumference showing correlations between positions spaced by a distance similar to the diameter of the enclosure (2 m in all radial directions). The number of contributing position pairs decrease from centre to periphery, rendering the outermost parts of the correlograms susceptible to random fluctuations in firing rate. The frequent increase in amplitudes along the circumference of the autocorrelograms (Fig. 2cd) and crosscorrelograms (Supplementary Sheet S5) may thus be a sampling artefact, although we cannot exclude that it also reflects a real alignment of the grid vertices to the wall of the cylinder.

The relation between recording location (distance from the postrhinal border) on one hand and grid spacing or field size on the other was determined by linear regression.

Histology

Electrodes were not moved after the final recording session. The rats received an overdose of Equithesin and were perfused intracardially with saline and either 4 % formaldehyde or 4 % paraformaldehyde. The brains were extracted and stored in formaldehyde, and frozen sagittal sections (30 µm) were cut. All sections were mounted on glass slides and stained with cresyl violet. With the use of a light microscope, equipped with a digital camera, the deepest position of the recording electrodes was determined. The exact position of the electrodes at recording was extrapolated in Adobe Photoshop using the read-out of the tetrode turning protocol, taking shrinkage as the result of histological procedures into account (~ 20 %). All electrodes in the entorhinal cortex were positioned in layer II or at the layer II-III border at the caudal end of the dorsolateral band of MEC[1]. No attempt was made to separate out recordings near the layer III as cells in layers II and III appear to have similar grid-like firing properties[4]. Distance along layer II from the recording position to the postrhinal border was measured by an experimenter who was blind to the physiological results.

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