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Materials and Methods

Subjects

41 adult male Lister Hooded rats were used for the experiments. Rats were individually housed in clear plastic cages (68 cm x 44 cm x 48 cm, W x L x H) and were kept on a 12:12 hour light:dark cycle at a controlled temperature (19-23 ^{0}C) and humidity (50-70 %). The rats weighed 350-450g on the day of surgery. They were maintained on a 90% of body weight food deprivation schedule where the most recent feeding was at least 12 hours before the experiments. Water was supplied ad libitum.

Surgery and electrodes

The rats were anaesthetised with 1-3 % of isoflurane in O_2 and a 0.01mg /100g im injection of buprenorphine. They were chronically implanted in the left hemisphere with a microdrive (Axona Ltd) loaded with four tetrodes (HM-L coated 90% platinum/ 10% iridium 17 micron diameter wire). The tetrodes were usually tightly glued together and cut at the same level. The electrode tips were plated to reduce the impedance to 150-300 kΩ at 1 kHz. Tetrodes were aimed at the most dorsal parts of the medial entorhinal cortex (mEC, 39 rats) and adjacent parasubiculum (PaS, 2 rats) (4.3-4.5 mm lateral to the midline; 0.2-0.5 mm anterior to the sinus; angled forwards in the sagittal plane at 0^0-10^0 and 1.5 mm below the

pia). 5-6 screws were threaded into the skull and the microdrive anchored to them with dental cement. A jeweller's screw fixed to the skull over the opposite hemisphere was used as a ground screw. After surgery the rats were given at least 7 days to recover before the experiments were started.

Histology and electrode localization

After completion of the recording sessions the rats were sacrificed using an overdose of sodium pentobarbital and perfused transcardially with saline followed by 4% of paraformaldehyde (PFA). The brain was cryoprotected in 4% PFA and 20% sucrose solution and sliced into 30 microns parasagittal sections using a freezing microtome. Sections were mounted and Cresyl-Violet Nissl-stained to allow visualization of the electrode track. Recording sites were determined by measuring backwards from the deepest point of the track and ensuring that the total length of the electrode track corresponded to the amount of electrode movement as measured from the microdrive screw turns (Extended Data Fig. 1-2). 20% shrinkage was allowed for. mEC layer II cells were identified as a densely packed strongly stained superficial layer of cells. mEC layer III cells were less intensely stained and less densely packed. mEC layer V cells were intensely stained cells in the pyramidal cell layer adjacent to the lamina dissecans. mEC VI layer were less intensely stained and adjacent to layer V. PaS superficial layer cells were identified as densely packed cells located in a dorsal-caudal area above the superficial layers of mEC which showed less staining and did not show a clear laminar structure.

Data Collection

Rats were allowed a period of at least 7 days of recovery after surgery at which point screening for cells began. Tetrodes were lowered 50 microns or less per day at the end of each recording session until the first cells of interest were found. Single unit data were acquired using a proprietary data acquisition system (DACQ system, Axona Ltd, UK). Two infrared light emitting diodes (LEDs) of different intensities and separated by 7 cm were fixed on the animal's head in order to track the animal's position and head orientation. The (x, y) coordinates of the LEDs were

acquired at 50 Hz by an infrared camera attached to the ceiling above the centre of the environment.

Single cell isolation from multi-unit recordings

The experimenter was blind to the cell firing patterns during data collection and cell cluster cutting. Isolation of single units was performed by manual cluster-cutting. Clusters were isolated in a feature space where all possible combinations of pairs of spike amplitudes recorded by four tetrodes were plotted against each other. Unit isolation was further refined by taking into account additional properties of spike waveforms such as the existence of positive pre-potentials. Clusters recorded on different trials were assigned to the same cell if: a) the tetrode had not been moved between trials; b) there was minimal change in spike waveforms; c) the position of the target cluster and neighbouring clusters remained similar in feature space. Despite having been recorded on several trials however, each cell was only included once in any analysis reported in this paper.

Training procedures and testing environments

Square enclosures. Cells were recorded as the animals foraged in one of a number of familiar square enclosures of the following dimensions (L x W x H): 0.6m x 0.6m x 0.5m; 0.9m x 0.9m x 0.5m; 1m x 1m x 0.5m; 1.2m x 1.2m x 0.5m; 1.3m x 1.3m x 0.5 m; 1.6m x 1.6m x 0.5m; 1.9m x 1.9m x 0.5m. The recording enclosures were always placed in the same position with respect to the laboratory during training and testing sessions. Distal visual cues (e.g. a white rectangular cue card, shelves, recording system etc) were available to maintain the same allocentric directional sense across sessions. The animals were trained to forage for sweetened rice thrown into random locations in the enclosure approximately every 10-20 seconds. At least two 15 min trials were recorded within a day. There were 10 min intervals between every trial during which the animal rested on a holding platform located near the testing environment.

Circular enclosure. Cells were also recorded in 9 animals in a 0.8m dia x 0.5m circular enclosure located in a different unfamiliar room. The animals had at least 6 training sessions over three days period prior to testing. Training and testing

were performed with black curtains around the recording circle and the only available landmark was a large well-illuminated white cue card (0.8m x 1.2m). The animals were disoriented before each session by slowly walking in the corridor and rotating the enclosed animal carrying box at ~0.1 Hz. Each session included two trials (10 and 15 min) with ~5-10 min break between them during which the animal rested in his carrying box inside the curtained environment.

Trapezoidal enclosures. Cells were also recorded in 8 animals in an isosceles trapezoidal enclosure (lengths of the shorter and longer parallel walls 0.2m and 0.9m respectively with angled walls equal to 1.9m; 0.5 m height). Each recording session consisted of 5 trials: a) the first 8-10 min trial in a $0.9m^2$ square; b) 15 min trial in the square; c-d) 20 min trial in the trapezoid; e) 15 min in the square. The animal had ~10-15 min breaks between trials. The animals were not disoriented between the trials. The square and the trapezoid were centred at the same position in the room below the tracking camera. The room was well illuminated with multiple cues positioned around the recording enclosures including one large well illuminated white card (1.13m x 0.83m) at the west side of the environment. Initial cell screening was carried out in the square enclosure until the first location-modulated cells were recorded and then the training in the trapezoid was begun. In two rats grid cells were recorded from the first exposure to both the square and the trapezoid to investigate the time course of scale changes with response to novelty and subsequent familiarisation.

Hexagonal enclosure. Two animals were also recorded on a familiar hexagonal platform (with length of each side equal to 0.83m) located in a third different experimental room. The hexagonal platform had no walls and was located in an environment with many visible visual cues.

All the first trials of the day (i.e. trial ‘a’) were excluded to minimise novelty effects.

Firing rate maps

A locational firing rate map was estimated by dividing the number of spikes fired in a given part of the environment by the time spent there. Position data and spike counts were sorted into 2.5cm x 2.5cm spatial bins. Unsmoothed firing rate maps

were obtained by dividing the spike count in each bin by the dwell time in that bin. The smoothed firing rate map was obtained by first applying adaptive smoothing (see ‘Adaptive Smoothing’) to the dwell time and spike maps and then dividing them. Colour bars represent firing rate in deciles of the range of firing rates (top 10 % in red, bottom 10 % in blue). Unvisited bins are shown in white.

Directional firing rate was estimated by dividing spike counts and dwell times into 3 degree bins. Adaptive smoothing was applied to spike and dwell time maps before dividing them.

Adaptive smoothing

Adaptive smoothing[1] was applied to the firing rate maps. In brief, to calculate the firing rate for a given bin the number of spikes assigned to that bin is defined by a circle centred at the bin with the radius r. The radius of the circle is expanded until

$$r \geq \frac{\alpha}{n\sqrt{s}} \qquad (1)$$

where $\alpha = 5000$, n is the number of bins and s is the number of spikes lying within the circle.

Criteria for cell classification

Grid cells were identified by comparing the gridness score to a threshold value calculated as the 95th percentile value of shuffled data. The data was shuffled following Wills et al[2] by wrapping the time-shifted spike train around the position data. The spike train was shifted by a random duration more than 20 s and less than the trial duration minus 20s. Each shuffling procedure was performed 200 times to estimate the gridness threshold. Grid cells were classified based on their gridness score in the square.

Identifying grid cells

Gridness was obtained as in Hafting et al[3]. We calculated the spatial autocorrelogram as

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

where $r\left(\tau_x,\tau_y\right)$ is the autocorrelation between bins with spatial offset τ_x and τ_y. f is the firing rate map of the cell with no smoothing applied; *n* is the number of overlapping bins in two offset copies of the map. The autocorrelogram was smoothed using a two-dimensional Gaussian kernel of 5 bins with standard deviation equal to 2 bins. The six local peaks of the autocorrelogram were defined as the six local maxima with $r>0$ closest to the central peak (excluding the central peak itself). Gridness was calculated by defining a mask on the spatial autocorrelogram centred on the central peak but excluding the peak itself bounded by a circle defined by the mean distance from the centre to the closest peaks multiplied by 2.5. This area was rotated in 30 degrees increments up to 150 degrees and for each rotation the Pearson product-moment correlation coefficient was calculated against the un-rotated mask. Gridness is then calculated taking the difference between the minimum correlation coefficient for rotations of 60 and 120 degrees and the maximum correlation coefficient for rotations of 30, 90 and 150 degrees.

The field size and orientation of the grid cells

The local spatial autocorrelogram was used to estimate grid cell orientations and the average field size. The local spatial autocorrelogram is defined as a central part of spatial autocorrelogram which includes six nearest peaks around the central peak. The orientations of all the six peaks were estimated as the angles between the

horizontal central axis and the line traversing the central peak and a surrounding peak (six orientations for all the six surrounding peaks) in an anti-clockwise direction.

The average field size was calculated using the diameter of the central field. The area of the central field was estimated as a sum of all the conjunctive bins within the centre fields after 20% threshold was applied.

Grid cell ellipticity

Grid ellipticity in trapezoids and squares was assessed by fitting an ellipse to the six central peaks of the local spatial autocorrelogram using a least squares method. Eccentricity *e* was used as a measure of ellipticity (with 0 indicating a perfect circle)[4,5]:

$$e = \sqrt{1 - \frac{b^2}{a^2}} \quad (3)$$

where *b* and *a* are the lengths of the smaller and longer axes of the ellipse respectively.

Grid cell orientation clustering in square enclosures

We identified all the grid cells recorded in each animal in a familiar square enclosure. When the same grid cell was recorded on more than one trial, the data from only one trial, chosen at random, was used. Eighteen animals had two different grid modules and one animal had three different grid modules. The average orientations from each module were calculated and displayed (Fig. 1d). We estimated the average deviation of grid orientation from the closest wall (vertical or horizontal) for each module (Fig. 1e).

We tested the degree of grid orientation clustering in the square by comparing the values of normalised Fourier power of the circular autocorrelogram of measured orientation distribution (Fig. 1d) vs. the distribution where the grid orientations were rotated by a random amounts. Circular autocorrelogram was calculated with the bin size of 2° (Extended Data Fig. 3a). Only half of autocorrelogram was included for Fourier analysis (i.e. from 0° to 180°) as the other half is symmetrical. The autocorrelogram was zero-padded from 90 bins to 2^{13} bins to enhance the resolution

of the one-dimensional Fourier spectrogram. A large peak was observed at 3Hz indicating clustering at 60° (Extended Data Fig. 3c). To test the possibility that the observed clustering could occur by chance, ten thousand shuffle sets were produced. In each shuffle a random direction was generated for each module and added to all three directions of the respective module to preserve the relative orientations. In each instance the one-dimensional Fourier spectrogram was calculated under the same condition as for the unshuffled data. The ratio of power within peak at 3±1Hz and total power (from 0 to 45 Hz) was calculated (called the normalized Fourier power) and compared with the same measure for unshuffled data. The hypothesis that clustering at 60° could be observed by chance was rejected with p=0.0438 (Extended Data Fig. 3b).

We also assessed how significant grid orientation clustering is in squares by comparing it against orientation clustering in a circle for the same 7 animals recorded in squares (12 modules) and circles (11 modules). If the geometry of the environment affects grid orientation, the clustering of orientations should be more variable in circles compared to squares. To test for clustering of orientations we found the distribution of three orientations modulo 60^o (the other three are symmetrical to the former ones) and compared whether standard deviations of these distributions were significantly different in squares vs. circles using a two-sample t-test.

Comparing behavioural biases in square vs. circular enclosures

We examined whether differences in grid cell firing patterns in squares vs. circles could be explained by systematic differences in directional and velocity profiles. To identify biases in the directional (or velocity) sampling of the environment we introduce a circularity score which is equal to the sum of squared distances between the measured sampling distribution and a perfect circle with radius equal to the mean of the sampling distribution.

$$m = \sum_i^n (r_i - \bar{r})^2 \qquad (4)$$

Here m is the circularity score, r_i is a mean value in a bin i, $\bar{r}$ is mean value and n is number of bins. In order to compare directional sampling between trials with different duration, m was normalized by n^2.

We calculated circularity scores for all trials with grid cells included in the current analysis (Extended Data Fig. 4). The average score was calculated for each module. A two-sample t-test was used to compare the degree of circularity.

Defining grid cell modules

A grid cell module was defined as a group of anatomically neighbouring grid cells which had similar scale and orientation[4].

Testing whether relative orientations between different grid modules are preserved across different testing conditions

We looked at 18 grid cells (3 rats, 6 modules in each rat) recorded in up to three different environments (Fig. 2c-e; Extended Data Fig. 5). The relative orientation is defined as the mean orientation difference between closest components of two simultaneously recorded modules. We asked if these modules kept their relative orientation across different testing arenas, i.e. the difference between the corresponding relative orientations should be close to zero. To test this we calculated the mean difference between the relative grid orientations in the square and in another testing environment (a circle or a hexagon; Supplementary Table 1) and estimated how likely it is to get such a mean difference by chance applying the Binomial test (the range of available differences is 0°-30°).

Non-homogeneity of grid cell firing pattern in trapezoids

We examined how homogeneous the grid cell firing pattern is in trapezoids vs. squares. To measure homogeneity we divided the enclosures into two parts (left and right) with equal areas (in the case of the trapezoid its horizontal axis is divided into segments 64% and 36% of the whole). We calculated local spatial autocorrelograms for the left and right parts of the trapezoid and square separately. To evaluate the degree of similarity between the left and the right parts of the environment (square or trapezoid) we calculated the Pearson product-moment correlation coefficient between them. If the sizes of local spatial autocorrelograms were different between the different

parts, the larger was cropped to the size of the smaller one. We also calculated the gridness score for each of them.

The orientation and scale on the left and right sides of the trapezoidal and square enclosures were evaluated from the local spatial autocorrelogram of each side (see *'The field size and orientation of the grid cells'* for details). The components closest to the horizontal axis were considered the first grid cell component, the second component was that at the closest angle in anti-clockwise direction followed by the third (Extended Data Fig. 6).

Can anisotropy of grid cell firing pattern in the trapezoidal enclosure result from inadequate behavioural sampling of the space on its left (narrower) part?

To examine whether the changes in grid cell properties on the left part of the trapezoid resulted from inadequate behavioural sampling, we generated a perfect homogeneous grid pattern in a square which had the same wavelength and orientation as grid cells recorded in the square environment as estimated from the six peaks of the local spatial autocorrelogram (Extended Data Fig. 7). We then transposed this idealised pattern onto the trapezoidal shape and measured the similarity and gridness of the left and right parts of the trapezoid and found no difference in gridness, demonstrating that the differences seen in the real data are not due to inadequate sampling on the left side of the trapezoid. We also found the orientations and wavelengths of the main three grid components of simulated grid patterns in the left and right sides of a trapezoid and a square and showed that they had much smaller differences than observed in our data and that they were not different across different directions.

Change in symmetry cannot be simply predicted by morphing the pattern from a square to a trapezoid

We tested whether the observed change in symmetry of grids recorded in a trapezoidal enclosure can be predicted by the simple morphing of the pattern from a square to a trapezoid (Extended Data Fig. 9). To estimate how grid pattern in a square would appear in a trapezoid we expanded the initial grid pattern in the square into a larger

square with the length equal to the long length of the trapezoid. Then we rescaled the pattern along the horizontal trapezoid axis to match the size of the original trapezoid (similar to[5]). The correlation coefficients between the predicted vs. recorded grid patterns were used to estimate how well our transformation matched the experimentally measured pattern. The distribution is not significantly different from the normal distribution with a mean equal to zero ($p=0.74$, $t=-0.33$, $df=36$, one-sample t-test).

Comparing behavioural biases in the left part of the trapezoid vs. the right

We used the same analysis as in '*Comparing behavioural biases in square vs. circular enclosures*' for comparing the sampling bias on the left side of the trapezoid with that on the right. We divided the trapezoid into two equal parts (area of half-trapezoid $0.51m^2$) and calculated the directional and velocity circularity scores for each part. A two-sample t-test revealed that on the whole both directional and velocity sampling distributions were different between the two parts (Extended Data Fig. 4). We then measured the correlation between the degree of similarity of the local grid pattern and the difference in sampling biases on the left and the right sides (Extended Data Fig. 4e-f). We showed that there is no significant correlation between these measures suggesting that observed grid non-homogeneity in the trapezoid did not result from the difference in directional or velocity sampling distributions.

References

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Table 1. The main grid cell properties in hexagonal, square and circular enclosures.

Rat No	Grid cell No	Grid Module No	Environment	Grid scale, cm	Grid orientation axis1, deg	Grid orientation axis2, deg	Grid orientation axis3, deg	Relative grid orientation between module1 and module2
2067	1	1	Hexagon	60	46.6	110.9	170.9	
2067	2	2	Hexagon	42	16.4	76.8	132.3	25.7
2067	3	1	Square	55	36.4	95.7	161.6	
2067	4	2	Square	35	11.3	53.1	121.0	23.9
2067	5	1	Circle	77	13.3	-	-	
2067	6	2	Circle	58	45.0	106.9	159.1	
2067	7	2	Circle	54	43.0	104.6	163.3	30.7
2121	8	1	Square	24	58.0	119.1	174.3	
2121	9	1	Square	24	63.4	114.0	174.3	
2121	10	2	Square	38	17.2	70.5	135.0	
2121	11	2	Square	38	14.9	75.1	132.3	17.0
2121	12	1	Circle	37	13.6	76.4	135.0	
2121	13	2	Circle	48	20.6	90.0	155.8	13.8
2183	14	1	Square	48	17.5	78.1	139.4	
2183	15	1	Square	54	23.2	81.5	146.3	
2183	16	2	Square	34	18.4	81.3	137.7	1.9
2183	14	1	Hexagon	59	40.4	108.4	180.0	
2183	16	2	Hexagon	33	48.0	104.0	167.0	3.3
2183	14	1	Circle	47	70.6	116.6	167.5	
2183	17	1	Circle	40	60.3	-	-	
2183	18	2	Circle	31	61.4	122.0	175.2	-3.0