A Code for Spatial Alternation During Fixation in Rat Hippocampal CA1 Neurons

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A code for spatial alternation during fixation in rat hippocampal CA1 neurons. J Neurophysiol 102: 556–567, 2009. First published May 6, 2009; doi:10.1152/jn.91159.2008. The classical notion of hippocampal CA1 “place cells,” whose activity tracks physical locations, has undergone substantial revision in recent years. Here, we provide further evidence of an abstract spatial code in hippocampal CA1, which relies on memory and adds complexity to the basic “place cell.” Using a nose-poking paradigm with four male Wistar rats, we specifically concentrated on activity during fixation, when the rat was immobile and waiting for the next task event in a memory-guided spatial alternation task. The rat had to alternate between choosing the right and left holes on a trial-by-trial basis, without any sensory cue, and relying on an internal representation of the sequence of trials. Twelve tetrodes were chronically implanted for single-unit recording in the right CA1 of each rat. We focus on 76 single neurons that showed significant activation during the fixation period compared with baseline activity between trials. Among these 76 fixation neurons, we observed 38 neurons that systematically changed their fixation activity as a function of the alternation sequence. That is, even though these rats were immobile during the fixation period, the neurons fired differently for trials in which the next spatial choice should be left (i.e., RIGHT-TO-LEFT trials) compared with trials in which the next spatial choice should be right (i.e., LEFT-TO-RIGHT trials), or vice versa. Our results imply that these neurons maintain a sequential code of the required spatial response during the alternation task and thus provide abstract information, derived from memory, that can be used for efficient navigation.

Introduction

It has long been established that the hippocampal memory system plays a crucial role in the organization and maintenance of episodic information in spatially defined contexts (Eichenbaum 2000). Researchers have focused on the function of “place cells” in the hippocampus, whose activity levels track locations and signal physical changes in the spatial environment (Anderson and Jeffery 2003; Gothard et al. 1996; Lee et al. 2004; Leutgeb et al. 2004; O’Keefe and Dostrovsky 1971; O’Keefe and Nadel 1978). Other factors, such as reinforcement and task demands, however, can also strongly influence the firing rates of these cells (Bower et al. 2005; Dayawansa et al. 2006; Ferbinteanu and Shapiro 2003; Frank et al. 2000; Fyhn et al. 2002; Griffin et al. 2007; Hölscher et al. 2004; Kobayashi et al. 1997; Lee et al. 2006; Markus et al. 1995; Wood et al. 2000). Here, we report neuronal activity that further highlights the sophistication of hippocampal spatial coding during well-learned navigation in a behavioral paradigm with stringent control of the animal’s spatial position.

Using a nose-poking paradigm with rats, we designed a memory-guided (delayed) spatial alternation task in which the animal could not rely on any sensory cue to discriminate the appropriate response direction. We were specifically interested in neuronal activity during the fixation period, when the rat was required to maintain its nose in the center hole while it waited for the next task event. At this time, the spatial position of the rat was effectively controlled. Differential activity during this fixation period as a function of the alternation sequence would represent strong evidence of an abstract memory-based spatial representation, which cannot be reduced to any physical property of the environment.

The existence of such differential activity was anticipated on the basis of recent work, showing that hippocampal lesions impair performance in a delayed alternation task, but not in a continuous alternation task (Ainge et al. 2007b). This finding implies that hippocampal involvement is specifically required when information must be maintained or kept accessible for retrieval from memory. Accordingly, we expected to find selective hippocampal activity when the rat was immobile and waiting for the next event in a behavioral paradigm with delay.

Several studies have described sequence-dependent hippocampal activity during delay periods. However, these studies relied on maze paradigms in which the rats were able to move around in a resting zone (Ainge et al. 2007b; Smith and Mizumori 2006). Such a maze paradigm may not be suited to establish the existence of an abstract, sequence-dependent spatial code that cannot be reduced to body position or any physical change in the environment. Instead, we used a nose-poking paradigm with a delayed spatial alternation task. Using a sustained nose-poking response for fixation, we achieve a well-controlled immobile condition, in which the rat is at the same location and unable to move, two factors whose variation is known to have a strong influence on hippocampal activity. Eliminating these factors, we are able to record an abstract, sequence-dependent spatial code.

Methods

Animals

The subjects were four male Wistar/ST rats (SLC, Hamamatsu, Japan) that weighed 280–420 g and were 4 to 6 mo old at the beginning of training. They were housed individually with a 14/10-h light/dark cycle. Lights were on from 7:00 a.m. to 9:00 p.m. All

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trappings and recordings were conducted during the light phase of the cycle. We fed the subjects individually 1 to 3 h after their daily sessions to maintain their body weights at >80% of their free-feeding weights. Water was freely available in their home cages. All procedures were in accordance with the National Institutes of Health guidelines for animal care and approved by the Tamagawa University Animal Care and Use Committee.

Apparatus

Behavioral training and neuronal recording were conducted in an experimental chamber constructed of Plexiglas with black wall papers to reduce the scattered reflection of lights on the headstage for position tracking (40 × 40 × 40 cm; Takahashi et al. 2002, 2007, 2009). The experimental chamber was fitted within a sound-attenuating box and illuminated by a 15-W light bulb as a house light. Two nose-poke holes were located 10 cm right and left from the center on the front wall of the chamber. The third was located on the center of the rear wall. Each nose-poke hole was 2 cm in diameter, 2 cm deep, and 4 cm above the floor. Light-emitting diodes (LEDs) at the rear of each hole were used as visual cues. Horizontal infrared photo-beam detectors in the hole were used to record the nose pokes. A food dispenser (PD-25D; O’Hara & Co., Tokyo) delivered a 25-mg food pellet (O’Hara & Co.) to a receptacle that was located 4 cm above the floor and at the middle of the front wall. A 0.5-s buzzer sound was presented at the time of reinforcer delivery. A charge-coupled device (CCD) camera was mounted on the ceiling of the sound-attenuating box for monitoring and position tracking. All events were controlled by customized software developed with Microsoft Visual C++ 6.0 on a Windows-based personal computer.

Behavioral task

The delayed spatial alternation task constituted the following sequence of events (see Fig. 1). A trial started when only the center hole light was illuminated. The rat was then required to make a nose-poking response, which had to be sustained for 1 s in the central hole (i.e., the fixation period). After 1-s sustained fixation, the central light was extinguished and after a delay of 1.5 s the right and left lights were simultaneously illuminated on the front wall. The rat had to alternate between choosing the right or left hole on a trial-by-trial basis and was rewarded with a 25-mg food pellet for each correct choice. If the rat made an erroneous response, no reward was given and the same trial repeated (i.e., a correction trial). The correct direction of the first trial of each session was determined randomly by the experimenter. The intertrial interval was 10 s. A training and recording session consisted of a maximum of 200 trials, not including correction trials, or until 60 min had elapsed. The training was completed when the rat could perform three consecutive sessions with an accuracy rate of >80% and a total of ≥100 correct trials.

Electrodes and surgery

After the completion of behavioral training, each rat was anesthetized with a mixture of ketamine (100 mg/kg, administered intraperitoneally [i.p]) and xylazine (7 mg/kg, ip) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Additional intramuscular injections of ketamine were given to maintain anesthesia. Twelve tetrodes made from four polyimide-insulated 12.7-μm nichrome twisted wires (Redlohm-800; Kenthal, Palm Coast, FL) and two reference wires were loaded into a 14-drive microdrive (Neuro-hyperdrive; David Kopf Instruments) that allowed independent vertical movement of each drive (Wilson and McNaughton 1993). Each tip of electrode wire was cut with sharp surgical scissors and gold plated to reduce its impedance to 300 kΩ at 1 kHz for multitunit recording. A small oval craniotomy was performed on the right parietal bone and the dura was removed from the exposed area. The hyperdrive was then secured in place with dental acrylic supported by eight anchor screws and a ground screw. The center of the electrode bundle was positioned at coordinates 3.6 mm posterior to bregma and 2.2 mm lateral to midline for the dorsal CA1 recording in accordance with the brain atlas by Paxinos and Watson (2004). Immediately after surgery, all electrodes were advanced about 1 mm. Rats were allowed ≥5 days to recover from surgery before resuming behavioral experiments.

Data collection

The neuronal data and the behavioral events were collected with the Cheetah 160 Data Acquisition System (Neuralynx, Bozeman, MT). The hyperdrive was connected to a headstage with 54 unity-gain preamplifiers and 10 color LEDs for position tracking (HS-54; Neuralynx). For spike recording, tetrode channels were band-pass filtered (600–6,000 Hz), differentially amplified (×2,000–5,000), and digitized at 32 kHz. When the voltage on any of the four channels of a single tetrode exceeded a threshold set by the experimenter, a 1-ms window of the spike waveform on each of the four channels on the tetrode was recorded to disk and time-stamped with microsecond resolution. For local field potential (LFP) recording, the most prominent channel of each tetrode was picked out, band-pass filtered (1 to 475 Hz), differentially amplified (×2,000), digitized at 2 kHz, and stored to disk. For position and head angle tracking, the position of the 10 LEDs on the headstage was detected by a CCD camera placed directly above the experimental chamber. The individual LED points and their median were calculated and recorded to disk at 60 Hz. The spatial sampling resolution was such that a pixel was approximately equivalent to 1 mm.

Each tetrode was advanced through the parietal cortex toward the hippocampus from 80 to 320 μm per day while monitoring the unit activity with an audio amplifier when the animal was located on an adjusting table (30 cm in diameter, 1 m above the floor) and was either asleep or quietly resting. The reference electrodes were positioned in corpus callosum or silent white matter. As the cell body layer of CA1 was approached, 125- to 250-Hz oscillations (i.e., ripples; Buzsáki et al. 1992; Suzuki and Smith 1988) could be observed. The tetrodes were then gradually advanced into the cell body layer of CA1 until multiple single units were detected. The rat was then returned to its home cage. When the neuronal activity was still present after 2 h, it was judged to be stable and suitable for recording (Sakurai 1994, 1996).

At this time the recording session was conducted. Note that because units were extracellularly recorded, the same neurons may in principle have generated different waveforms over days and the movement of other
tetrodyes may have affected the relative location of cells around the tetrode. For these reasons, it has been suggested that the precise number of recorded neurons cannot be definitively known (Ferbertante and Shapiro 2003; Kneiferim 2002). However, to prevent double counting of activity, we advanced each tetrode for a further approximately 40 μm before returning the rat to its home cage after a recording session.

Data analysis

SPIKE-SORTING METHODS. Our database included neurons from which activity was recorded during sessions of ≥100 trials at 80% accuracy. Spikes were clustered off-line into putative cells on the basis of their waveform properties using ML Clust 3.4 (Redish et al.: http://redishlab.neuroscience.umn.edu/MLClust/MLClust.html) with automatic preclustering using KlustaKwik 1.7 (Harris: http://klustakwik.sourceforge.net). The first to third principal components and the energy (i.e., sum of square values for each sampling points of the 1-ms waveform; Schmitzer-Torbert and Redish 2004) of their waveforms were used as the waveform features for spike sorting. To judge whether the calculated cluster actually consisted of only one neuron, we performed autocorrelation analysis for each cluster at 1-ms bin size to check the refractory period. If the autocorrelogram had no spike form, we calculated cluster actually consisted of only one neuron, we performed autocorrelation analysis for each cluster at 1-ms bin size to check the refractory period. If the autocorrelogram had no spike form, we regarded it a well-separated cluster and used it for further analyses.

A neuron was identified as a pyramidal cell if its waveform had a width of ≥270 μm (Smith and Mizumori 2006), if it showed a bimodal interspike interval (ISI) distribution that reflected complex spike bursting (Bower et al. 2005) and if it had a low mean firing frequency over the entire recording session (<2 Hz).

UNIT CATEGORIZATION. We defined three task periods for data analysis: prefixation (1 s before the onset of fixation), fixation (1 s during fixation), and postfixation (1 s after the offset of fixation). We divided recorded trials into two sequences: RIGHT-TO-LEFT trials in which the next spatial choice should be left and LEFT-TO-RIGHT trials in which the next spatial choice should be right. Excluded from analyses were data from error trials, correction trials, and trials in which the rat made premature fixation breaks or nose-poking responses before the trial start.

Neuronal activity was considered to be fixation related (i.e., fixation unit) if the firing frequency during the fixation period differed significantly from that of the session average (Wilcoxon signed-rank test; α = 0.05). Fixation-related neuronal activity was judged to be sequence dependent if the firing frequency during the fixation period showed a statistically significant difference between RIGHT-TO-LEFT and LEFT-TO-RIGHT trials (Mann-Whitney U test; α = 0.05).

Firing characteristics of fixation units were categorized into three types using the following indices

\[ I_{pre} = \frac{fr(PRE)}{fr(PRE) + fr(FIX)} \]

\[ I_{post} = \frac{fr(POST)}{fr(POST) + fr(FIX)} \]

where \( fr(X) \) denotes a firing rate during period \( X \), and PRE, FIX, and POST denote the prefixation period, the fixation period, and the postfixation period, respectively. For instance, \( I_{pre} > 0.5 \) indicates that the firing rate was larger during the prefixation period than during that of the fixation period. Three subtypes of fixation units were defined as follows: “Fixation Only” for fixation units whose \( I_{pre} \) and \( I_{post} \) were <0.5; “Fixation and Before” for fixation units whose \( I_{pre} \) was >0.5 but \( I_{post} < 0.5 \); and “Fixation, Before, and After” for fixation units whose \( I_{pre} \) and \( I_{post} \) were >0.5.

POPULATION HISTOGRAM AND FIXING TREND ANALYSIS. To examine activity at the population level for the hippocampal sequence-dependent fixation units during the fixation period, we made population histograms for every three subtypes. Spike data were sorted into a preferred versus nonpreferred sequence for each neuron (i.e., “preferred” sequence referring to the sequence that elicited the highest firing rate). Since the peak firing frequencies of the hippocampal pyramidal cells differed widely, the raw population histograms tended to mainly reflect the characteristics of a few high-frequency units. Therefore the spike count of each 0.1-s bin was normalized relative to the total firing rate during the fixation period. Because we focused on the differences in firing rates during the immobile fixation period, each bin of 0.1-s normalized firing rate was statistically compared between preferred and nonpreferred sequences using a t-test (two-tailed; α = 0.05) to check where the sequence dependence existed during fixation.

At the individual cell level, firing rates for the first (0 to 0.5 s) versus the second (0.5 to 1 s) half of the fixation period were compared to determine the firing tendency during the fixation period (two-tailed paired t-test, \( P < 0.05 \)) for each sequence-dependent fixation unit using the following index

\[ FI = \frac{fr(LATE) - fr(EARLY)}{fr(LATE) + fr(EARLY)} \]

where \( fr(X) \) denotes the firing rate during period \( X \), either \( EARLY \) or \( LATE \) referring to the first (0 to 0.5 s) and second (0.5 to 1 s) half of the fixation period, respectively.

HEAD ANGLE ANALYSIS. The head angle of the rat was extracted from the video tracking data sampled at 60 Hz (Neuralynx). The mean head angle was calculated for the 1-s fixation period in each trial. The zero degree was taken to be the y-axis direction of the CCD camera; this was the direction of vertical access to the center hole for the rat. The mean head angles in RIGHT-TO-LEFT versus LEFT-TO-RIGHT trials were statistically compared by a two-tailed paired t-test.

To investigate the relationship between the firing rate and the head angle on a trial-by-trial basis, we performed linear regression analysis

\[ (\text{Firing rate}) = a_0 + a_1 \times (\text{Head angle during fixation}) \]

The analysis was performed both at the population level and at the level of each individual unit. In the population analysis, all head angle data were recalculated on the basis of the mean head angle in the nonpreferred sequence trials and the direction was set so that the mean head angle in the preferred sequence trials should be positive.

LIA ANALYSIS. It is known that the hippocampal LFP shows large-amplitude irregular activity (LIA; Vanderwolf 1969) that contains the hippocampal sharp waves and ripples while the animal is quiet and immobile (Buzsáki et al. 1983, 1992). To examine whether such LIA occurred during the fixation period of the current paradigm, the fast oscillatory events were extracted using a procedure similar to that proposed by Csicsvari et al. (1999). For the extraction of fast oscillatory events, the original recorded data were digitally band-pass filtered (100–250 Hz). The power (i.e., root mean square) of the filtered signal was calculated by summing squared sample values within a 16-ms time window and calculating the square root of this sum. The mean and SD of the power signal were then calculated from all data collected within the session to calculate the z-score of the filtered LFP power and determine the detection threshold. Ripples were identified as epochs in which the z-score of the filtered LFP power increases to >7.

THETA-BAND LFP ANALYSIS. It has been proposed that there are two types of hippocampal theta rhythmic activity, identified as type 1 and type 2 by Kramis et al. (1975). Type 1 theta is that which generally accompanies motor movement such as walking, jumping, or rearing. It shows a relatively high frequency oscillation (8–12 Hz) and is resistant to the effects of centrally administered atropine. Type 2 theta is more rarely observed in the rat and occurs in behavioral immobility
or in an alert state. It shows relatively slower frequency oscillation (4–8 Hz) and is sensitive to atropine (Bland and Oddie 2001; Kramis et al. 1975). To confirm that we would obtain a reduction of type 1 theta and an increase of type 2 theta during fixation in our paradigm, the power of each type of theta-band activity was calculated and compared among three 1-s periods around fixation using the following procedure. LFP data that showed LIA during consummation (i.e., a period of 5 to 10 s following reward delivery) were considered to derive from hippocampal CA1 and selected for further analysis. Power spectral density (PSD) analysis was performed on the LFP database using the multitaper estimates method.

Since the overall signal amplitude can vary across recordings and animals due to factors such as electrode impedance and location, the data from each session were normalized so that the integral of the PSD over the range of 2–50 Hz was equal to one. This allowed us to make comparisons across sessions and animals (Russell et al. 2006). The normalized average power (i.e., the integral of the PSD over the target range divided by that of 2–50 Hz) of the type 1 theta band (8–12 Hz) and type 2 theta band (4–8 Hz) were calculated for the prefixation, fixation, and postfixation periods, respectively, and compared for each band separately, using one-way repeated-measures ANOVA, followed by Tukey’s honestly significant difference (HSD) for post hoc contrasts.

Firing Rhythm Analysis by Autocorrelograms. To understand the rhythmic firing of fixation units during the fixation period, autocorrelation analyses were performed for each fixation unit with a range of −500 to 500 ms and 10-ms bin width (Tabuchi et al. 2000). Statistical significance of the rhythmic firing was tested by comparison of the 99.5% confidence limits that were calculated using Neuro-explorer (Nex Technologies, Littleton, MA), with the assumption that the original spike train has a Poisson distribution. If there was a significant peak in the range of 4–12 Hz, the neuron was considered to have a theta-band rhythm in its firing during the fixation period (i.e., theta-rhythmic unit). To examine population-level properties of the theta-rhythmic and non-theta-rhythmic units, PSD analysis was performed for all four groups to check the firing power during the fixation period. The spike train was regarded as a sequence of binary (0 or 1) values and the calculation method for PSD estimates was similar to that for the LFP analysis described earlier. We also examined the peak frequency in each autocorrelogram and included the value in the probability distribution to examine the peak frequency locations. Many of the hippocampal pyramidal cells tend to show a complex probability distribution to examine the peak frequency locations. The population histogram shows a downward trend in the activity of this type of neuron. Figure 2B shows the trajectory and firing locations (colored dot) of 10 example trials used for the raster and histogram in Fig. 2A during the prefixation, fixation, and postfixation periods. The rat showed very similar trajectories across trials and the spikes occurred only around the center hole.

Note that the population histogram suggests a downward trend in the activity during fixation: from high activity at the beginning of the fixation period to lower activity at the end of the fixation period. The difference between the preferred versus nonpreferred type of trials, however, persisted throughout the fixation period. We obtained statistically significant differences between the preferred versus nonpreferred sequences in the normalized population data for every 0.1-s bin during the fixation period (Fig. 3, *P* < 0.01 for the 0- to 0.1-s and 0.9- to 1.0-s bins; the others are *P* < 0.001; two-tailed *t*-test).

Fixation and before

Twenty-two of 76 fixation units (28.9%) showed a significant increase of activity during the fixation period as well as during the 1-s prefixation period; 7 of these 22 units (31.8%) exhibited sequence-dependent activity. Six of these 7 sequence-dependent units preferred RIGHT-TO-LEFT trials, whereas the remaining one preferred LEFT-TO-RIGHT trials. Figures 4 and 5 show an example as well as a population histogram for this type of neuron in the same format as Figs. 2 and 3, respectively. Again the population histogram shows a downward trend in the activity during fixation. In this type of neuron, there also occurs an activity peak right before the fixation period. The population histogram further appears to show a smaller (nonsignificant) peak at the end of the fixation period. Statistical significance can be observed during 0- to 0.3-, 0.4- to 0.7-, and 0.8- to 0.9-s bins in the fixation period (*P* < 0.05).

Fixation, before and after

Five of 76 fixation units (6.6%) showed a significant increase of activity during the fixation period as well as during the 1-s prefixation period and during the 1-s postfixation period; 3 of

**RESULTS**

**Database**

We recorded a total of 248 hippocampal CA1 pyramidal neurons from four rats during 18 recording sessions, performing an average (±SD) of 130.4 ± 26.0 trials per session at a correct rate of 90.2 ± 4.9%. The mean (±SD) spike width of these neurons was 347.7 ± 42.6 μs and the mean (±SD) firing frequency throughout the session was 0.75 ± 0.55 Hz. We focused on neuronal activity during the fixation period and obtained a total of 76 fixation-related units (15 of 61 units from rat 1; 24 of 74 units from rat 2; 28 of 73 units from rat 3; 9 of 40 units from rat 4). We found that 38 of the 76 fixation units (50%) exhibited a sequence-dependent firing rate during the fixation period, preferring either RIGHT-TO-LEFT or LEFT-TO-RIGHT trials. All fixation units were further classified into three types according to additional firing properties around the fixation period (see METHODS).
these 5 (60%) exhibited sequence-dependent activity. Interestingly, all 3 of these sequence-dependent units preferred RIGHT-TO-LEFT trials. Figures 6 and 7 show an example as well as a population histogram for this type of neuron. As with the previous two types of neuron, the population histogram shows a downward trend in the activity during fixation, but in these fixation neurons with significant pre- and postfixation activity, there also appeared a conspicuous sequence-dependent peak immediately after the fixation period. In the activity peak at the beginning of the fixation period, on the other hand, there was no significant difference as a function of sequence. At the population level, with a sample of only 3 neurons for this type, we could not find any statistically significant difference as a function of sequence during the fixation period.

Head angle analysis

The head angles in RIGHT-TO-LEFT trials versus LEFT-TO-RIGHT trials showed a slight, but statistically significant difference ($P < 0.05$), with means and SDs of, respectively, $10.5 \pm 6.4$ and $-8.3 \pm 9.6^\circ$. To examine whether firing rate differences in the two trial types should be attributed to differences in head orientation, we performed linear regression analysis. In the population analysis for the “Fixation Only” units (see Fig. 8, top panels), the gradient of the fitted line ($a_1$) showed slight tilts for both sequences (0.00 for preferred sequences and

FIG. 2. Raster displays, firing rate histograms, and movement trajectories from an example neuron for sequence-dependent “Fixation Only” neurons. A: the neuronal activities around the fixation period ($n = 10$). The activity was aligned by the onset of the fixation. The yellow area indicates the fixation period. The top panels show an example of raster displays and firing histograms from a single neuron’s activity during the RIGHT-TO-LEFT trials; those below represent those during the LEFT-TO-RIGHT trials. B: the movement trajectories of the same example activity. Colored dots indicate the firing location of the neuron. C: the result of off-line cluster analysis. Each dot represents one neuronal spike. Three encircled clusters were recognized. The data used for this figure are colored red. D: superimposed waveforms recorded from 4 electrode wires (one tetrode). The thick colored line indicates the average waveform. E: an autocorrelogram for the current example neuron. A clear refractory period (<2 ms) indicates the activity was properly separated.

FIG. 3. Population histograms from sequence-dependent “Fixation Only” neurons. The data were normalized by the total firing during the fixation period. The yellow area indicates the fixation period. Neuronal activity in the preferred sequence is shown in blue and that in the nonpreferred sequence is shown in magenta. The bin width was 100 ms. The shaded area indicates SE ($n = 28$).

FIG. 4. Raster displays, firing histograms, and the movement trajectories from an example neuron for “Fixation and Before” neurons. All parameters and symbols are as in Fig. 2 except for the color of the target cluster, which is blue in D.
0.16 for nonpreferred sequences). However, both regression coefficients were very small ($R^2 < 0.0001$ for preferred sequences and $R^2 < 0.03$ for nonpreferred sequences), indicating that head angle variance cannot account for the firing rate during fixation. This trend applies to each sequence-dependent “Fixation Only” unit as confirmed by the individual analyses.

Analyses for the other two types of sequence-dependent units yielded similar results, discounting a role of head orientation in the neural activity. For the “Fixation and Before” units (see Fig. 8, bottom panels), $a_1$ and $R^2$ for preferred sequences were $-0.06$ and $<0.02$ respectively, whereas those for nonpreferred sequences were $0.01$ and $<0.003$ at the population level. At the individual level, the mean ($\pm$SD) $R^2$ for preferred sequences was $0.15 \pm 0.11$ and $0.01 \pm 0.01$ for nonpreferred sequences. Thus it appears that the head angle variation during the fixation period does not account for differences in the neural activity for any of the sequence-dependent units.

**Downward trend during fixation**

Every population histogram of the sequence-dependent fixation units exhibited a downward trend, especially in their preferred sequence during fixation, even though the rats stayed in place during this time. To examine the tendency at the single-neuron level, the firing rate in the first half and second half of each trial was statistically evaluated with a paired $t$-test ($P < 0.05$), separately for the preferred and nonpreferred sequence (see Fig. 9).

For the preferred sequence, 26 of 38 sequence-dependent fixation units (68.4%) exhibited a downward trend, that is, the
firing rate in the first half was significantly higher than that in the second half. In contrast, only 2 of these 38 units (5.3%) exhibited a climbing trend with a firing rate that was significantly higher in the second half than that in the first half. The activity of the remaining 10 units (26.3%) showed no difference between the two subperiods ($P > 0.05$). On the other hand, for the nonpreferred sequence, 13 of 38 units (34.2%) exhibited a downward trend, one unit (2.6%) exhibited a climbing trend, and 24 (63.2%) were neutral.

**LFP analysis**

The hippocampus is known to show three types of activity, depending on the behavioral state of the animal, each of which shows distinctive LFP: active exploration with type 1 theta; immobility while alert, with type 2 theta; and consummation, with LIA. The three types are thought to be governed by different molecular mechanisms such as acetylcholine, γ-aminobutyric acid, and so forth. To understand which type corresponds to the current target activity during the fixation period, we conducted hippocampal LFP analyses.

An example of behavioral and LFP traces from one representative trial is shown in Fig. 10. Figure 10A shows LFP traces of raw (1–475 Hz), theta-band (2–20 Hz), and LIA-band (100–250 Hz) signals around the fixation period. Figure 10B shows the movement trajectory of the animal. The shades of gray, in accordance with the scale provided at the bottom of Fig. 10E, provide a visual impression of the movement in time (from darker to lighter shades). Figure 10C shows the animal’s movement velocity. Figure 10D shows the LFP power in the LIA band. There is no evidence of any LIA during the fixation period, although the movement is completely stopped. Figure 10E shows the spectrogram in the lower-frequency band including the theta band, which clearly indicates the presence of theta-band activity during moving periods, that is, 1 s before and after the fixation period, as well as a reduction of theta-band activity during the fixation period. These tendencies are further analyzed in the following sections.

**LIA analysis**

To examine whether the fixation-specific activity was due to LIA, LFP data during fixation were investigated using a criterion similar to that proposed by Csicsvari et al. (1999; see METHODS). We did not obtain any LIA during the fixation period in any of the recording sessions, indicating that the fixation-specific firing in the current paradigm was not contingent on LIA, which tends to emerge as a result of coincidental spike bursts among large groups of pyramidal cells. Eight example traces of the filtered LFP and their LIA powers are shown in the left panels of Fig. 11. For comparison, the filtered LFP examples and their LIA powers are shown during consummation in the right panels of Fig. 11 (i.e., for a period of 10 s after reward delivery). At this time, LIAs (with $>7$ z-score activity) could be observed 1 to 2 s after the rat had stopped exploration.

**Theta-band LFP analysis**

The electrode wires from which we could obtain LIA during consummation were likely positioned in the hippocampus. We selected the data from these wires for theta-band analyses ($n = 173$). Example traces from one session are shown in Fig. 12A. A reduction of amplitude and relative desynchronization could be observed in every example trace during the fixation period. To understand these trends systematically, we calculated the normalized PSDs for the prefixation (PRE), fixation (FIX), and postfixation (POST) periods. An example of the PSD distributions calculated from the data shown in Fig. 12A is depicted in Fig. 12B. From this figure, it is clear that the peak values of PRE and POST were very similar, whereas that of FIX had a lower value. In addition, it appeared that the peak frequency of FIX shifted to a lower-frequency band. To check these tendencies statistically, the average power of type 1 theta band (8–12 Hz) and that of type 2 theta band (4–8 Hz) were calculated in the PRE, FIX, and POST periods (Fig. 12C). The mean ($\pm$SE) values were $0.389 \pm 0.016$, $0.257 \pm 0.011$, and $0.367 \pm 0.016$, respectively, for the type 1 band activity for PRE, FIX, and POST; for the type 2 band activity, the values were $0.163 \pm 0.006$, $0.198 \pm 0.004$, and $0.129 \pm 0.004$, respectively. A one-way repeated-measure ANOVA was performed for each type of band separately, showing a significant effect for type 1 band [$F(2,344) = 135.06$, $P < 0.001$] as well as for type 2 band [$F(2,344) = 121.30$, $P < 0.001$]. Post hoc Tukey HSD tests indicated that all pairs showed significant differences during the 1-s period, for both types of theta band ($P < 0.05$). Thus the type 1, motor-related theta was significantly reduced during the fixation period and the type 2, immobility-related theta increased significantly during the fixation period.
To examine whether the theta-band firing rhythms remained while the rat fixated at the center hole, even though the theta-band LFP power was reduced during that period, we performed autocorrelation analysis using the spike trains of every fixation unit during the fixation period. An example autocorrelogram is shown in Fig. 13A. This example showed a peak at the 7.7-Hz band, indicating that the neuron had a rhythmic firing pattern in the theta band during the fixation period. A total of 22 sequence-dependent fixation units (57.9%) exhibited significant theta rhythmic firing during the fixation period.

### Firing rhythm analysis by autocorrelograms

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#### FIG. 11. Results of the LIA band analyses.

- **A**: 8 examples of 5-s LFP traces (filtered 100 to 250 Hz) around the fixation period.
- **B**: the power of the LIA band (top) and the movement velocity (bottom) of those 8 examples. In A and B, all data were aligned by the onset of the fixation.
- **C**: shows 10-s LFP traces from the same trial of A during the consummation period (from 0 to 10 s after the reward delivery onset).
- **D**: the power of the LIA band (top) and the movement velocity (bottom) of those examples. In C and D, all data were aligned by the onset of the reward delivery.

#### FIG. 12. Results of the theta-band analyses.

- **A**: 8 examples of 5-s LFP traces (filtered 2 to 20 Hz) around the fixation period.
- **B**: power spectral density (PSD) estimates of PRE, FIX, and POST periods normalized by the total power of the 2- to 50-Hz range.
- **C**: mean theta powers of type 1 and type 2 bands during PRE, FIX, and POST periods (n = 173).

#### FIG. 13. Results of the firing rhythm analyses during the fixation period.

- **A**: an example autocorrelogram, which indicates a significant theta-band (a peak at 7.7 Hz) rhythmic firing. The bin width is 10 ms. The gray horizontal lines indicate 99.5% statistical significant limits.
- **B**: the population trends for the sequence-dependent fixation units (n = 38); the top panel indicates the normalized PSD estimates; the bottom panels indicate the distributions of the peaks from autocorrelograms of theta rhythmic units (n = 23) and nontheta rhythmic units (n = 15). C: the population trends for the sequence-independent fixation units (n = 38). All parameters and symbols are as in B (theta rhythmic units: n = 18; nontheta rhythmic units: n = 20).
fixation period, whereas 14 sequence-independent fixation units (36.8%) exhibited this tendency. The results of PSD analyses (see METHODS) are shown in the top panels of Fig. 13, B and C, with peaks around the theta band (8 Hz). As in the neurons that are categorized as “nonsignificant theta rhythmic” units, they show weaker but similar 8-Hz rhythmic firing during this period. Peak analyses were also performed for each type of unit (see bottom panels of Fig. 13, B and C)—again the main peak frequencies were around 8 Hz. In sequence-dependent fixation units, most of the peaks were concentrated around 8 Hz, whereas in the sequence-independent fixation units, the peak distributions were broader. For either type, we could not observe other peaks out of the theta-band range (4–12 Hz) among the “significant theta rhythmic” units.

**Histology**

Recording coordinates were reconstructed from the final location of each electrode tip and its track on the stained brain specimen and were confirmed to lie in the CA1 area of the hippocampus. Two examples are shown in Fig. 14. The arrow marks indicate final positions of the tips of the electrode.

**DISCUSSION**

Using a delayed spatial alternation task with a nose-poking paradigm, we found sequence-dependent activity in hippocampal CA1 neurons during the fixation period, when the rat was immobile and waiting for the next task event. Physical factors whose variation is known to have a strong influence on hippocampal activity were effectively controlled during this period and thus we can exclude trivial alternative explanations of the modulation in terms of position, sensory processes, or motor activity. We propose that the modulation reflects an abstract, memory-based spatial representation as a function of the type of trials (i.e., RIGHT-TO-LEFT vs. LEFT-TO-RIGHT trials), providing a basis for sequential behavior in a delayed alternation task. This result extends previous findings concerning spatial sequence-dependent activity of hippocampal neurons (Ainge et al. 2007a,b; Bower et al. 2005; Dayawansa et al. 2006; Fernbiteanu and Shapiro 2003; Frank et al. 2000; Griffin et al. 2007; Johnson and Redish 2007; Lee et al. 2006; Shapiro et al. 2006; Smith and Mizumori 2006; Wood et al. 2000; but see also Holscher et al. 2004; Lenck-Santini et al. 2001) and further indicates that such sequence dependence exists not only in the active exploring periods but also in a completely immobile, fixation period.

![Sample recording positions verified by histology. Arrowheads indicate microlesions located in the dorsal CA1 cell layer.](http://jn.physiology.org/)

A possible mechanism for the fixation-specific firing

Having established the existence of fixation-specific hippocampal firing in the CA1 neurons, we can shift the focus to the mechanisms that underlie the immobility-related hippocampal activity. One potential explanation would be in terms of differential mechanisms among active running (as observed in place cell studies), being immobile, but fully alert (in the current study; see also Ainge et al. 2007b; Hok et al. 2007; Smith and Mizumori 2006) and quietly resting (e.g., forward and/or reverse replay of the running sequence by place cells; Diba and Buzsáki 2007; Foster and Wilson 2006). It is known that active running versus immobility (especially, when quietly resting) leads to differential concentration of acetylcholine (Hasselmo 1999, 2006). During active running, theta (and gamma) rhythm-based retrieval may be important for recall of a well-learned sequence (Hasselmo 2006; Jensen and Lisman 2005), whereas the theta rhythm in the hippocampus tends to decrease when movement slows down and sometimes even vanishes when movement stops (Vanderwolf 1969). To remember sequences during stationary periods, then, the brain might rely on complementary mechanisms.

We also obtained a reduction of theta power during the fixation period, especially in the type 1 band. Interestingly on the other hand, the power of the type 2 theta band tended to increase during this period. As is generally assumed that the type 2 theta is related to attention processes (Kramis et al. 1975), one possible explanation for our data would be that “attention” here refers to “cognitive effort” or the rat’s internal state when retaining the sequence. In any case, the finding that several neurons still keep theta rhythmic firing during the fixation period (Fig. 13) suggests that the type 2 theta might have an active role in maintaining sequential information during the immobile period.

In addition, we confirmed that during the fixation period there was no LIA (i.e., sharp-wave and ripple), which is normally thought to occur when the acetylcholine concentration is low in hippocampus. The absence of LIA suggests that the hippocampal system had not been switched completely to a “quiet resting” state. This seems reasonable because the cholinergic switch likely depends on metabotropic muscarinic acetylcholine receptor action, which usually takes several seconds to complete. Accordingly, the LIA that we did observe during consummation emerged at around 2 s after the rat stopped moving (Fig. 11). Foster and Wilson (2006) and Diba and Buzsáki (2007) reported several CA1 place cells that showed episode-dependent forward and/or reversal replay during ripple waves. Thus two types of episode-dependent (i.e., sequence-dependent) hippocampal activities were previously reported: one during active running, the other during quiet resting. Our findings fill the gap between those two periods—the sequence-dependent hippocampal activity during immobile, but non-LIA period, with potentially relevant type 2 attention-related theta. The deficit in delayed spatial alternation in a maze paradigm, following hippocampal lesion (Ainge et al. 2007b), then, might be explained by the loss of sequence-dependent activity, which might constitute an attentional/cognitive type 2 theta mechanism.
Retrospective coding during fixation

What is the precise function of the sequence-dependent activity in hippocampal CA1 neurons? Given that the activity reflects an abstract, memory-based code, we now need to explore how this information is used during the control of behavior. In this regard, one important question is whether the information reflects retrospective coding (e.g., a memory-retrieval signal, representing trial sequence) or prospective coding (e.g., the preparation of an action or reward expectancy; for a detailed articulation of the importance of this question, see Rainer et al. 1999). One relevant finding in relation to this question is the downward trend observed in individual neurons as well as at the population level in CA1 (see Fig. 3, 5, 7, and 9)—starting with high activity at the beginning of the fixation period and then exhibiting a gradual reduction in the activity level—even though the rat remained stationary throughout this period and all physical factors were controlled. In accordance with the framework proposed by Rainer et al. (1999), this observation would suggest retrospective coding reflecting a retrieval or memorial process in relation to the context of the alternation sequence.

Another way to explore whether the firing during fixation reflects retrospective or prospective coding would be to investigate the relationship between the firing during fixation and that at the succeeding and the preceding choice ports. However, after performing this analysis, we found that only one unit among the 38 sequence-dependent fixation units showed an increased firing rate (>2 Hz) at a choice port, particularly for a succeeding rightward choice. The data from this neuron suggest prospective coding during the fixation period; however, it would be inappropriate to generalize from one exceptional result. More work is needed to establish the precise content of the abstract code during fixation. For instance, it might be useful in future research to compare the neural representations for other types of sequences (i.e., “alternation” + “repetition,” as in a sequence of LEFT/LEFT/RIGHT/RIGHT/VS. “alternation only,” as in the current experiment).

In the meantime, however, it is clear that the brain must somehow coordinate between retrospective information and decision-making processes for future actions to perform the delayed alternation task. Although the hippocampal CA1 neurons provide a likely mechanism for the retrospective coding during the immobile period, researchers should also consider how this information is used to extract relevant prospective codes in hippocampal CA1 or other neural structures.

Translating retrospective to prospective coding

How are prospective codes derived from the mnemonic information in hippocampal CA1? Several hippocampal place cell studies have pointed out that some of the neurons show a prospective code for their next location or event (Ainge et al. 2007a; Ferbinteanu and Shapiro 2003; Frank et al. 2000; Hok et al. 2007; Johnson and Redish 2007). In two of the neuron types that we observed in the present study—i.e., the sequence-dependent “Fixation and Before” and “Fixation, Before and After” neurons—there appeared a second component at the end of the fixation period, with a climbing trend toward the next event or action (Figs. 5 and 7). Nevertheless, the dominant activity during the stationary period did not show such a prospective characteristic (Fig. 9).

Intriguingly, the notion that there is less dominant prospective coding in hippocampal CA1 neurons during the stationary period—and that this period primarily involves type 2 theta—fits nicely with a theoretical framework proposed by Bland and Oddie (2001), according to which the hippocampal type 2 theta either provides the motor systems with a “readiness” signal for the preparation for movement or signals the intensity with which a movement should be initiated. Thus type 2 theta is likely to be observed under reward expectancy (Hok et al. 2007), aversive conditioning (Balleine and Curthoys 1991; Whishaw 1972), and action preparation (Bland et al. 2006).

The simplest hypothesis would be that hippocampal retrospective codes during the type 2 theta dominant mode of information processing in the stationary period are sent to other neural structures, which derive prospective codes for the purpose of decision-making and context-dependent reward expectancy (these prospective codes could then possibly be fed back to hippocampus). There is already some empirical evidence from neurophysiological investigations with monkeys suggesting that prefrontal cortex (Rainer et al. 1999) and striatum (Lauwereyns et al. 2002) exhibit this type of prospective activity. Hok et al. (2005) previously showed a trend reminiscent of place cells in rat medial prefrontal cortex, which suggested a goal-oriented function and might have been derived from a hippocampal retrospective code.

Poucet et al. (2004) proposed a model that solves the problem of transferring retrospective to prospective information on the basis of a neural circuit including hippocampus, prefrontal cortex, and the striatum. Anatomically, there are in fact strong connections from hippocampus to prefrontal cortex, and striatum, especially nucleus accumbens (Ongür and Price 2000; Thierry et al. 2000). Goto and O’Donnell (2001) reported that membrane potential transitions in nucleus accumbens neurons are correlated with electrical activity in the ventral hippocampus (suggested that hippocampal activity can determine the activity of ensembles of accumbens neurons). In turn, nucleus accumbens projects to substantia nigra and the ventral tegmental area, that is, the core structures of the dopaminergic system. It is quite plausible, then, that the hippocampus contributes to prospective coding in prefrontal cortex and striatum, directly and/or indirectly via the dopaminergic projections. Not only ripple-wave studies but also theta-wave studies have already shown that hippocampal activity certainly does affect cortical and striatal activity (Berke et al. 2004; DeCoteau et al. 2007; Gengler et al. 2005; Pennartz et al. 2004; Siapas et al. 1998, 2005).

Nose-poking paradigm

Future studies that manipulate the level of reward expectancy in a spatial alternation task may be particularly useful to gain further insights into the role of hippocampus in the conversion of memory-based contextual information into prospective codes during decision-making and action preparation. Such experiments would allow one to focus on the covariation between neurophysiological measures and behavioral indices for decision making (Carpenter 2004; Smith and Ratcliff 2004). Indeed, one additional advantage of the current nose-poking paradigm (when adapted to speeded choice tasks) will be that it affords the analysis of decision-making processes in terms of response bias and perceptual sensitivity by consider-
ing the shapes of reaction-time distributions (Carpenter and Williams 1995; Lauwereyns and Wisenewski 2006). In addition, the nose-poking paradigm provides stringent control of the rat’s position and its movement velocity. Combined with head angle tracking, the paradigm also provides a straightforward opportunity for off-line analysis, to examine the relationship between head angles during fixation and firing rates. To further improve the paradigm, we are currently piloting a method for on-line control of head angle, setting head angle criteria for the rat during the performance of the nose-poking task.

Concluding remarks

The current study focused on the hippocampal involvement in an abstract spatial alternation code during well-controlled fixation using a nose-poking paradigm. We obtained episode-dependent activity of hippocampal CA1 neurons likely to reflect retrospective codes during fixation. During this period, there was no LIA and type 1 movement-related theta was reduced, whereas type 2 attention-related theta increased. Our data represent the final piece of evidence required to establish that episode-dependent activities occur in all three internal modes of hippocampus: 1) active exploration, as documented in place cell studies with strong type 1 theta activity; 2) quiet resting with intermittent LIA, as documented with forward and/or reversal replay (Diba and Buzsáki 2007; Foster and Wilson 2006); and 3) quiet concentration, fully alert, as shown in the current study. Our data, combined with those from a hippocampal lesion study (Ainge et al. 2007b), suggest this type of activity, crucial for the performance of delayed sequential behavior, to be specific to hippocampus.

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