

Firing rate suppression of the medial septum during spatial working memory maintenance without navigation

Malek Aouadi · Nicola Solari · Johanna Petra Szabó · Bálint Király · Balázs Hangya

Received: 13 January 2026 / Accepted: 19 August 2026

© The Author(s) 2026

Abstract

The medial septum (MS) was shown to participate in working memory (WM) processes: MS lesions led to deficits in spatial WM performance, and hippocampal theta oscillations, known to depend on the MS, were linked to WM maintenance. However, what activity features of the MS may support working memory has not been explored. Therefore, we directly compared MS single neuron activity in mice performing a 2-alternative forced choice task with and without a WM component. Importantly, we minimized the locomotion component of the task to dissociate spatial navigation and WM processes. We found that the dominant response during stimulus processing and the subsequent delay period was a suppression of activity. WM engagement was characterized by stronger responsiveness of MS neurons compared to control, and especially a stronger firing rate suppression in the second half of the delay period. Moreover, we found fewer strongly theta-rhythmic neurons in the MS during WM performance, and these neurons were more suppressed during the delay period when WM was engaged. Based on these results, the MS may have an enabling rather than an active role in hippocampal WM processing.

Introduction

Pharmacological and lesion studies identified the medial septum as an important regulator of WM¹, likely through its hippocampal projections^{1,2}. Small MS lesions caused an impairment in WM performance of rats in a spatial alteration task, while leaving those tasks that were solvable through reference memory mechanisms intact³. The local anesthetic tetracaine, infused locally to the MS, decreased rats' performance in a T-maze alternation WM task, along with a reduction of the hippocampal theta rhythm. Similar effects were caused by infusing either the GABAergic agonist muscimol or the cholinergic antagonist scopolamine⁴. Importantly, the acetylcholinesterase inhibitor physostigmine did not rescue WM deficits caused by MS lesions in rats⁵, suggesting that it was not cholinergic mechanisms alone that contributed to MS control of working memory, unlike what was proposed based

This Article in Press is shared early to give you faster access to new research. It is citable and carries a permanent DOI. The final edited version will replace it automatically.

Malek Aouadi and Nicola Solari contributed equally to this work.



on human pharmacology studies⁶. Recently, the dependence of WM mechanisms on MS activity was confirmed by optogenetic suppression of the MS in mice⁷.

The MS is a known regulator of the 4–12 Hz theta oscillation in the hippocampus^{2,8–13}. Specifically, long-range projecting GABAergic neurons of the MS expressing parvalbumin (PV)¹⁴ and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel¹⁵ generate hippocampal theta rhythms¹⁶ by rhythmic disinhibition of hippocampal pyramidal neurons via local interneurons¹.

Theta oscillations have been associated with WM processes: in rodents, WM deficit was correlated with the disturbance of hippocampal theta during pharmacological manipulations of the MS⁵, and neuronal phase coupling to theta rhythms coded items held in WM in humans^{17,18}. It was proposed that theta-frequency band synchronization between the hippocampus and prefrontal cortex^{19,20}, and the modulation of faster gamma oscillation amplitude by theta phase (theta-gamma phase-amplitude coupling) are crucial mechanisms of WM maintenance^{21–23}.

Therefore, it is conceivable that the MS participates in WM processes through theta generation mechanisms. Indeed, selective GABAergic MS lesion impaired spatial WM in rats²⁴, and chemogenetic inhibition of MS PV-expressing, but not calbindin-expressing GABAergic neurons impaired spatial WM in mice²⁵.

However, theta oscillations have been strongly linked to exploratory behaviors and spatial navigation as well^{26–28}, raising the question to what extent spatial WM deficits were attributable to disturbed navigation in standard spatial WM paradigms, where these two processes were interrelated. Optogenetic scrambling of hippocampal theta oscillations by disrupting MS GABAergic activity impaired WM retrieval while leaving hippocampal spatial coding largely intact²⁹, suggesting that septo-hippocampal theta mechanisms supporting working memory may be dissociated from navigation-related spatial processing, in contrast with earlier lesion studies that emphasized a predominant role of the MS in spatial, rather than non-spatial, working memory processes^{30,31}. However, how local processing changes in the MS in support of WM processes is not known, since MS neuron activity has not been recorded during WM performance.

To fill this knowledge gap, we recorded single neuron activity from the MS while mice performed a delayed response spatial working memory task where navigational components were minimized, which was compared to a control task that required the same behavioral response but without a WM component. The MS was strongly engaged in the WM task with higher firing rates and larger magnitude firing rate responses. Furthermore, the WM task was characterized by sustained firing inhibition in the second half of the delay period along with a selective inhibition of the strongly theta-rhythmic MS population, suggesting that the MS has an enabling role of hippocampal WM processing during the response delay rather than a specific enhancement of rhythmic theta generation.

Materials and methods

Animals

Adult (over 2 months) male ChAT-IRES-Cre mice were used. Experiments were performed according to the regulations of the Hungarian Act of Animal Care and Experimentation (1998; XXVIII, section 243/1998, renewed in 40/2013) in accordance with the European Directive 86/609/CEE and modified according to the Directive 2010/63/EU. Experimental procedures were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest and by the Committee for Scientific Ethics of Animal Research of the National Food Chain Safety Office of Hungary.

Mice were randomly assigned to the WM or Control task groups prior to training. For the WM task, 11 mice were assigned. Four mice died during surgery or the pre-surgical training period, two mice failed to learn the task, and one mouse was excluded because of insufficient electrophysiological signal-to-noise ratio, resulting in a final sample of four mice included in the analyses (Supplementary Table S1). For the control task, seven mice were assigned. One mouse died a few days after surgery, and three mice were excluded because of insufficient

electrophysiological signal quality, targeting error, or unsuccessful histological verification, resulting in a final sample of three mice included in the analyses (Supplementary Table S1). The final sample sizes are consistent with previous electrophysiological studies in this field^{12,16,32}.

Surgery

Mice were anesthetized with a mixture of ketamine and xylazine (83 mg/kg ketamine and 17 mg/kg xylazine, dissolved in 0.9% saline) injected intraperitoneally. The scalp was shaved, disinfected (Betadine), and imbued with local anesthetic (Lidocaine). The animals were put in a stereotaxic frame (Kopf Instruments), their eyes were protected with eye ointment (Corneregel, Bausch & Lomb), their skin was opened, their skull was cleared and levelled. A craniotomy was drilled over the MS. Virus injection (AAV2/5.EF1a.Dio.hChR2(H134R)-eYFP.WPRE.hGH; dorsoventral 3.95, 4.45 and 5.25 mm, 200 nl at each depth) was performed according to standard techniques^{33,34}. The fluorophore expression was used for subsequent histological reconstruction (see below), while the ChR2 expression was used for conducting optogenetic cell type identification sessions. However, the number of identified cholinergic neurons remained low and thus they were not further analyzed. Since the combination of this mouse line and construct has been used in a large number of studies without any reports of adverse effects, in line with our own observations^{35,36}, this did not impact the conclusions of this study. A custom-built microdrive^{33,35} housing 8 tetrodes was lowered into the MS with a 10° lateral angle (MS 10°, antero-posterior + 0.90 mm, lateral, 0.90 mm) using the stereotaxic arm. Two additional holes were drilled over the bilateral parietal plates for placing the ground and reference electrodes. The craniotomies were filled with a silicone elastomer sealant (Kwik-Cast, World Precision Instruments) to protect the brain surface, and the implant was secured by dental cement (C&B MetaBond, Parkell Inc. and dental acrylic, Lang Dental). Mice received postoperative analgesics (Buprenorphine, 0.1 mg/kg) and local antibiotics (Neomycin). Mice were closely monitored after surgery and were allowed 10–14 days of recovery before starting behavioral training.

Behavioral training

We trained mice on a working memory task with minimized navigational component in a freely behaving delayed response 2-alternative forced choice paradigm (DR-2AFC WM). Mice were water-restricted according to a standard water scheduling protocol^{33,34,37}. At the beginning of each training session, mice were placed in a custom-built sound attenuated recording chamber equipped with precise sound stimulus, water reward and air puff punishment delivery systems in three adjacent water ports³⁸. Task events were controlled by a micro-controller-based behavior control system (BPod, Sanworks, US). An LED light above the central port indicated the availability of trial initiation. Mice reported in the central port and fixated for a required amount of time. Upon central port entry, one of two brief (0.2s) pure tones of different pitch (3–11 kHz at 62dB sound pressure level) predicted the availability of reward in the left or right-side port. Switching off the LED light above the central port and simultaneously switching on the LED lights above both side ports indicated the end of the fixation period and the availability of reward in one of the side ports, after which mice were free to leave the central port and report to the side ports. Entering the correct side port triggered the delivery of a small amount of reward (5 µl) through a waterspout. After mice finished water consumption, they disengaged from the side port, and the next trial began. If mice entered the incorrect side port, they received a brief puff of air from the port that they reportedly perceived as aversive³⁴. During training, the length of the mandatory central port fixation (cue presentation and delay period) was gradually increased and reached 0.9–1.5 s before the recording sessions were started. We introduced micro-rewards (1.5 µl water from the central port three times during the delay period) to maintain sustained central fixation during longer delay intervals. These rewards were substantially smaller than the final reward associated with correct task completion and served to support engagement during the delay period. We did not observe indications that the micro-rewards interfered with task performance or reinforced non-target behaviors. Mice could switch between the central and side ports with minimal body movement, thus eliminating any navigational

components typically present in WM tasks (e.g. spatial alteration, delayed match to sample, delayed non-match to sample^{39,40}). In a part of the sessions ($n = 56/111$; Supplementary Table S1), an optogenetic tagging protocol preceded behavioral training. We applied 1 ms laser pulses (473 nm, Sanctity) at 20 Hz for 2 s, followed by 3 s pause, repeated 20–30 times. We employed low light intensities, calibrated to preferentially activate neurons within the local electrophysiological recording range while minimizing broader network effects using the OPETH plugin⁴¹. To directly assess possible lingering effects of photostimulation, we aligned neuronal firing to the light pulses and found that photostimulation-induced firing changes did not outlast the stimulation period itself. We therefore do not expect persistent network or behavioral effects of the tagging protocol on the subsequent recordings.

We trained another cohort of mice in a control task in which the cue tone was on for the entire delay period, but training was otherwise the same, including the shaping procedure, reinforcement delivery in the side ports and micro-reward delivery in the central port (DR-2AFC Control), thus removing the working memory component of the task. This allowed us to directly compare neuronal responses in the delayed response tasks with or without engaging working memory.

Electrophysiology recordings

Neuronal activity was recorded with 8 moveable tetrodes twisted from polyimide-coated nichrome wires (12.7 μm diameter, Sandvik, Stockholm, Sweden). The tetrodes were gold-plated in a solution containing polyethylene glycol dissolved in deionized water to 1 g/l concentration (1.125 ml) mixed with gold plating solution (0.375 ml, Neuralynx, Bozeman, MT, US; see⁴² to impedances of 30–100 k Ω measured at 1000 Hz (NanoZ, Neuralynx) and dipped in DiI (ThermoFischer Scientific) red fluorescent dye to aid later track reconstruction efforts. The tetrodes were connected to a 32-channel connector (Omnetics) via an electrode interface board (Neuralynx) using gold pins. Ground and reference wires were inserted into the parietal cortex (30 AWG insulated copper wires). A 32-channel head stage (RHD2132, Intan) was connected to the Omnetics connector before each recording. Data was acquired by a Data Acquisition System (Open Ephys), digitized at 30 kHz. The electrophysiology signals were synchronized with behavioral events (cue and feedback) using TTL pulses. The tetrodes were advanced 40–100 μm after each recording session.

Histology

After the recordings, mice were deeply anesthetized with a mixture of ketamine and xylazine (83 mg/kg ketamine and 17 mg/kg xylazine, dissolved in 0.9% saline). An electrolytic lesion was performed to mark the tip of the tetrodes (5 μA current for $\sim 5\text{s}$ on 2 leads/tetrode, Supertech IBP-7c). Transcardial perfusion was performed with saline for two minutes, followed by 4% paraformaldehyde (PFA) solution for 20 min. Following perfusion, the brain was carefully removed and postfixed in 4% PFA overnight. Section (50 μm thick) were cut with a vibratome (Leica 2100 S). Special care was taken to section the brain perpendicular to brain surface, so that resulting sections could be easily aligned with coronal atlas images. The sections were mounted on microscopy slides using a mounting medium (Aquamount) and covered with coverslips. Fluorescent images of the sections were taken with a Nikon C2 confocal microscope. Green fluorescence marked the cholinergic neurons of the MS and red fluorescence indicated the DiI tracks of the electrodes. Atlas images⁴³ were aligned to the light micrographs of the brain sections using Euclidean transformations. These alignments were copied to fluoromicrographs taken with the same field of view. Coordinates of electrode entry points and deepest points in the brain were marked by DiI tracks and the tip of the electrode was localized using small electrolytic lesions (see above). Coordinates of electrode endpoints were read from the atlas image and were used to interpolate the recording locations referenced to the atlas coordinates, based on experimental logs of the protrusion of the electrode^{44,45}. Only those recordings where the electrodes were localized in the MS were further analyzed.

Data analysis

Data analysis was performed using Matlab R2023a. Analysis code is available at <https://github.com/hangyabals/MS-WM-analysis>.

Spike sorting and firing rates

The continuous electrophysiology data were band-pass filtered between 600 and 6000 Hz and threshold-discriminated to find spike times. Spike waveforms were then isolated and spike-sorted based on peak-to-valley amplitude and waveform energy manually using MClust 3.5 (A.D. Redish). Putative single cells with an isolation distance over 20 and an L-ratio under 0.15 were included (Supplementary Table S1). Auto-correlograms were inspected and putative single cell clusters with substantial refractory violations were removed. Firing rates were calculated in event-aligned time windows. Correct and error trials were only differentiated when reward- or punishment-related firing rate changes were analyzed, since firing rates were similar in the delay period in all response categories irrespective of outcome (Supplementary Fig. S12 e–f). Aborted trials were not included in the analyses. Distribution of raw firing rates was used to statistically demonstrate absolute firing rate differences between the behavioral conditions. Baseline-subtracted firing rate distributions were used to statistically analyze changes of neural firing during behavioral trials relative to their individual baseline firing rates. The following task phases were defined relative to cue tone onset: $[-1.6\ 0]$ s baseline, $[0\ 0.2]$ cue, $[0.2\ 0.9-1.5]$ delay; the delay period was considered from 0.2 to 1 s for statistical comparisons across WM and control tasks to ensure consistency across tasks.

PETH analysis

We computed event-aligned raster plots and peri-event time histograms (PETHs) for all recorded units, aligned to behavioral events. Trials for which the delay period was lower than 0.8 s were excluded. Spike times were binned at 1 ms resolution and convolved with a Gaussian kernel (standard deviation = 25 ms) to smooth the firing rate estimates. Baseline activity was calculated over a pre-cue window (-1.6 to 0 s), and individual PETHs were z-scored by the mean and standard deviation of this baseline window to normalize firing rates across neurons with different baseline activities. In contrast with raw and baseline-subtracted firing rate distributions (see above), z-scoring was used to provide visual summary and average firing rate statistics where the representation is balanced over the data set.

Accelerometer data analysis

Mice could potentially express their upcoming choice through subtle body or head movements during the fixation period, thereby reducing working memory demands. Therefore, we analyzed accelerometer signals recorded from the headstages. Accelerometer channels were resampled to 1 kHz, bandpass-filtered between 0.5 and 20 Hz, and combined into a movement-magnitude signal. For each trial, the fixation period (from fixation onset to fixation end) was divided into early and late halves, and the root-mean-squared (RMS) movement amplitude was computed for each epoch. We then compared left- versus right-choice trials at the session level using non-parametric statistics. In the DR-2AFC WM task, only a relatively small fraction of sessions showed significant left-right differences in movement signals during the early fixation period (12/111 sessions, $p < 0.05$, Mann-Whitney U-test, FDR-corrected), whereas such differences were more common in the DR-2AFC Control task (36/112 sessions, $p < 0.05$, Mann-Whitney U-test, FDR-corrected). We performed delay-period firing-rate analyses after excluding sessions with significant left-right RMS differences (Supplementary Fig. S5).

Auto-correlation

Auto-correlograms (ACG) were computed at a 1 ms resolution. Burst index (BI) was calculated as the normalized difference between the maximum ACG value for 0–10 ms lags and the mean ACG value for 180–200 ms lags. The normalizing factor was taken as the greater of the two values, resulting in an index bounded between -1 and 1 ^{36,37}.

Theta-index

Theta index was calculated from the ACG as the normalized difference between the mean ACG amplitude around the first theta peak (± 25 ms around the peak within 100–200 ms) and the mean ACG amplitude in a window corresponding to the trough before the first theta peak (65–85 ms). Normalization was performed by dividing the difference by the larger of the two values, yielding an index between -1 and 1 .

LFP analysis

We analyzed local field potential (LFP) signals across different behavioral epochs. LFP signals were downsampled at 1 kHz and low-pass filtered at 100 Hz. Continuous wavelet transformation was performed using Morlet wavelet transform, and wavelet power (in decibels) was normalized to a pre-fixation baseline. For each trial, wavelet power time-series were aligned to behavioral events, and variable-duration epochs (e.g. Delay) were standardized. We averaged wavelet power in the theta frequency band (4–12 Hz) across trials, sessions, and animals for each task variant.

ROC analysis

ROC analysis was performed to test if the activity during the delay period was different between MS neurons recorded in the DR-2AFC WM task and the Control task for each response category. A sliding window approach was used, with 50 ms bins shifted every 25 ms across the delay period (0.2 to 1 s after cue onset). For each neuron, spike counts were extracted within each window and normalized by subtracting the mean of baseline activity (-1.6 to 0 s). For each time window and response category, we computed the area under the ROC curve (auROC) and statistical significance by bootstrap permutation tests with 5000 resamplings.

Response clustering

We performed k-means clustering of z-scored PETHs aligned to cue onset for all well-isolated neurons recorded in the DR-2AFC WM task and we used their cluster centroids to assign each neuron recorded in the Control task to the nearest centroid by computing Euclidean distances. Three clusters were defined for each of the task variants based on the first 3 principal component of the PETHs restricted to the delay period (0.2 to 1 s after cue onset). Z-scored PETHs were sorted by maximum activity during the delay period for visualization in PETH heatmaps and were averaged to generate cluster-averaged PETHs.

Spike-shape clustering

We performed k-means clustering ($k=3$) of z-scored spike widths to classify well-isolated neurons based on their intrinsic electrophysiological properties. Spike width was defined as the interval from the peak to the subsequent trough, calculated with sub-sample precision via parabolic interpolation.

Statistics

Because neurons recorded from the same animal are not fully independent observations, some shared variance due to common biological and recording factors may be present. Nevertheless, consistent with common practice in electrophysiological and large-scale neural recording studies, individual neurons were treated as statistical units, as within-animal neuronal variability is generally considered sufficiently large to justify neuron-level analyses^{32,46,47}.

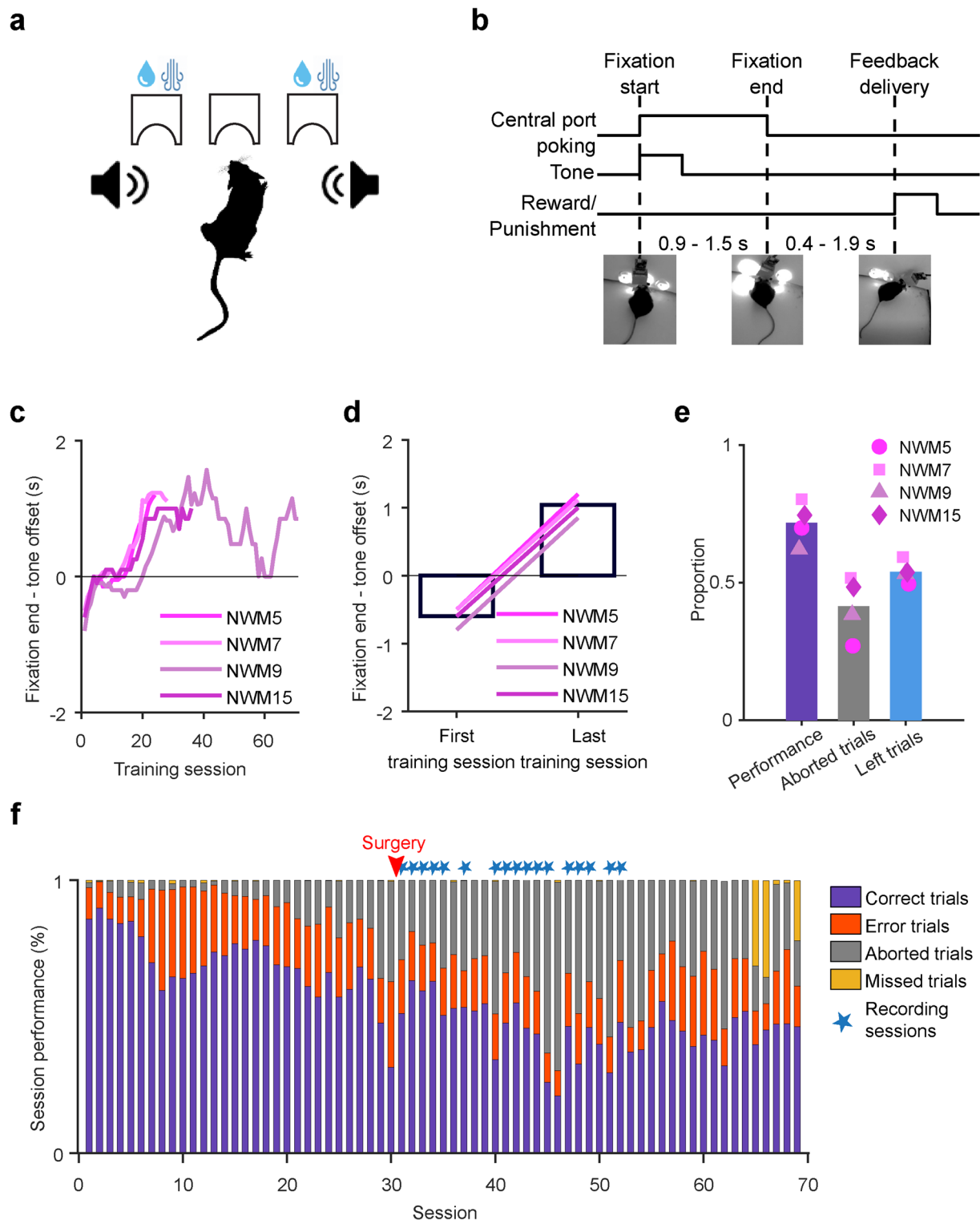
Since normality of data distributions could not be assumed, we performed non-parametric Mann-Whitney U-tests (two-sided, $p < 0.05$) for comparing central tendencies of two distributions. We performed chi-square tests of homogeneity to compare distributions of categorical variables ($p < 0.05$). Firing rate responses within a 1-second-long window from cue onset were tested for significant changes compared to a baseline window (− 1.6 to 0 s). Baseline firing rate was determined by mean firing probability in the baseline window. Next, the time course of firing rate decrease or increase was assessed by crossings of the half-distance between the extreme and the baseline firing rate before and after the minimum or maximum, respectively. This temporal window of inhibition or activation was then used to find corresponding intervals around local extremes in the baseline window. Spike counts were then compared using Mann-Whitney U-test (due to the asymmetric null hypothesis, one-sided p -value was calculated; we used $p < 0.01$ significance level). To address the hierarchical nature of the data, we performed (i) linear mixed-effects models with recording session nested within animal as random effects and (ii) analyses based on session-level averages. Matlab built-in functions `ranksum.m`, `signrank.m` and `fitlme.m` were used for Mann-Whitney U-test, Wilcoxon signed-rank test and linear mixed-effects model analyses, respectively. See Supplementary Table S2 for a summary of statistical comparisons.

Results

Training mice in a non-spatial working memory task

We aimed to test the role of MS neurons in working memory independent of navigation, given the known role of the septo-hippocampal system in the generation of the navigation-associated theta rhythm^{8,9,27}. Therefore, we trained mice on an auditory delayed response two-alternative forced choice task (DR-2AFC, $n = 4$ mice; Supplementary Table S1), in which freely behaving mice had to poke in a central port and hold fixation while they heard a brief (0.2 s) sound that indicated the availability of reward either in the right or the left side port, depending on the pitch of the tone. Mice then had to keep central fixation for an additional delay period (0.7–1.3 s in duration, see Methods) while the tone was off, holding the trial information in working memory, after which a binary decision between the left- and right-side port was made. Correct choices were rewarded by a drop of water, while incorrect choices were punished by a puff of air through the port, which mice perceive as aversive³⁴. The three ports were placed in close vicinity within a small training chamber, so that mice could switch from center to side ports with minimal body movement, thus eliminating any navigational component of the task (Fig. 1a–b).

Mice managed an increasing length of response delay, as indicated by the learning curve in Fig. 1c, showing the time difference of fixation end and tone offset. Initial negative values indicate that the tone was still on while the mouse disengaged from the central port (no WM component), while increasing positive values corresponded to increasing response delays (WM component; Fig. 1c–d). While mice tended to abort a fraction of trials by early withdrawal from the center port (mean $\pm$ SE in preoperative sessions, 0.171 ± 0.0016), they maintained a relatively high performance on completed trials (mean $\pm$ SE in preoperative sessions, 0.733 ± 0.0021 ; Fig. 1e–f, Supplementary Fig. S1a–c). Neither the performance nor the fixation dynamics changed significantly with drive implantation surgery, whereas the proportion of aborted trials showed a gradual increase throughout training sessions (performance, mean $\pm$ SE in postoperative sessions, 0.693 ± 0.0031 ; fraction of aborted trials, mean $\pm$ SE in



postoperative sessions, 0.436 ± 0.0025 ; Supplementary Fig. S1a–c). Nevertheless, behavioral parameters remained stable throughout the recording sessions (Supplementary Fig. S1d–g).

We trained another cohort of control mice that performed the same task, but we left the tone on for the entire delay period, removing the WM component from the task (Supplementary Fig. S2; $n=3$ mice; Supplementary Table S1). Mice performed well on this task version, managing an increasing length of central fixation, now with

Fig. 1 Training mice on a non-spatial working memory task. **(a)** Schematic of the experimental setup. The animals performed the task in a training box with 3 ports: 2 side ports for Reward/Punishment delivery and a central port for fixation, and 2 speakers for tone delivery. Mouse illustration by Prof. Julie McMurry (Pixabay, CC0). **(b)** Timeline of the DR-2AFC WM task. When mice poked in the central port ('Fixation start'), a brief cue tone was played indicating the availability of reward at the left or the right-side port, while the animal was required to hold central fixation. Once the animal was allowed to poke out ('Fixation end'), it visited one of the side ports and received water reward for correct or air puff punishment for error choices. **(c)** Learning curve for $n=4$ mice trained on the DR-2AFC WM task. Progress is indicated by increasing positive delays between cue tone offset and fixation end by training days. **(d)** Average difference between the mandatory fixation and the tone duration on the first and last days of training. **(e)** Performance of mice, aborted, and left choice trials for recording sessions. **(f)** Performance of an example mouse throughout all sessions. Recording sessions are marked with blue stars.

the tone played continuously (Supplementary Fig. S2b). Therefore, we could directly compare MS neuronal activity in the delay period, either with or without a WM component.

Recording medial septal single neurons during the working memory task

We recorded $n=539$ neurons from the MS of 4 mice (111 sessions; median and IQR of number trials per session: 314, 179) performing the DR-2AFC WM task (WM mice) and $n=538$ MS neurons from 3 mice (112 sessions; median and IQR of number trials per session: 225.5, 37.5) performing the DR-2AFC Control task (Control mice) using moveable tetrode implants (Fig. 2a–d; Supplementary Table S1). Mice were well-trained by the start of the recordings, thus being familiar with task structure and reward contingencies.

We tested if MS neurons were equally active in the task regardless of the presence or absence of a working memory component. When overall firing rate was considered, we found that MS neurons were on average more active in the task that featured a WM component, firing at significantly higher rates (WM mice, mean FR, 16.136 ± 0.608 Hz; Control mice, 12.961 ± 0.652 Hz, $p=2.497 \times 10^{-10}$, Mann–Whitney U-test; Fig. 3a).

We next assessed whether this firing rate difference was specific to some of the task phases or occurred uniformly throughout task performance. We found that increased firing rates during the WM task occurred already before fixation ('baseline', $[-1.6\ 0]$ s relative to tone onset, WM mice, 16.338 ± 0.616 Hz; Control mice, 13.149 ± 0.654 Hz, $p=8.107 \times 10^{-10}$, Mann–Whitney U-test; Fig. 3b) and continued during the cue presentation ($[0\ 0.2]$ s relative to tone onset, WM mice, 16.095 ± 0.723 Hz; Control mice, 12.706 ± 0.705 Hz, $p=5.422 \times 10^{-8}$, Mann–Whitney U-test; Fig. 3c), the delay ($[0.2\ 1]$ s relative to tone onset; WM mice, 14.828 ± 0.592 Hz; Control mice, 11.97 ± 0.629 Hz, $p=6.375 \times 10^{-9}$, Mann–Whitney U-test; Fig. 3d), and the feedback delivery (reward, WM mice, 15.147 ± 0.622 Hz; Control mice, 12.029 ± 0.614 Hz, $p=2.586 \times 10^{-8}$, Mann–Whitney U-test; punishment, WM mice, 16.804 ± 0.678 Hz; Control mice, 14.15 ± 0.749 Hz, $p=1.246 \times 10^{-7}$, Mann–Whitney U-test; Fig. 3e–f).

Conserved response categories of MS neurons during the response delay period

The higher firing rates of MS neurons in the DR-2AFC WM task suggest that they may be more engaged in task performance when there is a WM component. However, this can conceal potential diversity of the firing patterns of MS neurons throughout the task, which may also depend on the presence of a WM component.

Therefore, we assessed the significant firing rate changes of MS neurons in the delay period of the DR-2AFC WM task compared to a pre-stimulus baseline (Fig. 4). Most MS neurons (82.1%, Mann–Whitney U-test, $p<0.01$) changed their firing rates significantly throughout the behavioral trials, demonstrating a strong engagement of the MS in the task. We found that most MS neurons were inhibited during the delay, and this inhibition typically started already at cue onset (54.5%, Fig. 4a–c; Supplementary Fig. S3). Nevertheless, a substantial population of MS neurons responded with activation during the delay period (26.7%), while the remaining MS neurons showed no significant firing rate change (18.7%). Next, we performed the same analysis for MS neurons recorded in the DR-2AFC Control task (Fig. 4b). We found that the above response categories were surprisingly

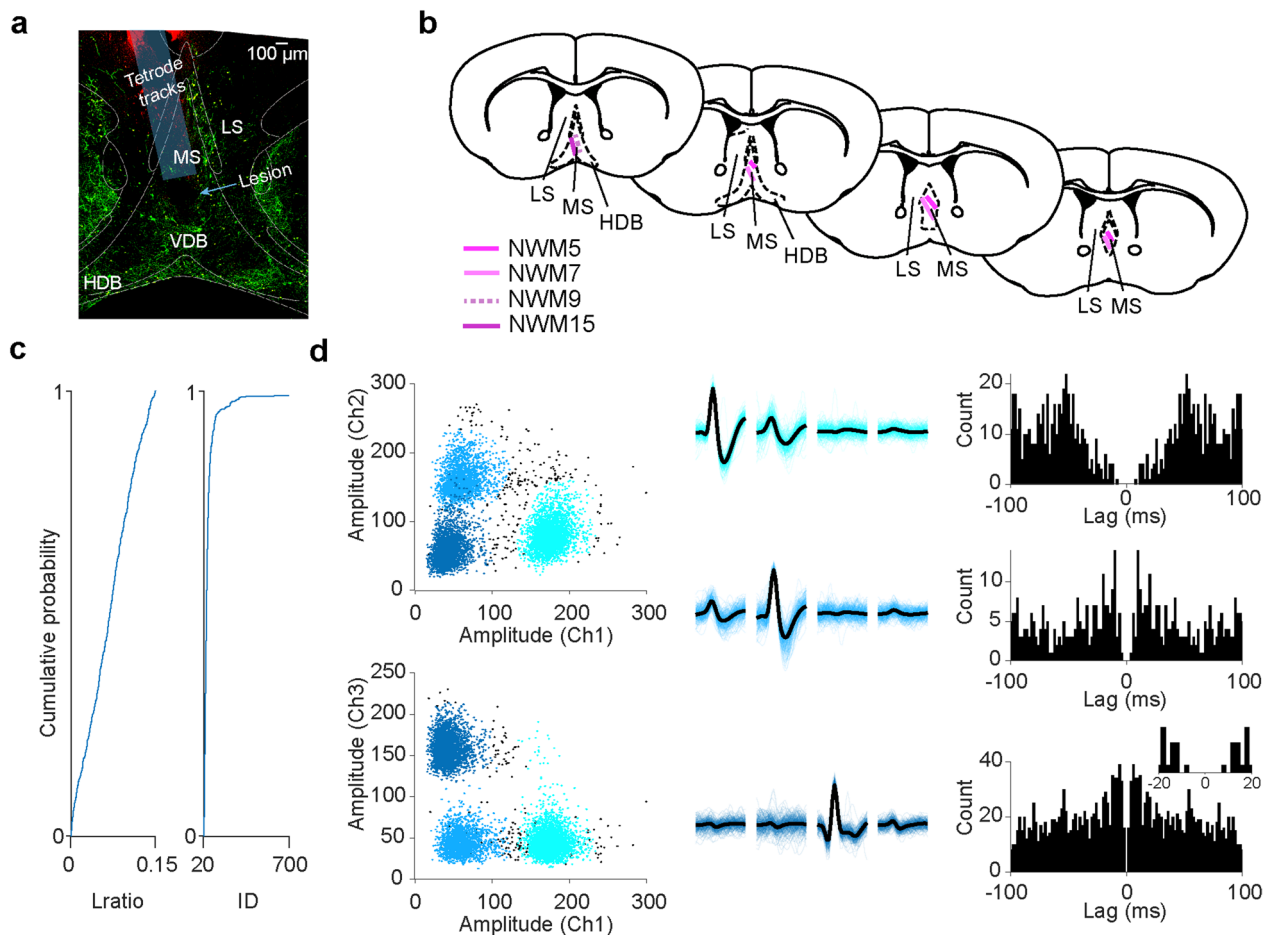


Fig. 2 Recording medial septal single neurons during the working memory task. **(a)** Coronal section from a ChAT-Cre mouse showing the tetrode tracks marked with a blue rectangle (DiI, red; ChAT+, green) through the MS. **(b)** Reconstructed location of the electrode tracks. Only those sessions that were conducted strictly within MS boundaries based on post hoc histological reconstruction were included. **(c)** Cumulative probability distribution of L-ratio and isolation distance parameters for well-isolated clusters (L-ratio < 0.15 and isolation distance > 20). **(d)** Example recording. Left, all spikes are shown in two-dimensional projections of the feature space. Three neurons were identified. Middle, individual (colored) and average (black) spike waveforms of the three neurons on the four tetrode leads separately. Right, autocorrelograms of the neurons show clean refractory periods.

conserved across the two tasks: we found no significant difference in MS neuron response distributions between WM and Control mice ($p=0.217$, Chi-square test; Chi-square statistic, 3.056; Degrees of freedom, 2). Response strength was larger for longer fixation, suggesting a correlation between MS neuronal responses and WM demand (Supplementary Fig. S4a-b), while correct and error trials were associated with similar response distributions (Supplementary Fig. S4c-d). An analysis of movement signals derived from accelerometer recordings suggested that anticipatory motor strategies were not likely to substantially contribute to the observed response distributions (Supplementary Fig. S5). Similarly, additional analyses showed that pre-training photostimulation (see Methods) neither had a lasting impact on firing rates, nor did it change the delay-period response distributions of MS neurons (Supplementary Fig. S6).

Moving away from pre-determined neuronal response categories, we also carried out unbiased clustering of MS neuronal responses during task performance^{47–49} (see Methods; Supplementary Fig. S7–8). This analysis clustered the MS neurons into similar response categories overall that could be described as strong inhibition (cluster #1), transient activation (cluster #2), and weak activation (cluster #3). While we observed some differences in the

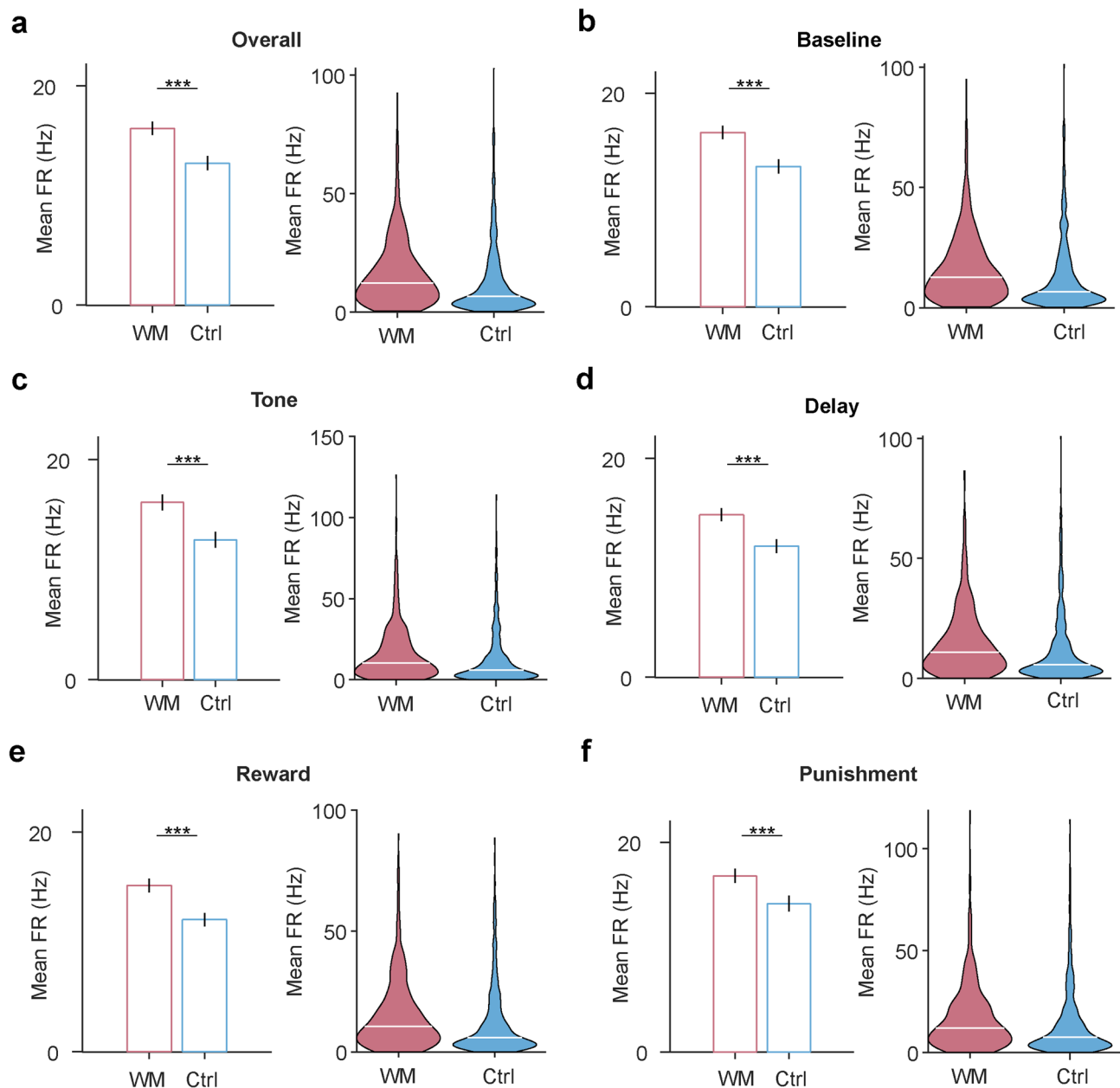
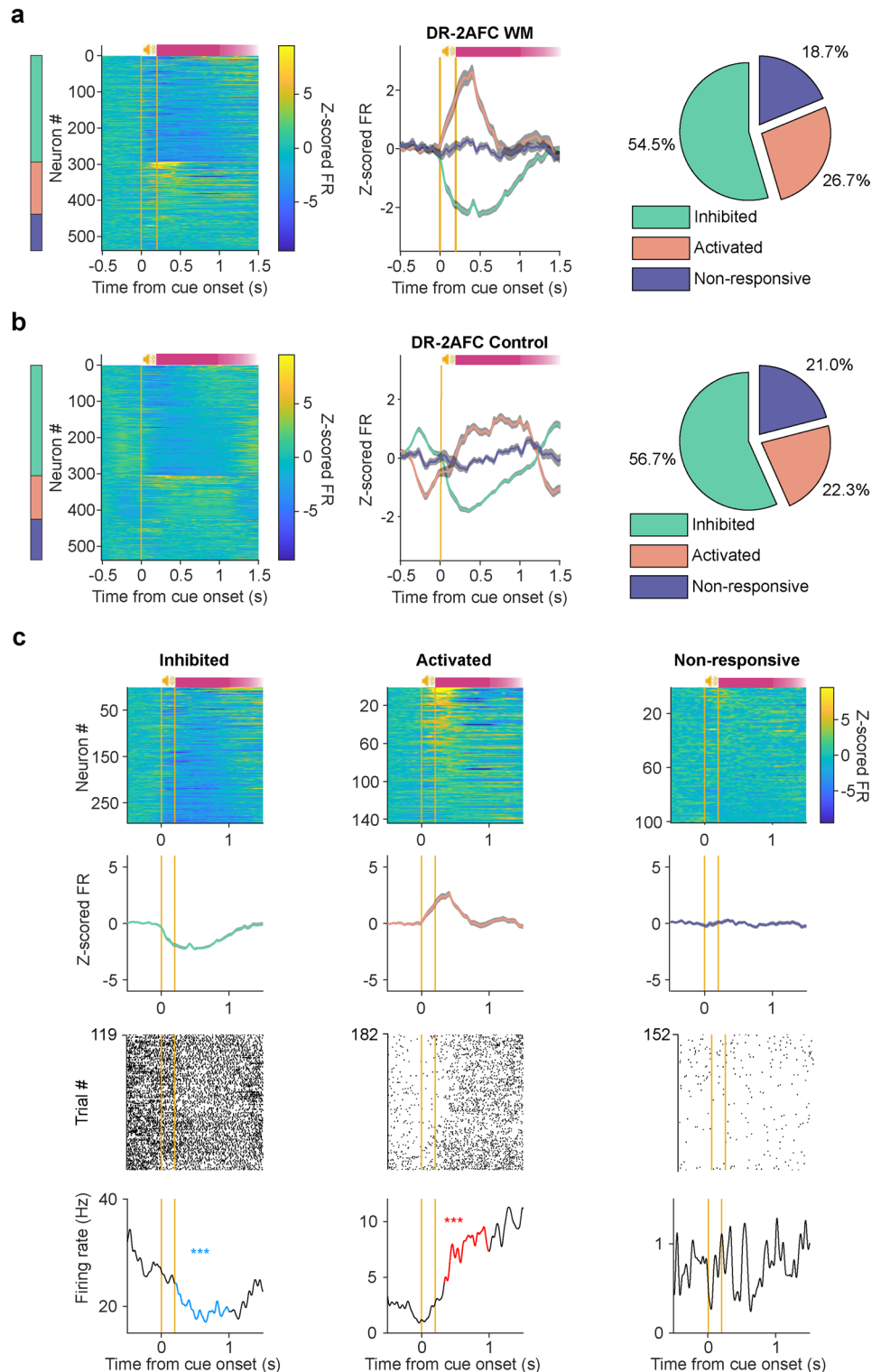


Fig. 3 MS neurons were more active in the task that featured a WM component. **(a)** Left, mean firing rate comparison between DR-2AFC WM (red) and Control mice (blue). Right, firing rate distribution (white line, median) of WM (red) and Control mice (blue). **(b)** The same as in panel a but restricted to a -1.6 to 0 s time interval before tone onset. **(c)** The same as in a, restricted to the tone presentation time period. **(d)** The same as in a, restricted to the delay period. **(e)** The same as in a, restricted to reward delivery. **(f)** The same as in a, restricted to punishment delivery. ***, $p < 0.001$; Mann–Whitney U-test.

time course of the cluster averages, which we unpack below, the distribution of MS neurons into these categories was similar in the DR-2AFC WM and Control tasks ($p = 0.74$, Chi-square test; Chi-square statistic, 0.603; Degrees of freedom, 2; the higher firing rate of MS neurons in DR-2AFC WM task was largely present across the main response categories, Supplementary Fig. S11b–g). We speculate that response categories of the MS neurons in the DR-2AFC are conserved and potentially linked with cell type specificity and anatomical features such as afferent connectivity, independent of whether WM mechanisms are involved in the task. Nevertheless,

Fig. 4 Conserved response categories of MS neurons during the response delay period. **(a)** Left, smoothed PETHs of all MS neurons in the DR-2AFC WM task grouped by statistically significant activity changes in the delay period, shown as a heatmap (yellow, high firing; blue, low firing). The purple bar indicates the delay period (from 0.2 to 0.9–1.5 s from cue onset). Middle, PETH averages per response group. Right, pie chart representation of response distribution. **(b)** The same as in panel a, but for MS neurons recorded in the DR-2AFC Control task. **(c)** MS neurons recorded in the DR-2AFC WM task in each response group. Top, PETHs of all neurons and average PETH. Bottom, spike raster plot and average PETH of a single example neuron in each group. Red, significant activation; blue, significant inhibition; **, $p < 0.01$; ***, $p < 0.001$; Mann-Whitney U-test.



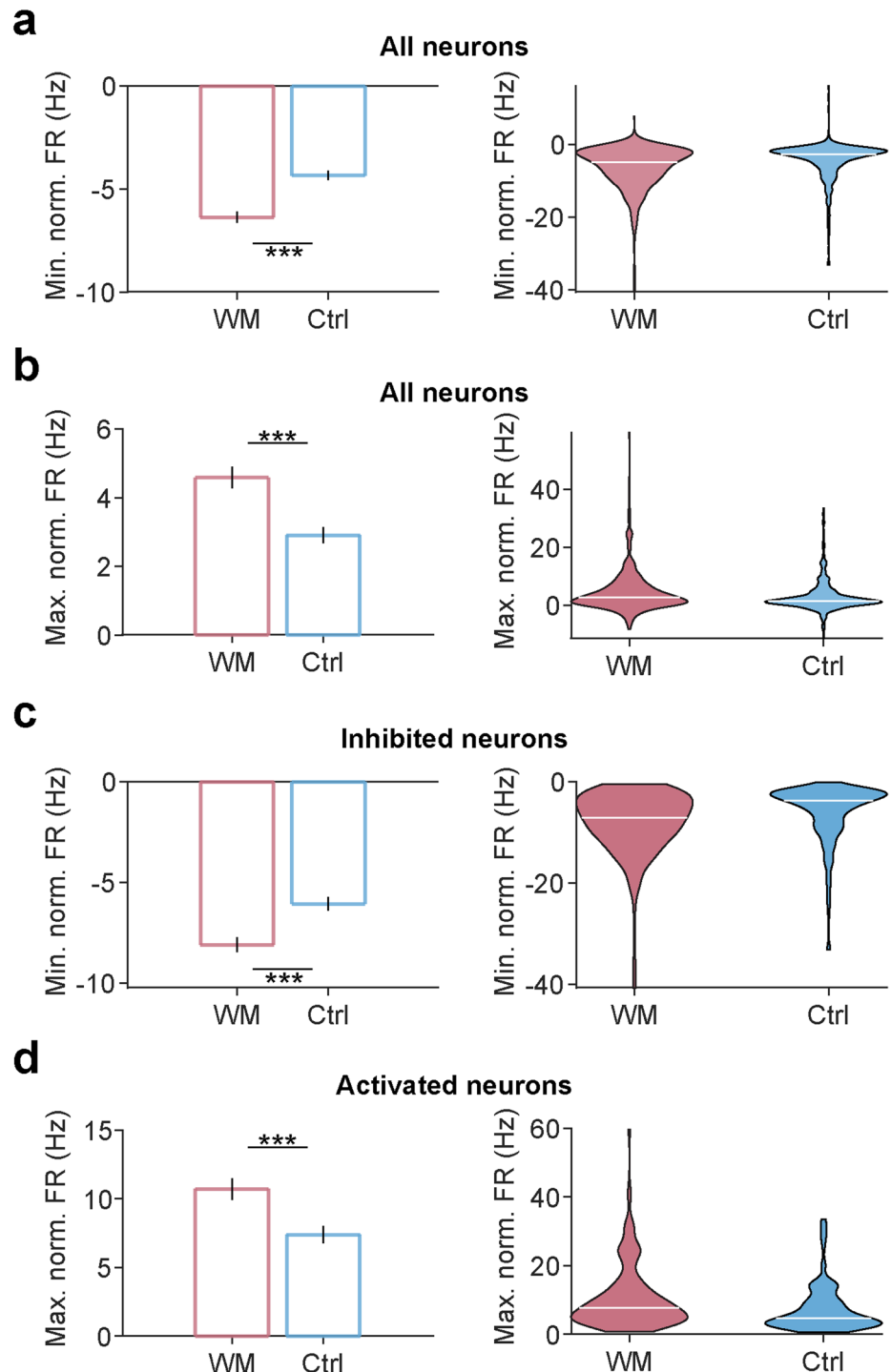
analyses of spike shapes did not reveal significant delay response differences across three groups of MS neurons (Supplementary Fig. S9–10).

Larger firing rate responses of MS neurons during WM engagement

Visual inspection of Fig. 4a–b (left) suggests that firing rate changes in the delay period led to more extreme values, manifested as darker yellows and blues corresponding to larger magnitude firing rate decreases and increases, respectively, in the DR-2AFC WM task compared to the Control task. Similar differences could be observed by comparing clusters #1 and #2 across behavioral conditions (Supplementary Fig. S7–8). To more quantitatively follow up on this observation, we compared the minima and maxima of the firing rate of MS neurons in the delay period. We found that, in line with the above intuition, MS neurons showed larger magnitude firing rate decreases and increases with respect to their baseline firing rate during the delay period when the task contained a WM component (change in average minimum firing rate, delay, WM mice, -6.373 ± 0.257 Hz; Control mice, -4.344 ± 0.225 Hz; $p = 7.934 \times 10^{-12}$, Mann-Whitney U-test; change in average maximum firing rate, delay, WM mice, 4.583 ± 0.308 Hz; Control mice, 2.902 ± 0.231 Hz; $p = 3.052 \times 10^{-6}$, Mann-Whitney U-test; Fig. 5a–b, Supplementary Fig. S12a–d). This was due to a larger magnitude firing rate decrease in MS neurons inhibited during the delay, and larger firing rate increases in MS neurons activated during the delay (change in average minimum firing rate, delay, inhibited neurons, WM mice, -8.105 ± 0.364 Hz; Control mice, -6.068 ± 0.332 Hz; $p = 1 \times 10^{-7}$, Mann-Whitney U-test; session-level analysis: $p = 2.7 \times 10^{-4}$; change in average maximum firing rate, delay, activated neurons, WM mice, 10.709 ± 0.782 Hz; Control mice, 7.395 ± 0.621 Hz; $p = 0.0002$, Mann-Whitney U-test; session-level analysis: $p = 3.3 \times 10^{-6}$; Fig. 5c–d, Supplementary Fig. S12a–d).

A closer inspection of the time course of firing rate changes in the DR-2AFC WM and Control tasks (Fig. 4a–b) also suggests larger task-related differences in the second half of the delay period: more sustained firing rate inhibition and less sustained activation when the task included a WM component. We quantified this by comparing firing rates relative to the average baseline firing of each MS cell across the two tasks, separately during the first and second half of the delay period. Indeed, while relative MS firing rates were not significantly different in the first half, they were significantly lower in the second half of the delay during the WM task (change in average firing rate, 1st half of the delay period, WM mice, -1.032 ± 0.26 Hz; Control mice, -1.779 ± 0.199 Hz, $p = 0.061$; 2nd half of the delay period, WM mice, -1.985 ± 0.212 Hz; Control mice, -0.578 ± 0.189 Hz, $p = 6.335 \times 10^{-8}$, Mann-Whitney U-test; Fig. 6a–b). We repeated this analysis within each response category and found that inhibited MS neurons were more inhibited and activated MS neurons were less activated in the second half of the delay in the DR-2AFC WM task, underlying the overall lower relative MS firing rates in this task version (change in average firing rate, 1st half of the delay period, inhibited neurons, WM mice, -4.131 ± 0.267 Hz; Control mice, -3.857 ± 0.255 Hz, $p = 0.156$; activated neurons, WM mice, 4.432 ± 0.531 Hz; Control mice, 1.95 ± 0.369 Hz, $p = 3.571 \times 10^{-5}$; 2nd half of the delay period, inhibited neurons, WM mice, -3.48 ± 0.275 Hz; Control mice, -2.375 ± 0.223 Hz, $p = 3.966 \times 10^{-5}$, Mann-Whitney U-test; activated neurons, WM mice, -0.074 ± 0.48 Hz; Control mice, 3.282 ± 0.422 Hz, $p = 1.641 \times 10^{-7}$, Mann-Whitney U-test; non-responsive neurons, WM mice, -0.359 ± 0.171 Hz; Control mice, 0.171 ± 0.151 Hz, $p = 0.024$, Mann-Whitney U-test; Fig. 6c–d). A direct comparison of MS firing rate distributions across the two tasks in the first and second half of the delay confirmed the larger task-related differences in the second half, where the WM component was more prominent, especially for the inhibited group of MS neurons (Figs. 2 and 6e-way ANOVA, Task type $\times$ Delay period epoch; significant interaction: $p = 0.037$ for inhibited neurons and $p = 4.113 \times 10^{-9}$ for activated neurons after Holm-correction; LME, Task type $\times$ Delay period epoch interaction: $F(1, 1194) = 2.75$, $p = 0.097$ for inhibited neurons and $F(1, 524) = 33.32$, $p = 1.3 \times 10^{-8}$ for activated neurons; session-level analysis for inhibited neurons: $p = 0.156$ and for activated neurons: $p = 3.5 \times 10^{-10}$; Fig. 6f, ROC analysis: $p < 0.01$ for inhibited neurons in the window 0.47–0.973 s and for activated neurons in windows 0.2–0.47 s, with larger firing rates in the WM task, and 0.7067–1 s, with larger firing rates in the Control task). Thus, while the dominant response in the MS during the delay was a

Fig. 5 Larger firing rate responses of MS neurons during WM engagement. **(a)** Mean ($\pm$ SE) (left) and distribution (right) of minimum firing rates in the delay period (relative to baseline) for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). **(b)** Mean ($\pm$ SE) (left) and distribution (right) of maximum firing rates in the delay period (relative to baseline) for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). **(c)** Mean ($\pm$ SE) (left) and distribution (right) of minimum firing rates in the delay period (relative to baseline) for inhibited MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). **(d)** Mean ($\pm$ SE) (left) and distribution (right) of maximum firing rates in the delay period (relative to baseline) for activated MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). ***, $p < 0.001$; Mann–Whitney U-test.



suppression of firing rates in both tasks, this delay-specific inhibition of firing was both larger and more sustained when the trial type information had to be maintained in working memory.

Theta-rhythmic MS activity is suppressed during WM engagement

The MS contains a population of neurons that show rhythmicity with a frequency within the theta band⁵⁰. These neurons are mostly GABAergic and they are thought to drive the hippocampal theta oscillation through rhythmic disinhibition of hippocampal pyramidal neurons^{1,9,16}.

We tested whether theta-rhythmic activity within the MS depended on the presence of a WM component in the response delay period. We first characterized MS neurons as non-theta-rhythmic, moderately theta-rhythmic and strongly theta-rhythmic using an autocorrelation-based rhythmicity index (Theta Index, see Methods; Supplementary Fig. S13a-b, Fig. 7a). We found that there was a larger MS population that showed strong theta rhythmicity in the DR-2AFC Control experiment than in the WM task (6% vs. 2%, $p=0.0145$, Chi-square test; Chi-square statistic, 8.466; Degrees of freedom, 2).

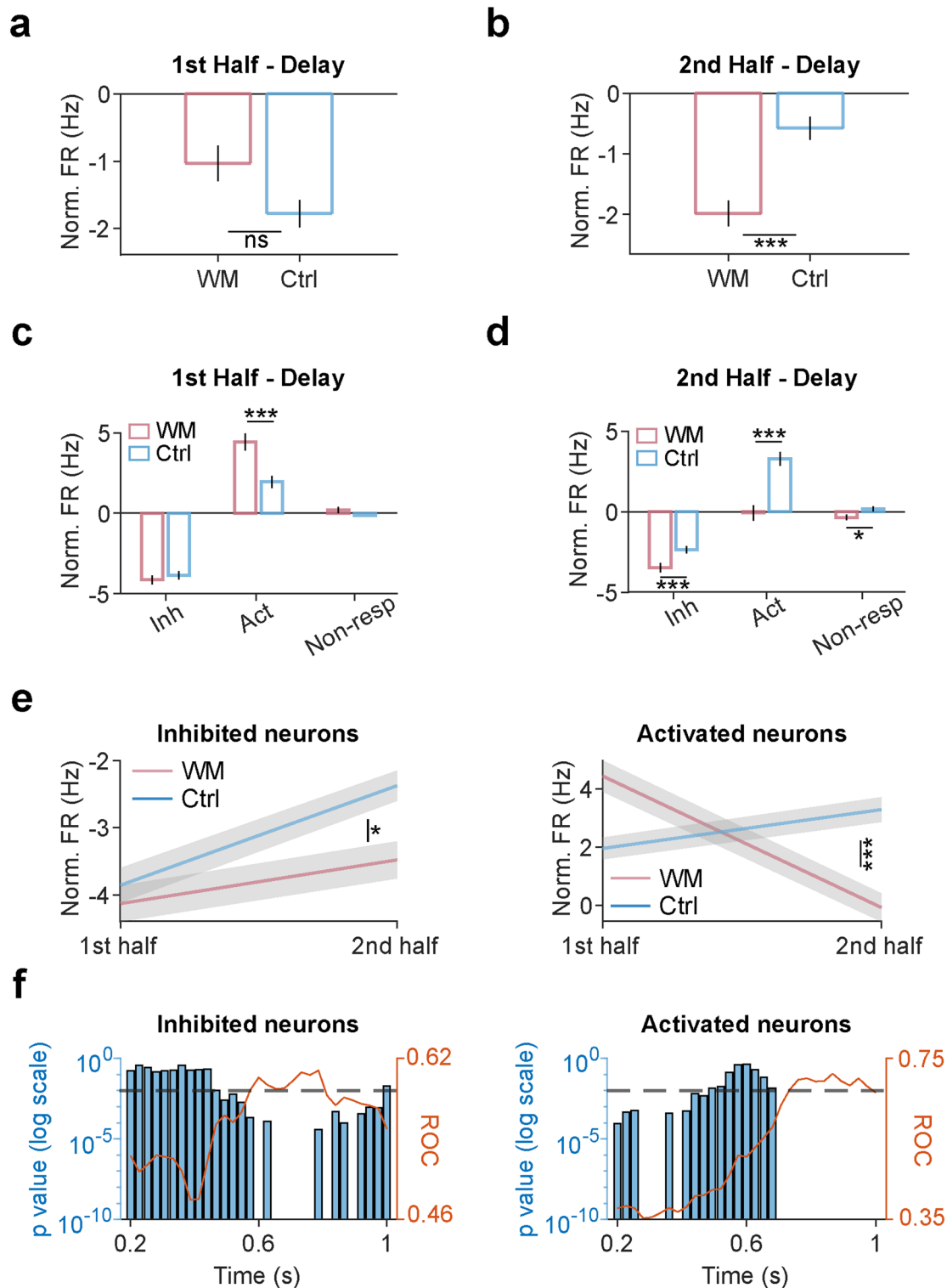
Next, we tested the activity of MS neurons partitioned according to their theta rhythmicity. While most groups were dominated by firing rate inhibition during the delay in both task versions like the overall MS population (see Figs. 4, 5 and 6), the most prominent task-related difference was observed in the strongly theta-rhythmic group. Specifically, this group showed firing rate suppression during the delay period when the task contained a WM component, whereas they were moderately activated in the Control task (change in average firing rate, delay period, WM mice, -1.903 ± 0.343 Hz; Control mice, 0.423 ± 0.173 Hz, $p=4.87 \times 10^{-6}$, 1st half of the delay period, WM mice, -1.438 ± 0.394 Hz; Control mice, 0.22 ± 0.174 Hz, $p=4.342 \times 10^{-4}$; 2nd half of the delay period, WM mice, -2.367 ± 0.342 Hz; Control mice, 0.625 ± 0.208 Hz, $p=2.35 \times 10^{-6}$, Mann-Whitney U-test; Fig. 7b-d; Supplementary Fig. S14). The suppression of activity of strongly theta rhythmic MS neurons in the DR-2AFC WM task was consistent across recording sessions and correct, incorrect and aborted trials (Supplementary Fig. S15). Task-related theta power changes in the MS local field potential (LFP) revealed a suppression of theta-band power during the delay period in both tasks, consistent with the observed reduction in theta-rhythmic neuronal activity when the larger pool of moderately theta-rhythmic neurons was considered (Fig. 7b-c; Supplementary Fig. S16).

Thus, there were more strongly theta-rhythmic neurons, which were also more active during the delay when the task did not engage WM, suggesting that MS theta-rhythmic activity is suppressed during WM engagement.

Discussion

The hippocampal formation was shown to play a critical role in spatial WM processes^{51–53}. It was proposed that the hippocampal theta oscillation is central to these functions, by orchestrating bouts of faster beta/gamma oscillations through cross-frequency coupling that is required to hold multiple items in WM⁵⁵. Therefore, it was assumed that the medial septum, providing strong subcortical input to the hippocampus that plays a major role in pacing theta oscillation, is also necessary to the WM functions of the hippocampus. Indeed, lesions to the MS or pharmacological inhibition of its cholinergic system lead to an impairment of WM maintenance^{3,5,7,54}. Moreover, we recently demonstrated that the MS is a critical regulator of hippocampal beta/gamma oscillations as well⁵⁵, likely influencing the theta-gamma cross-frequency coupling thought to underlie working memory functions⁵⁶.

However, the hippocampal theta oscillation is also deeply linked to spatial navigation and exploration⁵⁷, complicating the interpretation of studies where navigational and WM components are not separated, that is, an animal must translate between distant locations while solving the WM task. Nevertheless, a recent study demonstrated that spatial coding and WM may be separable at the level of the septohippocampal system, suggesting that these two cognitive functions can be investigated independently²⁹. Therefore, to address the neural mechanisms of WM independent of navigation, we designed a behavioral task where mice engaged in a sound-cued spatial WM task



without the need for running from A to B, solving the task with minimal full-body movement. We compared this to a control task that was the same in all aspects except for the WM component, since the auditory cues remained available until the animals made their choices. We recorded the activity of MS neurons while mice performed these tasks to address the neural mechanisms whereby the MS contributes to hippocampal WM functions.

Fig. 6 Lower relative MS firing rates in the second half of the delay during the WM task. **(a)** Mean ($\pm$ SE) of firing rates relative to baseline in the first half of the delay period for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). **(b)** Mean ($\pm$ SE) of firing rates relative to baseline in the second half of the delay period for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue). **(c)** Mean ($\pm$ SE) of firing rates relative to baseline in the first half of the delay period for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue), as a function of response category. **(d)** Mean ($\pm$ SE) of firing rates relative to baseline in the second half of the delay period for MS neurons in DR-2AFC WM task (red) and DR-2AFC Control task (blue), as a function of response category. **(e)** Comparison of mean ($\pm$ SE) firing rates relative to baseline for inhibited MS neurons (left) and activated MS neurons (right) in DR-2AFC WM task (red) and DR-2AFC Control task (blue) between first half and second half of the delay period (2-way ANOVA test, Task type $\times$ Delay period epoch). Interaction p-values (Holm-corrected) show significant divergence in temporal dynamics between conditions. *, $p < 0.05$; ***, $p < 0.001$. **(f)** ROC and p values (on log scale) for each time bin during the delay period, comparing firing rates (relative to baseline) of inhibited (left) and activated (right) MS neurons in the DR-2AFC WM task vs. the DR-2AFC Control task. The dashed blue line represents $p = 0.01$. *, $p < 0.05$; ***, $p < 0.001$; Mann-Whitney U-test.

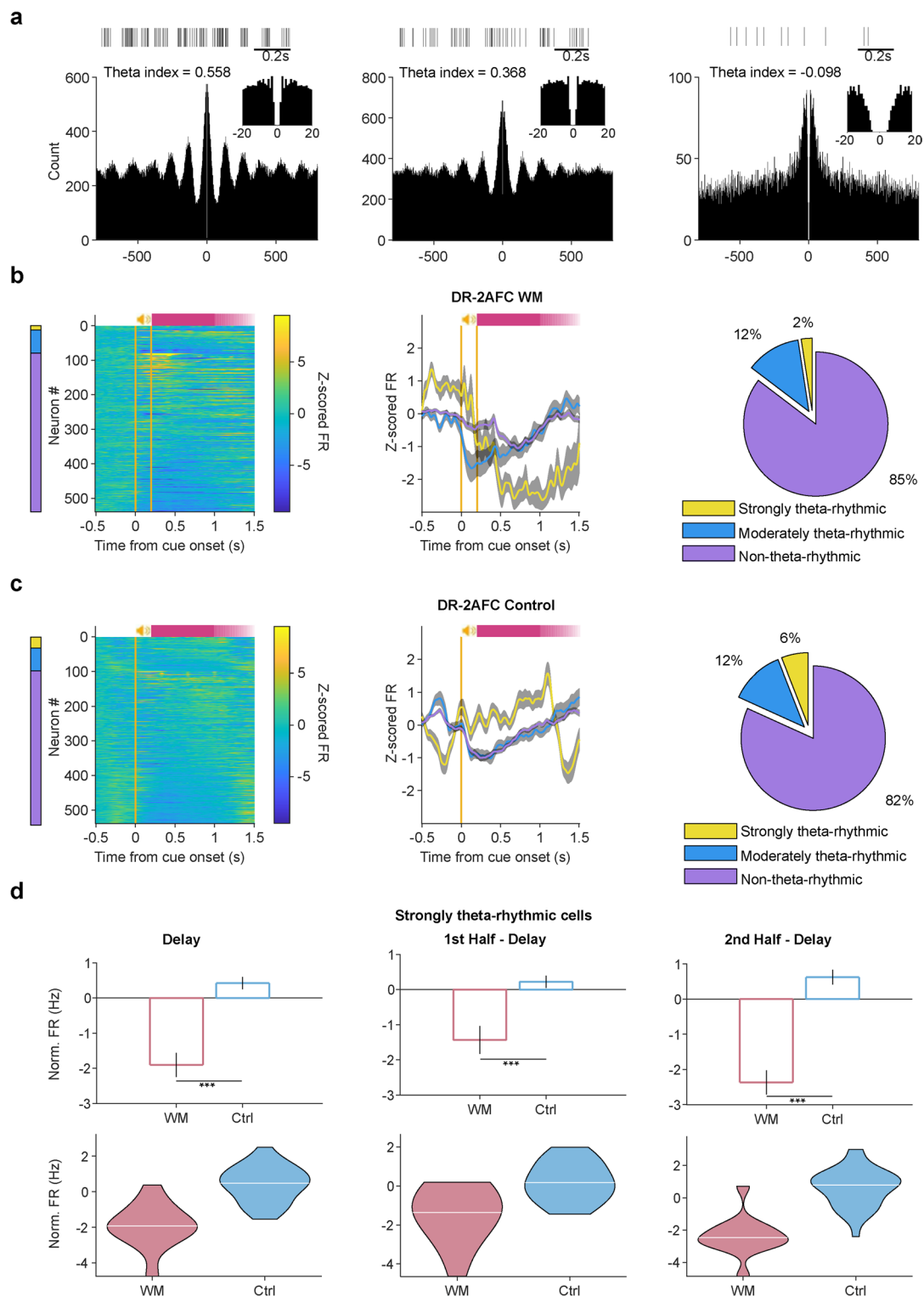
First, we found that MS neurons were overall more active in the task version with WM component. However, this was not restricted to the delay period when WM was engaged but continued through all trial phases. It is conceivable that the higher activity of MS neurons in the WM task was a consequence of overall higher arousal due to the substantial cognitive load compared to the control task. Since the MS is part of the ascending arousal system receiving prominent inputs from the locus coeruleus, the reticular pontis oralis and the brainstem nucleus incertus, such an effect was not unexpected^{58–62}. Alternatively, given the limited number of mice recorded, these overall firing rate differences could also be attributed to potential sampling effects.

In contrast, we found that during stimulus processing and WM delay the dominant MS neuronal response was a suppression of activity. This may suggest that the MS has an enabling function in WM processing, shifting the balance of hippocampal computations from septal to other, probably entorhinal inputs. This is supported by the findings that WM needs active coordination of hippocampal, entorhinal and prefrontal processes^{19,20,63,64}. In line with this, the inhibitory MS responses were sustained and maintained until about the end of the delay period in the WM task, while they were shorter and more transient in the control task. This also rules out the possibility that these firing rate changes represented a simple stimulus response evoked by the auditory cues, since the cues stayed on during the entire delay period in the Control but not the WM task, opposite to the duration of the inhibition. However, we note that the response of the smaller activated population of MS neurons could be related to stimulus-evoked firing.

A recent report demonstrated that MS cholinergic firing can induce brain state-dependent suppression of other MS neurons⁶⁵; therefore, it is conceivable that a task phase-dependent local cholinergic effect contributed to the observed MS firing rate differences (but see⁶⁶). Glutamatergic MS neurons were shown to control locomotion^{12,67}, and thus, the MS firing suppression may be related to the mouse holding still in the center port during the delay period. However, this cannot explain the difference between the WM and the Control task, which should therefore be related to the WM component.

When examining specific response types in the MS, we again confirmed the dominance of inhibitory responses but also found that about a third of MS neurons were activated during the delay period. We observed a surprising level of conservations of response type distributions across MS neurons, both by comparing statistically-defined response types and response clusters based on an unbiased PCA clustering approach. This may suggest that response types correspond to cell types, input connectivity or other anatomical features that remain stable across different tasks. Along these lines, one might speculate that the inhibited cells are glutamatergic, being the most numerous MS cell type^{68,69}.

The overall higher firing rates in the WM task were present across all major response categories of MS neurons (inhibited, activated, non-responsive), arguing for a role of an activating bottom-up input that targets multiple MS cell types, like the arousing afferents mentioned above^{58–60}. However, we also found that both the inhibited and the activated groups of MS neurons showed larger magnitude responses in the WM task. This may suggest that either cell-type specific inputs or local connections of the MS were also engaged by the WM component^{69–71}.



A comparison of the first and second half of the delay period revealed a stronger divergence of MS activity between the two tasks in the late delay period when the WM component was more prominent. Specifically, firing-rate suppression was more sustained in the WM task than in the Control task, with activated neurons exhibiting greater suppression relative to baseline during the late delay period. It is conceivable that the GABAergic hippocampo-septal feedback projections mediate this effect^{72–74}, relaying WM ‘needs’ to the MS that in turn

Fig. 7 Theta-rhythmic MS activity was suppressed during WM engagement. **(a)** Auto-correlograms of DR-2AFC WM example cells of strongly theta rhythmic cells (left), moderately theta rhythmic cells (middle), and non-theta rhythmic cells (right) with insets showing an example spike train and autocorrelation refractory period. The theta index for each example cell is displayed in the auto-correlograms. **(b)** Left, smoothed PETHs of all MS neurons in the DR-2AFC WM task grouped by theta index shown as a heatmap (yellow, high firing; blue, low firing). The purple bar indicates the delay period. Middle, PETH averages per group. Right, pie chart representation of theta index groups distribution. **(c)** The same as in panel b, but for MS neurons recorded in the DR-2AFC Control task. **(d)** Mean ($\pm$ SE) (top) and distribution (bottom; white line, median) of firing rates relative to baseline of strongly theta-rhythmic MS neurons in the delay period (left), first half of the delay period (middle), and second half of the delay period (right) in the DR-2AFC WM task (red) and the DR-2AFC Control task (blue). ***, $p < 0.001$; Mann–Whitney U-test.

enables WM processing through hippocampal-neocortical loops^{19,20,63,64}. However, this is not supported by our recent finding that hippocampo-septal feedback suppresses the generation of theta-nested gamma oscillations in the hippocampal CA1⁷⁵, potentially related to WM processes^{56,75}.

Finally, we analyzed the strongly theta-rhythmic MS population. Based on earlier literature, this population likely consists predominantly of parvalbumin-expressing GABAergic neurons^{9,14,15} that pace hippocampal theta by providing rhythmic disinhibition to the network^{1,9,27} through a Huygens-synchronization mechanism¹⁶. We found that fewer MS neurons showed strong theta-bursting, and they were also less active during the delay in the WM task. This was surprising in the context that hippocampal WM processes were shown to be linked with theta oscillation^{19,22,23,56}, but consistent with our previous assumption that the MS firing suppression may largely involve MS glutamatergic neurons that provide necessary excitatory drive for theta-bursting^{16,71}. Moderately theta-rhythmic MS neurons showed a firing rate suppression during the delay in both tasks, consistent with a theta-band power decrease observed in MS LFP recordings. These findings underscore the importance of separating spatial navigation and WM functions by our task²⁹, as movement-related theta could have masked this effect and could have been erroneously attributed to WM processing. It is conceivable that both the larger MS firing rates in the late delay period and the activation of strongly theta-rhythmic MS neurons in the control task are related to sensory processing induced by the continuously presented cue tone. Of note, these results do not contradict theta-related hippocampal activity being important to WM, since theta-rhythmicity remained strongly present in the MS during WM delay (see the moderately theta-rhythmic group in Fig. 7).

In summary, we investigated MS neuronal firing during WM delay and found specific changes compared to an equivalent task without WM engagement. Most notably, MS neurons showed strong task-related modulation that was dominantly manifested as a decrease of activity during WM delay, accompanied by a suppression of theta-rhythmic MS activity. As a limitation of our study, we did not identify genetically defined MS cell types; however, theta-rhythmic firing properties and numerosity considerations provided tentative hints regarding neurotransmitter identity. An important next step will be to relate the observed firing patterns to neurotransmitter-defined MS cell types. Combining the present behavioral approach with cell type-specific recording and manipulation strategies will provide mechanistic insight into how neurotransmitter-defined MS populations contribute to working memory. An additional limitation of the present study is the between-subject design used to compare the WM and control tasks. While this approach avoided potential confounds associated with sequential training on multiple paradigms, it leaves open the possibility that differences in neural sampling, behavioral strategies, or other inter-animal factors contributed to the observed effects. Advances enabling stable longitudinal tracking of identified MS neurons across multiple behavioral paradigms may help resolve these issues more directly. Furthermore, future studies combining MS and hippocampal recordings may provide more specific information on septo-hippocampal integration during WM processing through analyses of hippocampal LFP dynamics and oscillatory coupling. In addition, establishing a direct causal relationship between MS activity and non-spatial WM performance in the present behavioral framework will be an important direction. In this context, recent optogenetic studies have already demonstrated that perturbation of MS-dependent theta coordination can impair non-spatial WM performance²⁹, supporting the idea that MS activity contributes to WM processes beyond navigation-related functions.

The MS has hitherto mostly been investigated in the context of theta genesis, and comparatively little is known about behavior-specific activity of MS neurons^{76–78}. The present study provides a departure point for investigating MS functions across different cognitive behaviors while simultaneously considering its specialized role in oscillogenesis.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-026-68172-8>.

Acknowledgements We thank Katalin Lengyel for technical assistance in anatomical methods. We thank the FENS-Kavli Network of Excellence for fruitful discussions. This work was supported by the Hungarian Brain Research Program NAP3.0 (NAP2022-I-1/2022) of the Hungarian Academy of Sciences, the “Lendület” LP2024-8/2024 grant by the Hungarian Academy of Sciences, the NKFIH K147097 grant of the National Research, Development and Innovation Office and the European Union project RRF-2.3.1-21-2022-00004 within the framework of the Artificial Intelligence National Laboratory.

Author contributions B.H. and N.S. conceived the project. N.S. and J.P.S. performed the experiments. M.A., N.S., J.P.S. and B.K. analyzed the data. B.H. supervised the project and obtained funding. B.H. and M.A wrote the manuscript. All authors reviewed and revised the manuscript.

Funding This work was supported by the Hungarian Brain Research Program NAP3.0 (NAP2022-I-1/2022) of the Hungarian Academy of Sciences, the “Lendület” LP2024-8/2024 grant by the Hungarian Academy of Sciences, the NKFIH K147097 grant of the National Research, Development and Innovation Office and the European Union project RRF-2.3.1-21-2022-00004 within the framework of the Artificial Intelligence National Laboratory.

Data availability The datasets generated and/or analysed during the current study are available in the Figshare repository, at <https://figshare.com/s/32ab0875c4ee2d710faf>(<https://figshare.com/s/32ab0875c4ee2d710faf>).

Declarations

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. Freund, T. F. & Antal, M. GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature* **336**, 403–405 (1988).
2. Vertes, R. P. & Kocsis, B. Brainstem-diencephalo-septohippocampal systems controlling the theta rhythm of the hippocampus. *Neuroscience* **81**, 893–926 (1997).
3. Thomas, G. J., Brito, G. N., Stein, D. P. & Berko, J. K. Memory and septo-hippocampal connections in rats. *J. Comp. Physiological Psychol.* **96**, 339–347 (1982).
4. Givens, B. S. & Olton, D. S. Cholinergic and GABAergic modulation of medial septal area: Effect on working memory. *Behav. Neurosci.* **104**, 849–855 (1990).
5. Parsons, D. S., Peagler, A., Barlow, T. S. & Harrell, L. E. Failure of chronic physostigmine to ameliorate working memory deficits after medial septal lesions. *Exp. Neurol.* **96**, 456–461 (1987).
6. Furey, M. L., Pietrini, P., Haxby, J. V. & Drevets, W. C. Selective effects of cholinergic modulation on task performance during selective attention. *Neuropsychopharmacology* **33**, 913–923 (2008).
7. Gemzik, Z. M., Donahue, M. M. & Griffin, A. L. Optogenetic suppression of the medial septum impairs working memory maintenance. *Learn. Mem.* **28**, 361–370 (2021).
8. Petsche, H., Stumpf, C. & Gogolak, G. The significance of the rabbit’s septum as a relay station between the midbrain and the hippocampus I. The control of hippocampus arousal activity by the septum cells. *Electroencephalogr. Clin. Neurophysiol.* **14**, 202–211 (1962).
9. Hangya, B., Borhegyi, Z., Szilagy, N., Freund, T. F. & Varga, V. GABAergic neurons of the medial septum lead the hippocampal network during theta activity. *J. Neurosci.* **29**, 8094–8102 (2009).
10. Viney, T. J. et al. Shared rhythmic subcortical GABAergic input to the entorhinal cortex and presubiculum. *eLife* **7**, 1–35 (2018).
11. Takeuchi, Y. et al. The medial septum as a potential target for treating brain disorders associated with oscillopathies. *Front. Neural Circuits* **15**, 1–24 (2021).
12. Mocellin, P. et al. A septal-ventral tegmental area circuit drives exploratory behavior. *Neuron*. <https://doi.org/10.1016/j.neuron.2023.12.016> (2024).
13. Zaborszky, L., van den Pol, A. & Gyengesi, E. The basal forebrain cholinergic projection system in mice. In *The Mouse Nervous System* (eds Watson, C., Paxinos, G. &uelles, L.) 684–718 (Elsevier, Amsterdam, 2012). <https://doi.org/10.1016/B978-0-12-369497-3.10028-7>.

14. Borhegyi, Z. et al. Phase segregation of medial septal GABAergic neurons during hippocampal theta activity. *J. Neurosci.* **24**, 8470–8479 (2004).
15. Varga, V. et al. The presence of pacemaker HCN channels identifies theta rhythmic GABAergic neurons in the medial septum. *J. Physiol.* **586**, 3893–3915 (2008).
16. Kocsis, B. et al. Huygens synchronization of medial septal pacemaker neurons generates hippocampal theta oscillation. *Cell. Rep.* **40**, 111149 (2022).
17. Kamiński, J., Brzezicka, A., Mamelak, A. N. & Rutishauser, U. Combined phase-rate coding by persistently active neurons as a mechanism for maintaining multiple items in working memory in humans. *Neuron* **106**, 256–264e3 (2020).
18. ten Oever, S., De Weerd, P. & Sack, A. T. Phase-dependent amplification of working memory content and performance. *Nat. Commun.* **11**, 1832 (2020).
19. Jones, M. W. & Wilson, M. A. Theta rhythms coordinate hippocampal–prefrontal interactions in a spatial memory task. *PLoS Biol.* **3**, e402 (2005).
20. Stout, J. J., George, A. E., Kim, S., Hallock, H. L. & Griffin, A. L. Using synchronized brain rhythms to bias memory-guided decisions. *eLife* **12**, 1–23 (2024).
21. Ursino, M., Cesaretti, N. & Pirazzini, G. A Model of working memory for encoding multiple items and ordered sequences exploiting the theta-gamma code. In *Cognitive Neurodynamics*, vol. 17 (Springer Netherlands, 2023).
22. Daume, J. et al. Control of working memory by phase–amplitude coupling of human hippocampal neurons. *Nature* **629**, 393–401 (2024).
23. Brooks, H. et al. Assessing the longitudinal relationship between theta-gamma coupling and working memory performance in older adults. *Cereb. Cortex.* **32**, 1653–1667 (2022).
24. Roland, J. J. et al. Medial septum-diagonal band of broca (MSDB) GABAergic regulation of hippocampal acetylcholine efflux is dependent on cognitive demands. *J. Neurosci.* **34**, 506–514 (2014).
25. Schlesiger, M. I. et al. Two septal-entorhinal GABAergic projections differentially control coding properties of spatially tuned neurons in the medial entorhinal cortex II II Two septal-entorhinal GABAergic projections differentially control coding properties of spatially tuned n. *CellReports* **34**, 108801 (2021).
26. Grastyán, E., Karmos, G., Vereczkey, L. & Kellényi, L. The hippocampal electrical correlates of the homeostatic regulation of motivation. *Electroencephalogr. Clin. Neurophysiol.* **21**, 34–53 (1966).
27. Buzsáki, G. *Rhythms of the Brain* (Oxford University Press, 2006). <https://doi.org/10.1093/acprof:oso/9780195301069.001.0001>.
28. Buzsáki, G. Theta oscillations in the hippocampus. *Neuron* **33**, 325–340 (2002).
29. Etter, G., van der Veldt, S., Choi, J. & Williams, S. Optogenetic frequency scrambling of hippocampal theta oscillations dissociates working memory retrieval from hippocampal spatiotemporal codes. *Nat. Commun.* **14**, 410 (2023).
30. Yee, B. K. & Rawlins, J. N. P. A comparison between the effects of medial septal lesions and entorhinal cortex lesions on performance of nonspatial working memory tasks and reversal learning. *Behav. Brain. Res.* **94**, 281–300 (1998).
31. Kelsey, J. E. & Vargas, H. Medial septal lesions disrupt spatial, but not nonspatial, working memory in rats. *Behav. Neurosci.* **107**, 565–574 (1993).
32. Pastalkova, E., Itskov, V., Amarasingham, A. & Buzsáki, G. Internally generated cell assembly sequences in the rat hippocampus. *Sci. (New York N Y).* **321**, 1322–1327 (2008).
33. Kvitsiani, D. et al. Distinct behavioural and network correlates of two interneuron types in prefrontal cortex. *Nature* **498**, 363–366 (2013).
34. Hangya, B., Ranade, S. P., Lorenc, M. & Kepecs, A. Central cholinergic neurons are rapidly recruited by reinforcement feedback. *Cell* **162**, 1155–1168 (2015).
35. Laszlovszky, T. et al. Distinct synchronization, cortical coupling and behavioral function of two basal forebrain cholinergic neuron types. *Nat. Neurosci.* **23**, 992–1003 (2020).
36. Hegedüs, P., Heckenast, J. & Hangya, B. Differential recruitment of ventral pallidal e-types by behaviorally salient stimuli during Pavlovian conditioning. *iScience* **24**, 102377 (2021).
37. Hegedüs, P., Velencei, A., de Belval, C. H., Heckenast, J. & Hangya, B. Training protocol for probabilistic Pavlovian conditioning in mice using an open-source head-fixed setup. *STAR. Protocols.* **2**, 100795 (2021).
38. Solari, N., Sviatkó, K., Laszlovszky, T., Hegedüs, P. & Hangya, B. Open source tools for temporally controlled rodent behavior suitable for electrophysiology and optogenetic manipulations. *Front. Syst. Neurosci.* **12**, 18 (2018).
39. Lind, J., Enquist, M. & Ghirlanda, S. Animal memory: A review of delayed matching-to-sample data. *Behav. Process.* **117**, 52–58 (2015).
40. Dudchenko, P. A. An overview of the tasks used to test working memory in rodents. *Neurosci. Biobehavioral Reviews.* **28**, 699–709 (2004).
41. Széll, A., Martínez-Bellver, S., Hegedüs, P. & Hangya, B. OPETH: Open source solution for real-time peri-event time histogram based on open ephys. *Front. Neuroinformatics.* **14**, 1–19 (2020).
42. Ferguson, J. E., Boldt, C. & Redish, A. D. Creating low-impedance tetrodes by electroplating with additives. *Sens. Actuators A: Phys.* **156**, 388–393 (2009).
43. Paxinos, G. & Franklin, K. B. J. *Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates* (Academic, 2019).

44. Hegedüs, P., Sviatkó, K., Király, B., Martínez-Bellver, S. & Hangya, B. Cholinergic activity reflects reward expectations and predicts behavioral responses. *iScience* **26**, 105814 (2023).
45. Király, B. et al. In vivo localization of chronically implanted electrodes and optic fibers in mice. *Nat. Commun.* **11**, 4686 (2020).
46. Jun, J. J. et al. Fully integrated silicon probes for high-density recording of neural activity. *Nature* **551**, 232–236 (2017).
47. Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B. & Uchida, N. Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature* **482**, 85–88 (2012).
48. Sadacca, B. F., Jones, J. L. & Schoenbaum, G. Midbrain dopamine neurons compute inferred and cached value prediction errors in a common framework. *eLife* **5**, 1–13 (2016).
49. Király, B. & Hangya, B. Navigating the statistical minefield of model selection and clustering in neuroscience. *eNeuro* **9** (2022).
50. Ford, R. D., Colom, L. V. & Bland, B. H. The classification of medial septum-diagonal band cells as σ -on or σ -off in relation to hippocampal EEG states. *Brain Res.* **493**, 269–282 (1989).
51. Jeneson, A. & Squire, L. R. Working memory, long-term memory, and medial temporal lobe function. *Learn. Mem.* **19**, 15–25 (2012).
52. Yonelinas, A. P. The hippocampus supports high-resolution binding in the service of perception, working memory and long-term memory. *Behav. Brain. Res.* **254**, 34–44 (2013).
53. Aggleton, J. P., Hunt, P. R. & Rawlins, J. N. P. The effects of hippocampal lesions upon spatial and non-spatial tests of working memory. *Behav. Brain. Res.* **19**, 133–146 (1986).
54. Solari, N. & Hangya, B. Cholinergic modulation of spatial learning, memory and navigation. *Eur. J. Neurosci.* **48**, 2199–2230 (2018).
55. Király, B. et al. The medial septum controls hippocampal supra-theta oscillations. *Nat. Commun.* **14**, 6159 (2023).
56. Axmacher, N. et al. Cross-frequency coupling supports multi-item working memory in the human hippocampus. *Proc. Natl. Acad. Sci.* **107**, 3228–3233 (2010).
57. Buzsáki, G. & Moser, E. I. Memory, navigation and theta rhythm in the hippocampal-entorhinal system. *Nat. Neurosci.* **16**, 130–138 (2013).
58. Sweeney, J. E., Lamour, Y. & Bassant, M. H. Arousal-dependent properties of medial septal neurons in the unanesthetized rat. *Neuroscience* **48**, 353–362 (1992).
59. Berridge, C. W. Noradrenergic modulation of arousal. *Brain Res. Rev.* **58**, 1–17 (2008).
60. Zhang, G. W. et al. A non-canonical reticular-limbic central auditory pathway via medial septum contributes to fear conditioning. *Neuron* **97**, 406–417e4 (2018).
61. Szőnyi, A. et al. Brainstem nucleus incertus controls contextual memory formation. *Science* **364**, eaaw0445 (2019).
62. Ma, S. et al. Modulation of hippocampal theta oscillations and spatial memory by relaxin-3 neurons of the nucleus incertus. *Learn. Mem.* **16**, 730–742 (2009).
63. Dorian, C. C., Taxidis, J., Arac, A. & Golshani, P. Behavioral timescale synaptic plasticity in the hippocampus creates non-spatial representations during learning and is modulated by entorhinal inputs. *bioRxiv*. <https://doi.org/10.1101/2024.08.27.609983> (2025).
64. Li, J. et al. Theta–alpha connectivity in the hippocampal–entorhinal circuit predicts working memory load. *J. Neurosci.* **44**, e0398232023 (2024).
65. Mamad, O., McNamara, H. M., Reilly, R. B. & Tsanov, M. Medial septum regulates the hippocampal spatial representation. *Front. Behav. Neurosci.* **9**, 1–16 (2015).
66. Wu, M., Shanabrough, M., Leranth, C. & Alreja, M. Cholinergic excitation of septohippocampal GABA but not cholinergic neurons: implications for learning and memory. *J. Neurosci.* **20**, 3900–3908 (2000).
67. Fuhrmann, F. et al. Locomotion theta oscillations, and the speed-correlated firing of hippocampal neurons are controlled by a medial septal glutamatergic circuit. *Neuron* **86**, 1253–1264 (2015).
68. Gritti, I. et al. Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. *Neuroscience* **143**, 1051–1064 (2006).
69. Yang, C., Thankachan, S., McCarley, R. W. & Brown, R. E. The menagerie of the basal forebrain: how many (neural) species are there, what do they look like, how do they behave and who talks to whom? *Curr. Opin. Neurobiol.* **44**, 159–166 (2017).
70. Xu, M. et al. Basal forebrain circuit for sleep-wake control. *Nat. Neurosci.* **18**, 1641–1647 (2015).
71. Hajszan, T., Alreja, M. & Leranth, C. Intrinsic vesicular glutamate transporter 2-immunoreactive input to septohippocampal parvalbumin-containing neurons: Novel glutamatergic local circuit cells. *Hippocampus* **14**, 499–509 (2004).
72. Toth, K., Borhegyi, Z. & Freund, T. F. Postsynaptic targets of GABAergic hippocampal neurons in the medial septum-diagonal band of Broca complex. *J. Neurosci.* **13**, 3712–3724 (1993).
73. Damborsky, J. C. & Yakel, J. L. Regulation of hippocamposeptal input within the medial septum/diagonal band of Broca. *Neuropharmacology* **191**, 108589 (2021).

74. Mattis, J. et al. Frequency-dependent, cell type-divergent signaling in the hippocamposeptal projection. *J. Neurosci.* **34**, 11769–11780 (2014).
75. Lisman, J. The theta/gamma discrete phase code occurring during the hippocampal phase precession may be a more general brain coding scheme. *Hippocampus* **15**, 913–922 (2005).
76. Hangya, B. & Varga, V. Editorial The medial septum as a smart clock: New aspects of its function beyond pacemaking. *Front. Neural Circuits.* **16**, 1093711 (2023).
77. Mocellin, P. & Mikulovic, S. The role of the medial septum—associated networks in controlling locomotion and motivation to move. *Front. Neural Circuits* **15**, 1–14 (2021).
78. Griguoli, M. & Pimpinella, D. Medial septum: Relevance for social memory. *Front. Neural Circuits* **16**, 1–7 (2022).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Malek Aouadi^{1,2} · Nicola Solari¹ · Johanna Petra Szabó^{1,2,3} · Bálint Király^{1,4} · Balázs Hangya^{1,4,5}

✉ Balázs Hangya
balazs.hangya@meduniwien.ac.at

¹ MTA–HUN-REN KOKI Lendület “Momentum” Laboratory of Systems Neuroscience, HUN-REN Institute of Experimental Medicine, Budapest 1083, Hungary

² János Szentágothai Neurosciences Program, Semmelweis University School of PhD Studies, Budapest 1085, Hungary

³ Epilepsy Center, Clinic for Neurosurgery and Neurointervention, Semmelweis University, Budapest 1085, Hungary

⁴ Division of Neurophysiology, Center for Brain Research, Medical University of Vienna, 1090 Vienna, Austria

⁵ Subcortical Modulation Research Group, HUN-REN Institute of Experimental Medicine, Budapest 1083, Hungary