Supplementary Information for

Two distinct ways to form long-term object-recognition memory during sleep and wakefulness

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Supplementary methods

Animals. In all, 42 adult male Long-Evans rats (Janvier, Le Genest-Saint-Isle, France, 250–350 g, 10–12 weeks) were used for the experiments. Rats were housed in groups of 2–4 per cage, were kept on a 12-h light/12-h dark cycle (lights on at 6:00 h) and had unrestricted access to food and water throughout the experiments. Rats were handled daily for 5–10 min for at least 5 days before starting an experiment. All experimental procedures were performed in accordance with European animal protection laws and policies, and were approved by the Baden-Württemberg state authority.

Habituation procedure and behavioral apparatus. Before starting the NOR task, rats were brought into the test room once a day on three consecutive days to habituate them to the learning context and the sleep environment. The test room was equipped with masking noise (60 dB; light intensity between 20-30 lux). The habituation session started with an object-familiarization session (10 min) in which the rats were allowed to explore an object (not used for the proper experiments) positioned in the center of an empty animal cage. Then, the rats were placed in the empty, open field arena (80 cm × 80 cm, height of walls: 40 cm made of grey PVC) for 10 min and allowed to freely explore the open field and its surrounding distal cues (i.e. two posters on the North side, a white curtain on the East side, a black curtain on the South side, and two objects hanging on the West wall). Rats were then connected to the cannula infusion system and recording cable (only in the respective experiments), and left undisturbed for 2 h in a 'resting-box' (35 × 35 cm, height: 45 cm) made of grey plastic and containing some bedding material. The open field and resting-box were placed in the same experimental room, but were separated by a curtain. During the 1st and 2nd of these sleep habituation periods, three mock infusions (i.e., no substance administration) were performed during both sleep and wake epochs. For the last habituation, mock infusion was performed only once – upon the first occurrence of continuous sleep EEG activity. The procedure enabled substance administration to freely moving rats without disturbing ongoing activity (sleep or wake).

For the 'context-change' experiment (Figure 3a), the habituation procedure was similar, except that the rats habituated to two different contexts, with habituation to one context being followed by habituation to the other context throughout the three sessions. Specifically, after the 10-min object familiarization session, rats were introduced into the empty open field in context A for 10 min followed by 2 h in resting-box A. Then, the animals were placed in the empty open field in context B for 10 min, followed by a 2-h habituation session in resting-box B.

Enforced wakefulness. To enforce wakefulness in the 2-hour post-encoding period of the wake condition we used the standard gentle handling procedure which has been shown to minimize stress (1, 2). This is important because stress after encoding, through the accompanying release of the stress hormone corticosterone can alter consolidation processes (3). The gentle handling procedure involved gently tapping on the 'resting box' and, if necessary, gently shaking the box. No intense stimulation was used. Also, the rat and its bedding materials were not touched during the entire procedure. Arousal-interventions were introduced whenever the rat closed their eyes (with or without sleep posture) and was immobile for more than 5 sec. In general, the overall number of arousal-intervention to prevent the rat from sleeping during the 2-h post-encoding interval was < 20 per animal. Video recordings ensured that no signs of startle or freezing behavior occurred (see SI Appendix, Movie S1, Video for representative samples of gentle handling procedure). In fact, we and others showed that the gentle handling procedure, even when applied over periods longer than the 2-hour post-encoding period of the present experiments, does not induce substantial increases in blood levels of corticosterone, compared with undisturbed control animals (1, 2, 4–6), which excludes the presence of stress-related confounds in our wake group due to enforcing wakefulness in these animals.

Detection of slow oscillations and spindles. Slow oscillations (SOs) and spindles were identified in the EEG over the left frontal cortex. Procedures were adapted from Sawangjit et al. (2018). For identification of SOs, the animal's EEG signal during all SWS epochs was filtered between 0.3 and 4.5 Hz (Butterworth filter of 3rd order). An SO event was selected if the following

criteria were fulfilled: (i) two consecutive positive-to-negative zero crossings of the signal occurred at an interval between 0.8 and 2.0 s, (ii) of these events in an individual rat, the 35% with the highest negative peak amplitude between both zero crossings were selected, and (iii) of these events the 45% with the highest negative-to-positive peak-to-peak amplitude were selected. This algorithm resulted in the identification of SOs with negative peak amplitudes exceeding -120 μV and peak-to-peak amplitudes exceeding 220 μV. For the detection of spindle events the EEG was filtered between 10 and 16 Hz (Butterworth filter of 3rd order) and the Hilbert transform was calculated. The signal was then smoothed with a moving average (window size 200 ms). A spindle was identified when the absolute value of the transformed signal exceeded a threshold of 1.5 standard deviations of the mean signal during the animal's SWS epochs, for at least 0.4 s and for not more than 2.0 s.

Detected spindles were considered to be coupled with a SO when the maximum of the spindle amplitude was located within the two positive-to-negative zero crossings of the detected SO. In addition, time-frequency plots were calculated to further analyze the co-occurrence of SOs and spindles. For this, time-frequency analysis for frequencies between 5 and 20 Hz was performed in a window of ±3 s around the negative peak of all detected SOs, using the mtmconvol function of the FieldTrip toolbox (7). The analysis was done in steps of 0.5 Hz using a sliding Hanning tapered window with a variable, frequency-dependent length that always comprised 7 cycles. Time-locked power values for each frequency of each event were normalized by dividing the value by the average power during a prior baseline interval (-1.5 to -1.0 s relative to the negative SO peak) using the function ft_freqbaseline, baslinetype: "relative". Normalized values were then averaged across all events and animals (functions ft_timelockanalysis and ft_timelockgrandaverage).

Statistical analyses. To assess whether long-term memory in the NOR task differed between post-encoding Sleep vs. Wake conditions and between intrahippocampal Muscimol vs. Control conditions, a mixed-effects model was fitted with individual rats as random effect (random intercept

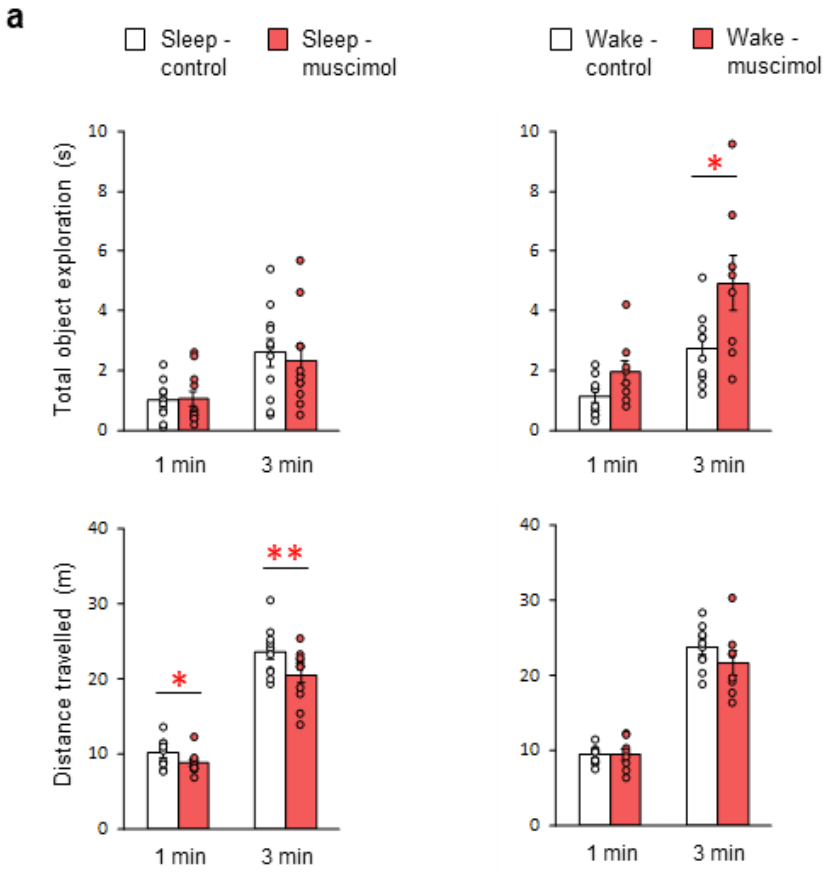
only) and Minute (1st, 3rd min of Retrieval phase), post-encoding interval (Sleep/Wake) and infused substance (Muscimol/Control) as fixed effects:

DR ~ (Minute * Sleep/Wake * Muscimol/Control + (1 |animal)

where *DR* indicates the discrimination ratio between familiar and novel object. Significance of the factors was assessed by removing the factor or interaction of two factors step by step from the model, and comparing the modified models with the original using likelihood-ratio tests. As testing of the nested models as described resulted in a significant Sleep/Wake x Muscimol/Control interaction, post-hoc tests were used to assess the differences between conditions. To this end, a mixed model with individual rats as random effect and Sleep/Wake and Muscimol/Control as fixed effects was fitted. In addition, Welch two-sample *t*-tests or paired *t*-tests were calculated to assess differences between Sleep vs. Wake and Muscimol versus Control conditions. To test discrimination ratios against chance level, one-sample *t*-tests were computed. The same mixed model approach was applied to parameters of exploratory rearing. For the 'context-change' experiment, the mixed model approach comprised Sleep/Wake and Minute (1st, 3rd min) as fixed effects and DR or percentage change in mean rearing duration as dependent variables. All post-hoc *t*-tests reported here were calculated two-sided.

Correlation analyses were performed between behavioral measures of memory on the one hand and sleep parameters (total time in SWS and REM sleep, numbers, density and power of spindles and SOs, numbers of coupled SO-spindle events and REM sleep theta power and energy) on the other. Spearman rank coefficients are reported to account for the small sample size and for their robustness against outliers. Coefficients were calculated for values of the 1st min of the Retrieval phase, unless otherwise indicated. No correction for multiple comparisons was introduced. In order to distinguish contributions of coupled SO-spindle events to memory performance from those associated with spindles that were not temporally linked to SOs, a linear regression model was fitted with the discrimination ratio as dependent, the number of SO-spindle events and the infusion condition (Muscimol/Control) as independent variables, and the number of non-coupled spindles as covariate. In this way, the effect of coupled SO-spindle events was assessed, while controlling for spindles that occurred in the absence of an SO.

Supplementary figures


a
Sleep - control
Sleep - muscimol
Wake - control
Wake - muscimol
Total object exploration (s)
Distance travelled (m)
1 min
3 min
*
**

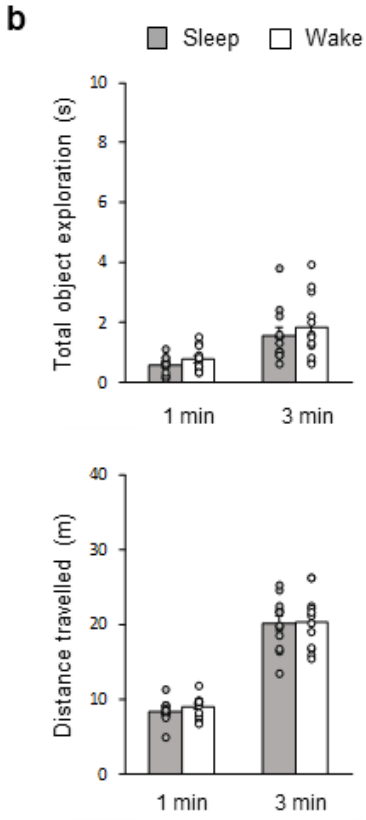
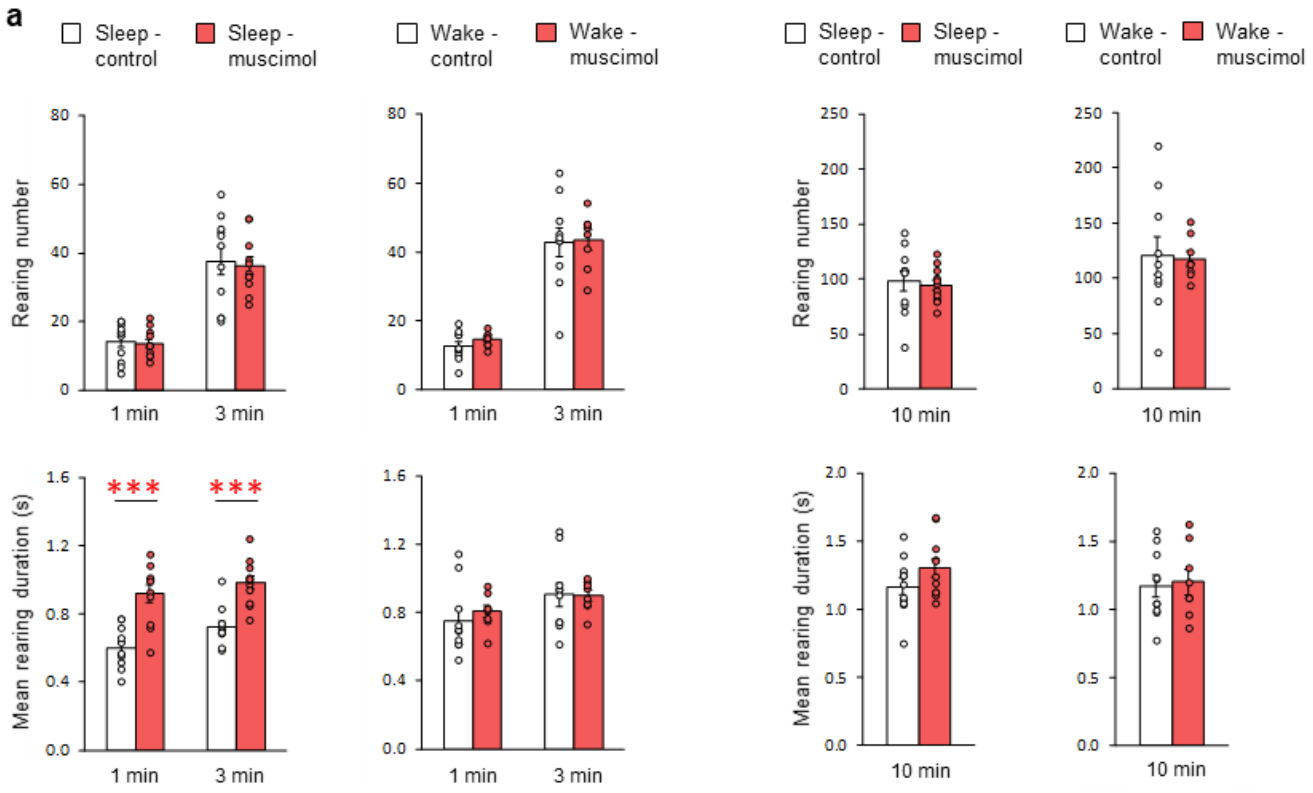

b
Sleep
Wake
Total object exploration (s)
Distance travelled (m)
1 min
3 min

Fig. S1. Control measures for NOR task performance. Mean (± s.e.m.) values (dot plots overlaid) for total object exploration (s) and total distance travelled (m) at the 1st and 3rd min of retrieval testing. a, For animals of the Sleep group (left) and Wake groups (right) with functioning hippocampus (empty bars) and following infusion of muscimol (red bars) during the 2-h post-encoding interval (corresponding to Figure 1b of the main text). n = 11, 11, 10, and 8 rats for Sleep control, Sleep muscimol, Wake control, and Wake muscimol groups, respectively. b, The same control parameters for animals of the Sleep (grey bars) and Wake groups (empty bars) in the experiments that tested NOR retrieval in a context different from that employed during encoding (corresponding to Figure 3b of the main text). n = 12 rats each for the Sleep and Wake conditions. **P < 0.01, *P < 0.05 for pairwise t-tests (two-sided) between Muscimol and Control groups.


a
Sleep - control
Sleep - muscimol
Wake - control
Wake - muscimol
Rearing number
Mean rearing duration (s)
1 min
3 min
10 min
***
***

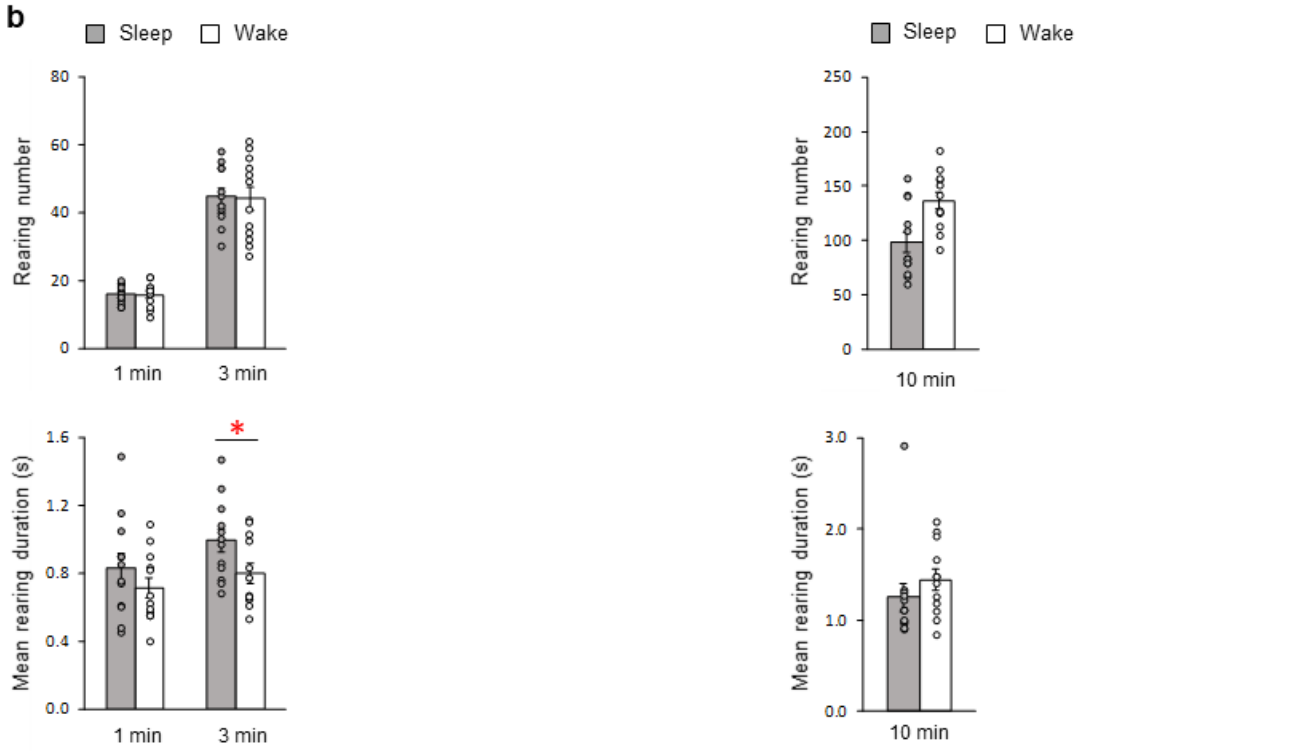

b
Sleep
Wake
Rearing number
Mean rearing duration (s)
1 min
3 min
10 min
*

Fig. S2. Exploratory rearing parameters. Mean values (± s.e.m., dot plots overlaid) for the number and mean duration (s) of rearing events at the 1st and 3rd min of retrieval testing (left panels) and the 10-min encoding phase (right panels). a, Values for animals of the Sleep group (left) and Wake group (right) with functioning hippocampus (empty bars) and following infusion of muscimol (red bars) during the 2-h post-encoding interval (corresponding to Figure 2b of the main text). n = 11, 11, 10, and 8 rats for Sleep control, Sleep muscimol, Wake control, and Wake muscimol groups, respectively. b, Values for the same control parameters for animals of the Sleep (grey bars) and Wake groups (empty bars) in the experiments testing NOR retrieval in a context different from that during encoding (corresponding to Figure 3b of the main text). n = 12 rats each for the Sleep and Wake conditions. **P < 0.01, *P < 0.05 for pairwise t-tests (two-sided) between Muscimol and Control groups.

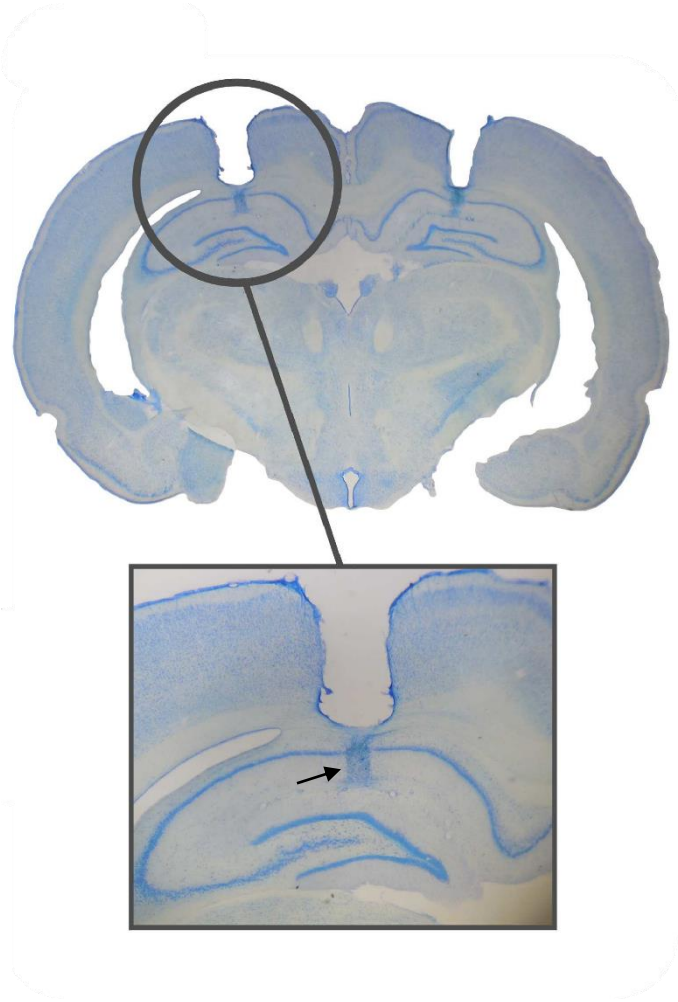


Fig. S3. Verification of cannula location. Coronal brain section showing the location of the cannula in both hemispheres. Inset shows the trace of the injection cannula in the dorsal hippocampus (black arrow).

Supplemental Tables

Table S1: Correlation analyses

	Vehicle		Muscimol	
	Object discrimination ratio	Mean rearing duration: Change from encoding (%)	Object discrimination ratio	Mean rearing duration: Change from encoding (%)
SWS duration (min)	*rho* = 0.571, *P* = 0.200	*rho* = - 0.536, *P* = 0.236	*rho* = 0.107, *P* = 0.840	*rho* = <0.01, *P* = 1
REM sleep duration (min)	*rho* = 0.721, *P* = 0.068	*rho* = - 0.216, *P* = 0.641	*rho* = 0.143, *P* = 0.783	*rho* = - 0.357, *P* = 0.444
Spindle number	***rho* = 0.782, *P* = 0.008**	*rho* = 0.146, *P* = 0.688	*rho* = 0.543, *P* = 0.105.	*rho* = - 0.030, *P* = 0.934
Spindle density (per min)	*rho* = 0.534, *P* = 0.112	*rho* = 0.273, *P* = 0.448	*rho* = 0.622, *P* = 0.054	*rho* = 0.273, *P* = 0.448
SO number	***rho* = 0.730, *P* = 0.017**	*rho* = <0.01, *P* = 1	***rho* = 0.665, *P* = 0.036**	*rho* = 0.200, *P* = 0.584
SO density (per min)	*rho* = 0.546, *P* = 0.103	*rho* = 0.285, *P* = 0.427	***rho* = 0.659, *P* = 0.038**	*rho* = 0.346, *P* = 0.331
SO mean amplitude (mV)	*rho* = 0.411, *P* = 0.238	*rho* = 0.103, *P* = 0.785	***rho* = 0.640, *P* = 0.046**	*rho* = 0.394, *P* = 0.262
Number of SO-spindle events	***rho* = 0.738, *P* = 0.015**	*rho* = -0.061, *P* = 0.868	***rho* = 0.706, *P* = 0.022**	*rho* = 0.237, *P* = 0.510
Theta Power (mV2/s)	*rho* = -0.055, *P* = 0.880	*rho* = - 0.212, *P* = 0.560	*rho* = 0.578, *P* = 0.133	*rho* = 0.095, *P* = 0.840

Summary of correlational analyses between memory measures (of the 1st minute) and sleep target variables, i.e. total duration of SWS and REM sleep, Spindle number and density, SO number, density and mean peak-to-peak amplitude, number of SO-spindle events, and mean theta power (n = 10). It should be noted that the reported correlational analyses are of exploratory nature given their low statistical power and, thus, cannot rule out existing correlations. For instance, none of the correlations between NOR recall or exploratory rearing during remote retrieval testing and duration of SWS or REM sleep reached statistical significance (*rho* < 0.721, *P* > 0.068), albeit showing a statistical trend for REM sleep duration ~ Object discrimination ratio in the control condition.

Supporting Movie

Movie S1. Gentle Handling Technique. Illustration of the gentle handle procedure, used to keep animals awake during the post-encoding interval in the wake conditions. The procedure involves gently tapping on the resting-box and, if necessary, gently shaking the box. Video snippets are shown separately for the first and second hour of the post-encoding interval, for both, the vehicle and muscimol conditions in one example animal.

SI References

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