Memory stabilization with targeted reactivation during human slow-wave sleep

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It is believed that neural representations of recent experiences become reactivated during sleep, and that this process serves to stabilize associated memories in long-term memory. Here, we initiated this reactivation process for specific memories during slow-wave sleep. Participants studied 50 object-location associations with object-related sounds presented concurrently. For half of the associations, the related sounds were re-presented during subsequent slow-wave sleep while participants underwent functional MRI. Compared with control sounds, related sounds were associated with increased activation of right parahippocampal cortex. Postsleep memory accuracy was positively correlated with sound-related activation during sleep in various brain regions, including the thalamus, bilateral medial temporal lobe, and cerebellum. In addition, postsleep memory accuracy was also positively correlated with pre- to postsleep changes in parahippocampal–medial prefrontal connectivity during retrieval of reactivated associations. Our results suggest that the brain is differentially activated by studied and unstudied sounds during deep sleep and that the thalamus and medial temporal lobe are involved in establishing the mnemonic consequences of externally triggered reactivation of associative memories.

consolidation | neuroimaging | EEG-functional MRI | replay

Memory consolidation involves the stabilization of memory traces for long-term retention (1–4). It is thought that sleep, especially deep sleep, has a facilitating role in this process, most likely through an internally orchestrated mechanism of reactivation, also known as replay (5, 6). Animal experiments have shown that, during periods of deep sleep, brain areas involved in the encoding and retrieval of recent experiences can show patterns of activation similar to those observed during active behavior (1–5, 7, 8). Additionally, several studies have produced evidence for the existence of reactivation processes in humans as well (9–13).

Recently, a study by Rudoy et al. used targeted reactivation with trial-unique auditory cues to improve memory for a selection of learned object-location associations (12). The specificity of the memory benefit, which was found only for cued associations, suggests that it is possible to externally trigger reactivation for selected experiences, an approach that would be very useful in educational and clinical settings (14).

The neural mechanisms behind the behavioral effects of externally triggered reactivation, however, are largely unknown. It seems likely that induced reactivation mirrors internally generated replay on the neural level and, as such, would involve brain regions initially involved in encoding and retrieval of learned material. This scenario would predict that a variety of sensory and associative areas would become reactivated depending on the sensory input used during encoding, in line with several findings from recent functional connectivity studies in humans (15–18). In addition, areas traditionally associated with declarative memory, such as the hippocampus and parahippocampus, could be expected to play a role in this process. This view is supported by data from several neuroimaging studies in humans (10, 11, 13).

According to these hypotheses, targeted reactivation would only be successful if the sensory input given to trigger the reactivation can reach sensory, associative, and mnemonic areas. This will largely depend on input processing in the thalamic system, the main relay of sensory information to the cortex (19, 20). During slow-wave sleep, the thalamic system undergoes phases of inhibition and activation aligned with the global up and down states of the brain, observed as characteristic slow oscillations during non-rapid eye movement (NREM) sleep (21). The periodic neural synchronization resulting from the phasic shifts between brain states is believed to provide windows for replay and intracortical dialogue (22–25). As such, the effectiveness of induced reactivation might be largely determined by the state of the thalamus at the moment that sensory input is provided.

Here, we adapted the object-location paradigm of Rudoy et al. (12) for use in an fMRI context. Participants were taught to associate pictures of objects with locations on a computer screen before an evening sleep opportunity. During the learning phase, each object was consistently presented with a characteristic sound (e.g., a cat with a meow and a kettle with a whistle), and participants were instructed to place the object as accurately as possible on its correct location. After object-location associations were learned to criterion, baseline placement accuracy was recorded. During the subsequent sleep period in the magnetic resonance (MR) scanner, auditory cues corresponding to half of the learned associations were presented during EEG-verified slow-wave sleep. Also, control stimuli were randomly intermixed so that auditory activation could be compared for studied and unstudied sounds. After the sleep period, placement accuracy was assessed once more.

We compared blood oxygen level dependent (BOLD) activity during presentation of studied and unstudied sounds in slow-wave sleep and additionally assessed whether functional connectivity in activated regions was modulated by this contrast. Subsequently, we investigated whether activity and connectivity during presentation of studied sounds could predict the behavioral outcome of our reactivation protocol.

Our hypothesis was that presentation of cue sounds during sleep would induce reactivation of the associated object-location pairs. Based on existing literature (10, 11, 13), we therefore expected that sound presentation would be correlated with activity in areas previously involved in acquisition and retrieval of the associative memory traces, primarily located in the medial temporal lobe. Moreover, we hypothesized that functional

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connectivity between sensory and associative areas at the time of sound presentation would be increased as the reactivation would reinstate the multimodal representations of the object-location-sound associations. Finally, we predicted that the mnemonic effects of reactivation would depend on activity in medial temporal regions, based on their proposed role in memory consolidation.

Results

Object Location Task: Learning and Presleep Test. In the evening, shortly before sleeping, participants memorized the locations of 50 pictures of everyday objects. First, participants passively viewed each object as it was sequentially shown on its correct location. Second, subjects were instructed to move the object from the center of the display to its original location by using a joystick (Fig. 1). Each object was consistently presented together with a characteristic sound (e.g., a picture of a cat would be presented with a meow). Feedback on an object's correct location was provided after each placement. We quantified memory performance by using the absolute distance between the participant's placement of each object and its correct location. Learning continued until subjects were successful in placing the objects within 4 cm of their original location on two consecutive rounds. Objects for which this criterion was met were removed from subsequent learning rounds. After reaching this criterion for all objects, participants performed a final presleep test without feedback to provide a baseline measure of memory accuracy. Stimuli were then divided in two sets of 25 ("cued" and "uncued") associations that were matched on accuracy for every participant individually. Correspondingly, baseline performance was not significantly different for subsequently cued and uncued associations [cued (error ± SEM): 2.66 ± 0.12 cm; uncued (error ± SEM): 2.79 ± 0.13 cm; paired t test t_{21} = −1.58, P = 0.13].

Reactivation During Sleep. This presleep test was followed by a sleep period of 2 h in the MR scanner during which the participant's sleep state was continuously monitored through polysomnography. It was essential to our paradigm that slow-wave sleep duration was sufficient to allow completion of the reactivation protocol. Participants were therefore partially sleep-deprived in the morning and started the sleep period in the late evening around their habitual bedtimes. Data were only included if at least 80% of the sound stimulation protocol could be completed in slow-wave sleep, the sound stimulation did not produce visible microarousals in the EEG, and participants were not aware that sounds had been presented during the sleep period (based on their responses during the debriefing). This selection resulted in inclusion of 22 participants (out of an original sample of 56) for all analyses reported here (for sleep parameters, see Table S1; for a more detailed description of data inclusion and exclusion, see *SI Materials and Methods*).

During the sleep period, sounds previously paired with object-location associations from the cued set ("cue sounds") were presented to participants through headphones. Sounds not presented during the learning phase ("control sounds") were additionally played to the subjects to allow comparison of evoked BOLD responses between studied and unstudied sounds.

Postsleep Vigilance and Attention. After the sleep period, participants had a short break and a shower before conducting a second memory test that was identical to the presleep test. This test thus provided a measure of postsleep memory accuracy. Considering the timing of this postsleep test (between midnight and 2:30 AM), time-of-day effects could potentially affect performance at this stage of the experiment. For this reason, participants executed a psychomotor vigilance task [PVT (26); see also *SI Materials and Methods*] at the start of the experiment and before starting the postsleep testing session. A comparison of pre- and postsleep reaction times and error rates on the PVT showed no significant differences in vigilance between the two moments of testing [presleep: mean RT (± SEM) = 304 (±17) ms, no. of errors = 1.38 (±0.30); postsleep: mean RT = 318 (±13) ms, no. of errors = 1.00 (±0.23); paired t test: $t_{21}(2.02)$, $t_{21}(1.07)$: both P > 0.05]. This result suggests that the time of testing had no apparent effect on vigilance or attention during the postsleep testing phase.

Object Location Task: Postsleep Test. We observed that performance decreased significantly from the pre- to the postsleep test for both cued and uncued associations: cued (Δerror ± SEM) = −0.44 ± 0.11 cm; uncued (Δerror ± SEM) = −0.33 ± 0.11 cm; paired t test: cued = $t_{21}(3.86, P = 0.001)$; uncued = $t_{21}(2.56, P = 0.018)$. There was no significant session × condition interaction ($F_{1,21} = 0.83$; $P = 0.374$), indicating that there was no overall effect of reactivation on participants' accuracy after the sleep period. A comparison of reaction times on cued and uncued trials before and after sleep showed no significant differences or interactions between conditions (all P > 0.05).

Object Location Task: fMRI. Next, our investigations focused on neural responses observed during the object-location test. To investigate which brain areas were involved in performing the object-location task, we contrasted BOLD activity during retrieval of object-location associations with that observed during the fixation period. This contrast showed that performance of the object-location task was related to increased activity in areas involved in visual and spatial perception, memory, and motor
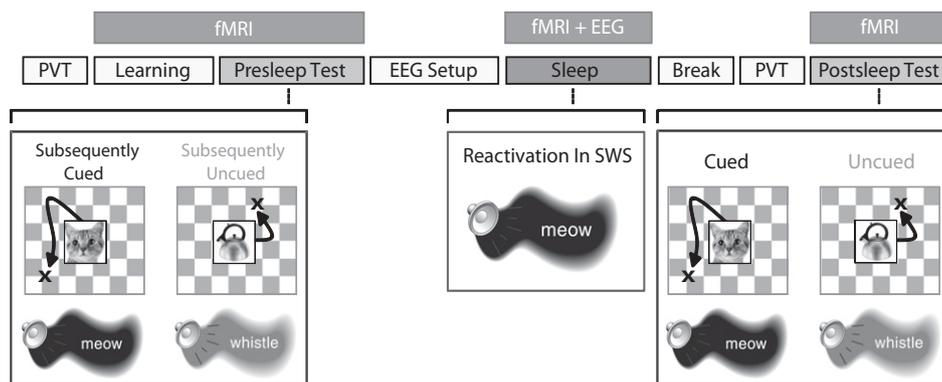
Fig. 1. Graphical depiction of the experimental design. Participants learned 50 object-location associations in the presence of typical object-related sounds. Twenty-five of these associations were cued by using their sounds during subsequent slow-wave sleep (SWS) in the MR scanner. Performance and BOLD responses during task performance and during sleep were assessed to investigate the behavioral and neural underpinnings of externally triggered reactivation.

action. Areas activated included primary visual cortex, fusiform gyrus, posterior parietal cortex, bilateral parahippocampal cortex, bilateral hippocampus, and left (pre)motor areas. (Fig. 2 and Table S2).

Reactivation: fMRI. We then investigated whether evoked responses in BOLD activity during slow-wave sleep differed during presentation of auditory stimuli from the object-location task (cue sounds) compared with task-unrelated stimuli (control sounds). Cue presentation was related to increased activity in the right parahippocampal cortex compared with presentation of control sounds (peak: [32 −42 −6]; Fig. 3A). No significant increase in activity was observed when contrasting the presentation of control with cue sounds.

Reactivation: Functional Connectivity. To probe whether sound presentation modulated functional connectivity of the parahippocampal cortex, we conducted a psychophysiological interaction analysis (PPI). This analysis was seeded from the right parahippocampal region that was differentially activated for cue sounds versus control sounds. Results indicate that right parahippocampal connectivity with posterior brain regions, including the fusiform and calcarine gyrus, precuneus, and primary and secondary visual areas, was selectively increased during presentation of cue sounds (Fig. 3B and Table S3). No significant increases in parahippocampal connectivity were observed during the presentation of the control sounds.

Reactivation: Behavioral Correlations. Although there was no overall behavioral effect of the reactivation protocol on postsleep memory performance, intersubject variability was high. We therefore reasoned that the event-related activity observed during presentation of cue sounds might predict the mnemonic effect of the reactivation protocol on the level of the individual participant. To test this hypothesis, we added a covariate to our group analysis of the contrast between cue and control sounds. This covariate contained for each participant the average change in performance from pre- to postsleep test for the cued associations. In other words, this covariate captured the subject-specific effect that reactivation had on accuracy for the selection of associations that were cued during sleep. This way, we could investigate how cue-related activity was correlated with the behavioral outcome of the reactivation procedure. We found that cue-related activity in bilateral thalamus, cerebellum, and medial temporal lobe (including both the hippocampus and parahippocampal
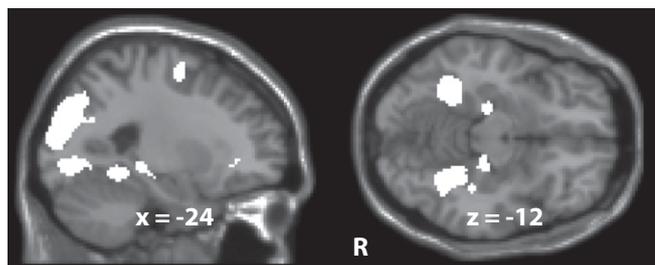
Fig. 2. Activity associated with retrieval of object-location associations. Shown is an overview of activity that increased during retrieval of object-location associations compared with a fixation baseline. Data shown here were acquired during the presleep test. Retrieval of object-location associations involved activity in bilateral hippocampus, parahippocampus, posterior visual and association areas, and (pre)motor regions. Significant clusters are superimposed on representative sagittal and axial slices of the canonical single subject T1 from SPM8. Results are obtained after initial thresholding at P < 0.001 uncorrected at the voxel level, followed by family-wise error (FWE) correction for multiple comparisons at the cluster level at P < 0.05. R, right.
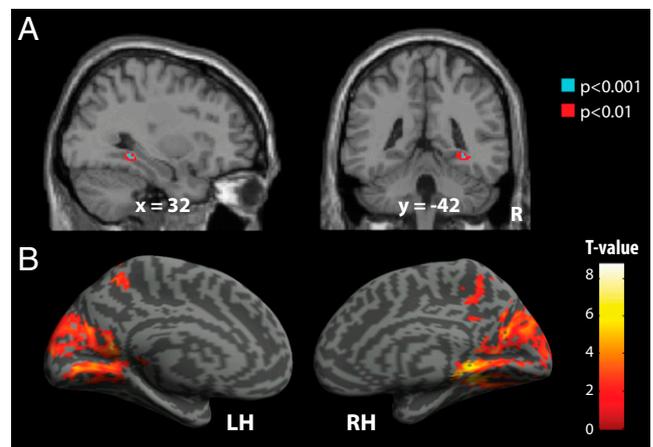
Fig. 3. (A) The right parahippocampal cortex is more activated during presentation of cue than control sounds in slow-wave sleep. The cluster shown here is superimposed on a representative sagittal and coronal slice of the canonical single subject T1 from SPM8. Activations are depicted at P < 0.001 (blue) and P < 0.01 (red) uncorrected at the voxel level. After thresholding at the P < 0.001 level, the blue cluster shown is significant (P = 0.035; FWE) after small volume correction using the peak coordinate of right parahippocampal activation observed in Fig. 2. (B) Parahippocampal connectivity is increased during presentation of cue compared with control sounds. Parahippocampal connectivity to the precuneus and posterior visual areas was shown to be significantly increased during cue sound presentation. Significant clusters are rendered by using the SPM8 surface renderer as provided by FreeSurfer. Results are obtained after initial thresholding at P < 0.001 uncorrected at the voxel level, followed by FWE correction for multiple comparisons at the cluster level at P < 0.05. LH, left hemisphere; RH, right hemisphere; R, right.

gyrus) was positively correlated with better retention for the corresponding object-location associations (Fig. 4A and Table S4).

A similar analysis was conducted for the parahippocampal PPI results. Here, our data suggest that parahippocampal connectivity with the precuneus at the time of cue presentation predicted improved retention for the related object-location associations (Fig. 4B and Table S5). We found no negative correlations between postsleep performance and either activity or parahippocampal connectivity at the time of cue presentation.

Object Location Task: Neural Changes from the Pre- to Postsleep Test. To test whether reactivation was related to changes in neural activity and functional connectivity during postsleep retrieval, we contrasted activity and parahippocampal connectivity for cued versus uncued associations at the pre- and postsleep test (see also *SI Materials and Methods* and Tables S6 and S7). Parahippocampal connectivity with a medial prefrontal region during retrieval of cued associations at the postsleep test was significantly correlated with retention of cued associations (Fig. 4C). In other words, participants with a more positive effect of the reactivation protocol showed stronger parahippocampal to medial prefrontal connectivity when retrieving cued associations. We subsequently tested whether this parahippocampal-medial prefrontal connectivity changed from the pre- to postsleep test. Using a region-of-interest analysis, our results show that parahippocampal connectivity with this medial prefrontal region changed significantly from the pre- to the postsleep test (Table S6). Importantly, we also found that this increase in connectivity over sleep was correlated with better retention of cued associations (Table S7).

Discussion

The results of this study are threefold. First, we showed that auditory cueing of object-location associations during slow-wave

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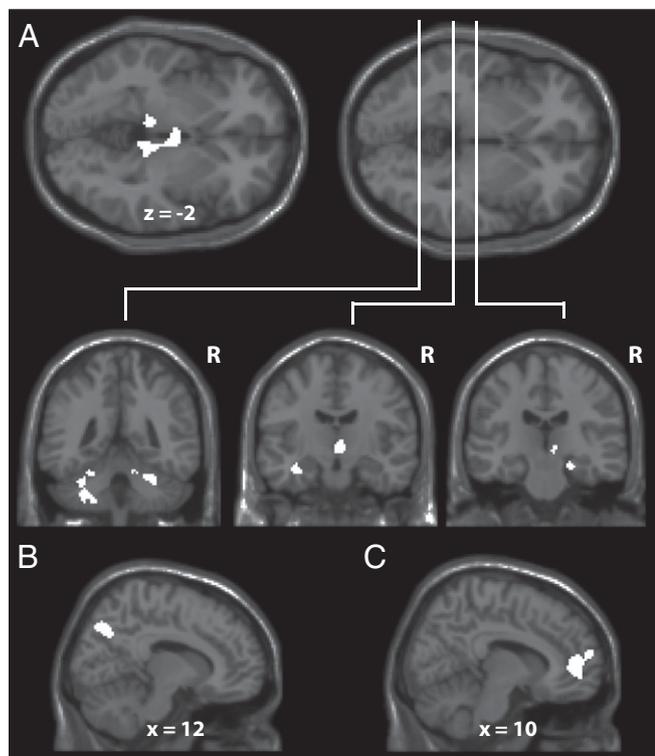
Fig. 4. Correlations between activity and connectivity and postsleep memory performance. (*A*) Cue-related brain activity (cue > control sounds) in slow-wave sleep that was correlated with a positive effect of the reactivation protocol across participants. Activity in bilateral thalamus*, cerebellum*, and medial temporal lobe** at the moment of cue-presentation predicted postsleep accuracy for cued associations. (*B*) Cue-related parahippocampal connectivity* (cue > control sounds) in slow-wave sleep that was correlated with a positive effect of the reactivation protocol across participants. Parahippocampal connectivity with the bilateral precuneus at the moment of cue-presentation predicted postsleep accuracy for cued associations. (*C*) Cue-related parahippocampal connectivity* (cued > uncued associations) during the postsleep test that was correlated with a positive effect of the reactivation protocol across participants. Better retention of cued associations was associated with stronger parahippocampal–medial prefrontal connectivity during postsleep retrieval. Results are obtained after initial thresholding at *P* < 0.001 uncorrected at the voxel level, followed by FWE correction for multiple comparisons at the cluster level at *P* < 0.05 (*) or FWE correction for multiple comparisons at the small volume level at *P* < 0.05 (**). Significant clusters are superimposed on representative slices of the canonical single subject T1 from SPM8. R, right.

sleep increased activity in the right parahippocampal cortex. This finding demonstrates that, even during deep sleep, sounds related to previously studied material can be processed differently from unstudied auditory stimuli. This outcome argues against the traditional view that our brain is shut off from the external world during deep sleep and is in line with recent electrophysiological findings in nonhuman primates (27, 28). Interestingly, the parahippocampal region that was active during sleep overlapped with the area that was involved in active retrieval of the object-location pairs during the presleep test. This result is therefore in line with our hypothesis that retrieval-associated areas can be reactivated during subsequent slow-wave sleep using auditory cues.

Second, we found that presentation of cue sounds during sleep modulated parahippocampal connectivity. Specifically, parahippocampal connectivity with posterior visual regions was increased during cue compared with control sound presentation. This result suggests that presentation of the sounds during slow-wave sleep induced network connectivity in nonauditory areas. The overlap between retrieval activity and cue-related connec-

tivity during sleep (Fig. S1) again supports the notion that our cueing protocol induced reactivation of the visual representations of the object-location associations and that the evoked response was not limited to the processing of the auditory stimuli themselves. There are findings from several recent studies indicating that functional connectivity during rest and sleep can be related to preceding learning experiences (15–18). This study extends these findings by reporting modulations of connectivity in relation to a reactivation protocol. Together, these results suggest that connectivity analyses could provide valuable insights into the interplay between brain areas at times of reactivation.

Third, our data indicate that cue-related changes in activity and connectivity can also predict memory stabilization over sleep. Specifically, activity in the medial temporal lobe, thalamus, and cerebellum at the moment of reactivation predicted better memory retention for the cue-related associations. In other words, those participants with the highest cue-related activity in these areas during sleep showed the best retention of cued object locations at the postsleep test. Moreover, parahippocampal connectivity with the precuneus at the time of cueing was similarly related to improved retention of location memory for cued objects after the sleep period. Finally, retention of cued associations was related to increased parahippocampal to medial-prefrontal connectivity at postsleep retrieval.

A role for medial temporal lobe regions in offline memory consolidation and reactivation has been proposed based on animal studies (1, 5) and was additionally demonstrated in a number of neuroimaging experiments in humans (10, 11, 13). With this study, we add to the existing body of research by showing that the medial temporal lobe is involved in establishing the behavioral consequences of cue-specific reactivation during slow-wave sleep.

Confirming our hypothesis, we found that thalamic activity at the time of cueing was positively correlated with the mnemonic effects of reactivation. We propose that cue-related activity within the thalamus could be used as a state-dependent proxy for sensory responsiveness, based on its involvement in the gating of sensory input (19, 21). The success of memory reactivation through auditory cueing would then depend on the thalamic activation in response to the presentation of the sounds. The thalamic response at this moment would determine whether sensory input is subsequently relayed to the cortex, where it could reinstate the object-location representations and work toward stabilization of the associated memory traces.

Although the role of the cerebellum in memory consolidation is unclear, previous studies have implicated this brain region in the overnight reorganization of procedural memories (29–31) and, additionally, in various forms of conditioning (32). The object-location task used here, besides teaching participants the association between objects and locations, also has a clear procedural component. Participants acquire extensive experience in placing the object at a particular place on the screen, and through feedback, learn to associate each object with a certain joystick movement. One could argue that the predictive value of cerebellar activity at the moment of cueing relates to the reactivation of motor patterns associated with the placement of the cue-related object. However, cerebellar activity was not increased during retrieval of object-location associations in the presleep test, so we have no further imaging results to support this idea. Moreover, reaction times did not differ between cued and uncued associations after the sleep period. Further research into targeted reactivation of motor sequences could shed more light on the role of the cerebellum in memory consolidation. Alternatively, reactivation protocols that use a declarative memory task without a rather sophisticated motor response could reveal whether the cerebellar involvement in stabilization processes observed here is specific to the task used.

Furthermore, the behavioral relevance of functional connectivity between the parahippocampal cortex and the precuneus fits well with recent literature. Several studies have shown that precuneus connectivity changes over time after learning and have therefore proposed a role for this area in processes of memory consolidation (18, 33, 34). Moreover, precuneus involvement in visuospatial imagery, self-referential processing, and the encoding and retrieval of explicit memories makes this region a likely node in the neural network used for the offline processing of learned experiences (35). We would suggest that the parahippocampal-precuneus connectivity signifies an internal retrieval process that might reinstate the object-location representations in the brain.

Lastly, the finding that parahippocampal to medial prefrontal connectivity is increased with better retention of the cued associations is in line with various consolidation studies in humans and nonhuman animals (36–40). The medial prefrontal cortex is believed to become more involved in memory processing as memories are consolidated. Our results support this idea by showing that increased medial prefrontal connectivity is associated with better performance for associations that have been stabilized during slow-wave sleep. This finding offers additional support for the idea that our protocol produces mnemonic effects through its impact on sleep-dependent memory consolidation.

Taken together, our results provide evidence that sensory input during sleep can change neural processing and cue reactivation of selected experiences, and that neural processing at the time of reactivation and during postsleep retrieval can be related to behavioral effects observed after sleep. Nevertheless, overall, reactivation did not significantly benefit postsleep memory performance, in contrast with the results from the original study (12). We suspect that a primary reason for this apparent discrepancy is the difference in sleeping environments. In the original study, participants slept in a quiet, sound-attenuated laboratory environment with a backdrop of unobtrusive white noise. In this study, participants had to adjust to the constant operational noise of the MR scanner to be able to sleep. We believe that participants who managed to sleep in our experiment likely had a higher threshold for noise disruptions than other subjects and/or could suppress ambient sounds more successfully. Auditory stimulation would therefore have a reduced impact on participants included in the analyses reported here compared with the participants in the original study. This reduced responsiveness to external stimuli is likely mediated through adaptive sensory gating at the level of the thalamus (41–43). The same suppression mechanism that allows participants to fall asleep in the MR laboratory environment might also decrease the chances for reactivation to be induced during subsequent slow-wave sleep by increasing the threshold at which external inputs might be processed in higher sensory and associative areas. According to this hypothesis, the effect of reactivation would scale with thalamic activity after sensory input, which is exactly what we observed here. Nevertheless, it is important to note that the neural responses that modulate the behavioral effect of our reactivation protocol are not necessarily identical to those that produce the consequences of reactivation in a noise-free setting.

We cannot exclude the possibility that the effect of reactivation depends on the participant's state during the active and resting phases of the experiment. In this study, participants were partially sleep-deprived and underwent the protocol in the late evening and night, in contrast with the original experiment in which participants were normally rested and had an afternoon nap. Possible effects of circadian-related factors on the outcome of the reactivation protocol have yet to be studied.

In summary, we have shown that inducing reactivation of object-location associations with auditory cues modulates the activity and connectivity of the parahippocampal cortex. Furthermore, our results indicate that cue-related activity in the medial temporal lobe, the thalamus, and the cerebellum, as well as the connectivity between the parahippocampal cortex and the precuneus, predicts the behavioral outcome of such a reactivation protocol. These findings shed more light on the neural mechanisms of induced reactivation and take us one step closer to understanding the offline consolidation of our daily experiences.

Materials and Methods

For an in-depth description of the experimental procedures, please see *SI Materials and Methods*.

Participants. Fifty-six participants were recruited from the Radboud University Nijmegen student population through an online research participant pool (age range 18–27; 14 males; 4 left-handed). Thirty-four participants were excluded from the final analysis (27 because of insufficient slow-wave sleep during the sleep period; 4 because of technical difficulties in EEG or fMRI acquisition; 2 because of excessive motion during the sleep period; and 1 because of illness), leaving 22 participants for the analyses reported in this article. The experiment was conducted in accordance with national legislation for the protection of human volunteers in nonclinical research settings and the Helsinki Declaration. Participants were given course credits or monetary compensation for participation.

General Procedures. The experiment began between 7 and 8 PM with the sleep period starting between 10 PM and midnight. Participants arrived at the laboratory, gave informed consent, and executed a PVT (26) to measure attentiveness. After the PVT task, participants were placed in the MR scanner. First, a test was conducted to set the sound volume to a subject-specific level at which the participant could clearly distinguish individual sounds while the scanner was operating. Subsequently, participants executed the object-location task. After a short break, subjects were prepared for polysomnographic recordings outside the scanner. Next, participants reentered the scanner room and were instructed to rest for 2 h. In this period, the participant's EEG was observed online. During intervals of stable slow-wave sleep, half of the sounds previously used to cue the object-location associations were played to the participant. In addition, sounds previously unassociated with the learned material were presented as control stimuli. The sleep period was followed by a short break. To ensure that participants were awake and alert after the sleep period, all participants took a shower before starting the postsleep testing session. To measure vigilance in the postsleep period, a second PVT was then administered. This task was followed by the postsleep testing session and an anatomical scan in the MR scanner, after which subjects were debriefed.

fMRI Data Acquisition and Analysis. fMRI data were recorded on a 1.5 T MR scanner (Avanto; Siemens Healthcare) by using a reduced-noise EPI sequence (44) and an eight-channel head coil. Preprocessing and statistical analysis of all scans was done in SPM8 (www.fil.ion.ucl.ac.uk/spm) by using a standard approach supplemented by custom methods for data quality optimization.

EEG Data Acquisition and Analysis. During the sleep period, electrophysiological recordings were obtained by using an MR-compatible BrainAmp MR Plus amplifier and an MR-compatible 32-channel electrode cap (Brain Products). Electrooculograms, the electrocardiogram, and the electromyogram were recorded throughout the sleep period according to AASM guidelines (45) using an MR-compatible "BrainAmp ExG MR" system in combination with bipolar AgCl electrodes (Brain Products). Artifact correction and sleep staging were subsequently done in BrainVision Analyzer 2.0 (Brain Products).

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