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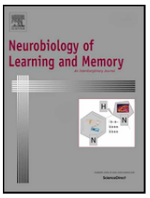

Deepened sleep makes hippocampal spatial memory more persistent

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ABSTRACT

Ample evidence has indicated a beneficial role of sleep, and particularly of slow wave sleep (SWS) in memory consolidation. However, how basic features of sleep, its depth and duration, contribute to this process remained elusive. Here, we investigated spatial object-place recognition (OPR) memory in rats, to systematically dissociate effects of sleep depth and duration on the formation of recent and remote hippocampus-dependent memory. Encoding of the spatial configuration was followed by an experimental post-encoding period of either 2 or 4 h, during which the rats had either "regular sleep", "deeper sleep", or were kept awake. A deeper sleep was achieved by an extended habituation of the rats to the sleep environment. Retrieval was tested either immediately after the 2-hour post-encoding period (recent memory test) or 1 week later (remote memory test). Deeper sleep expressed itself in a selective increase in the time spent in SWS, and in numbers of slow oscillations, spindles, and hippocampal ripples during SWS, whereas preREM and REM sleep were not affected. At the recent test, OPR memory was preserved only after sleep, but independent of its depth. At the remote test, however, OPR memory was preserved only after deeper sleep, whereas the wake and the regularly sleeping rats did not show remote OPR memory, even with the longer 4-h post-encoding period. Our results indicate that, rather than a longer duration, deeper sleep, i.e., a longer time in SWS together with enhanced oscillatory signatures of mnemonic processing during this sleep stage, occurring within a 2-hour window after encoding, is the factor that makes hippocampus-dependent memory more persistent.

1. Introduction

Sleep enhances the consolidation of memory (Rasch & Born, 2013; Stickgold, 2005; Tononi & Cirelli, 2014). This memory effect of sleep is thought to originate from a systems consolidation process which likewise captures hippocampus-dependent and non-hippocampus-dependent aspects of an episodic memory representation, and is essentially established during slow wave sleep (SWS) (Inostroza & Born, 2013; Klinzing, Niethard, & Born, 2019; Sawangjit et al., 2018). Specifically it is assumed, that during SWS newly encoded episodic memory features are reactivated primarily in hippocampal networks which leads to the transmission of the reactivated memory information and, more gradually, to the redistribution of the representation such that extrahippocampal connectivity is increasingly strengthened. The consolidation process is coordinated in time by the neocortical (~1 Hz) slow oscillation and thalamic (10–15 Hz) sleep spindles, both representing EEG oscillatory hallmarks of SWS. While the slow oscillations drive spindles; the spindles, in turn appear to synchronize ripples which enwrap neuronal reactivation in hippocampal networks. Thereby, reactivations occur during a window of increased excitability and plasticity allowing the redistribution of representations towards extrahippocampal storage sites (Latchoumane, Ngo, Born, & Shin, 2017; Niethard, Ngo, Ehrlich, & Born, 2018; Seibt et al., 2017; Staresina et al., 2015).

Although some knowledge about the detailed mechanism underlying memory consolidation during sleep has been elucidated, basic questions about how this memory process is linked to the sleep process itself, remained unanswered. Does a longer duration of sleep itself produce better long-term memory? Or is the efficacy of long-term memory formation during sleep after encoding mainly dependent on the depth of sleep? The few human studies on this issue have produced mixed results. For example, extending sleep from 40 min to 90 min after encoding benefited memory performance on a hippocampus-dependent visuo-spatial task (Diekelmann, Biggel, Rasch, & Born, 2012). However, in other studies, a short period of sleep was found to be as effec-

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tive in enhancing learning of texture discriminations and word-pair associations, respectively, as a whole night of sleep (Mednick, Nakayama, & Stickgold, 2003; Tucker & Fishbein, 2009). Also, memory improvements over sleep have been found to correlate with both the time spent in sleep and SWS (Takashima et al., 2006) as well as with the average EEG slow wave activity during non-rapid eye movement (Non-REM) sleep (Wilhelm et al., 2011, 2013) suggesting that both duration and depth are relevant for consolidation. Here, we approached this issue using a rat model of hippocampus-dependent spatial memory formation on an object–place recognition (OPR) task. Rats encoded a spatial configuration and then slept (or remained wake) for either 2 or 4 h, and retrieval was tested either immediately after or 1 week after the post-encoding sleep period. We experimentally deepened sleep by extending the duration of the habituation sessions of the rat to the sleep environment. This procedure is related to the well-known "first-night-effect" in humans and, here, was established in separate experiments in rats. Our findings identify the depth of sleep, i.e., a selectively increased time spent in SWS, rather than an overall increase in sleep duration as the primary factor supporting OPR long-term memory.

2. Methods

2.1. Animals

Seventy-five 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 rats per cage. They 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 5 days before starting an experiment. All experimental procedures were performed in accordance with the European animal protection laws and policies and were approved by the Baden-Württemberg state authority.

2.2. Experimental design

The main experiments examined the effect of sleep depth and duration on recent and remote object-place recognition (OPR) memory (Fig. 1A). Each experimental condition comprised an encoding phase, during which the rat was exposed to the task stimuli and allowed to explore them, a subsequent 2-hour or 4-hour post-encoding period, and a retrieval phase that occurred either 2 h (recent test) or 1 week (remote test) after the encoding phase. The groups differed according to the sleep depth during the post-encoding period ("regular" versus "deeper"), and the duration of the post-encoding period (2 versus 4 h). In the groups with a 2-hour post-encoding period, OPR was tested either 2 h or 1 week after encoding (recent versus remote). In the groups with a 4-hour post-encoding period, OPR was tested only 1 week after encoding. Thus, 6 experimental groups including a total of 64 rats resulted, i.e., 2 groups testing recent retrieval: "Regular sleep/2-hour period/recent" (n = 15), "Deeper sleep/2-hour period/recent" (n = 11), and 4 groups testing remote retrieval: "Regular sleep/2-hour period/remote" (n = 10), "Regular sleep/4-hour period/remote" (n = 10), "Deeper sleep/2-hour period/remote" (n = 11), and "Deeper sleep/4-hour period/remote" n = 10). Deeper (versus regular) sleep was induced by extending the animal's habituation to the sleep environment (resting cage) from 3 × 2 h/day (regular sleep) to 3 × 4 h/day (deeper sleep, see below). Each group of rats was tested on a sleep condition (regular or deeper) and a wake condition in which they stayed awake during the post-encoding period. The sleep and wake conditions were separated by a 2-weeks interval, with the order of conditions counterbalanced across animals in each group.

To characterize deeper versus regular sleep, in a further experiment in different groups of rats (n = 11) electrodes were implanted for recordings of the EEG and hippocampal local field potentials (LFP). The rats were assigned to two experimental groups in which EEG and LFP signals were recorded during a 2-hour post-encoding period filled either with regular sleep (n = 5) or deeper sleep (n = 6). The experimental procedures were the same as described in the main experiments except that the rats did not perform the retrieval phase.

In all experiments, animals were randomly assigned to the experimental groups and conditions before the experiment. The experimenters were not blinded to the animal group or condition during data collection. However, all behavioral and electrophysiological recordings were analysed offline, with the experimenters blinded to the experimental groups and conditions.

2.3. Behavioral procedures

The behavioural procedures were the same as described in Sawangjit et al. (2018). In brief, animals were first habituated to the task and sleep environment before tested on the OPR task. For habituation, the rats were brought into a test room once a day on three consecutive days. The habituation session started with an object familiarization phase where the rats were placed into an empty cage with an object (not used for experiments) positioned in the center of the cage. They were allowed to explore the object for 10 min. Then, the rats were placed into the empty open field for 10 min to freely explore the open field and its distal cue contexts. Afterward they were left undisturbed in a plastic box (35 × 35 cm, height: 45 cm) serving as 'resting-box', for 2 h in the regular sleep condition. In order to deepen sleep in the deeper sleep condition, the time the rat spent habituating to the resting box was increased to 4 h.

Twenty-four hours after the last habituation session, the rats were again brought into the test room for the encoding phase of the OPR task. They were placed into the open field containing two identical objects and were allowed to explore the objects for 10 min. The rats were then placed into the 'resting-box' for the post-encoding period condition (sleep or wake) and the duration (2 h or 4 h) according to the assigned experimental groups. For retrieval testing on the OPR task (2 h or 1 week after encoding), one of the two objects of the encoding phase was displaced to a different location. During the retrieval phase, the rats were allowed to explore the arena for 5 min.

Sleep was assessed using video recorded behavior according to standard procedures (see below). In the wake condition, wakefulness was enforced using gentle handling (Inostroza, Binder, & Born, 2013). This procedure is known to minimizes stress and confounding influences of locomotion (Hagewoud et al., 2010; Palchykova, Winsky-Sommerer, Meerlo, Dürr, & Tobler, 2006). It involved tapping on the 'resting-box' and, if necessary, gently shaking the box. No intense stimulation was used, and video records ensured that signs of startle or freezing behavior did not occur. The rats were brought to their home cages after the post-encoding period and kept under routine conditions until retrieval testing.

The OPR task was performed in a room with masking noise. The open field (80 cm × 80 cm, height of walls: 40 cm) was made of grey PVC. The rats could see the distal cues through the open upper side of the arena. Objects were made of glass, with different shapes and colors, and heavy enough not to be moved by the rat (height: 15–30 cm; base diameter: 7–12 cm). They were placed at least 10 cm equidistant from the walls. Pilot studies ensured that the rats could discriminate among the different objects and did not show any preference for one of the objects. The locations of objects during the encoding and retrieval phases were counterbalanced across the retention conditions. After each phase, the objects and arena were cleaned with water containing 70% ethanol. The exploratory behavior of rats was monitored by a video camera and analysed offline by an experienced researcher using ANY-maze software (Stoelting Europe, Dublin, Ireland). All experiments took place

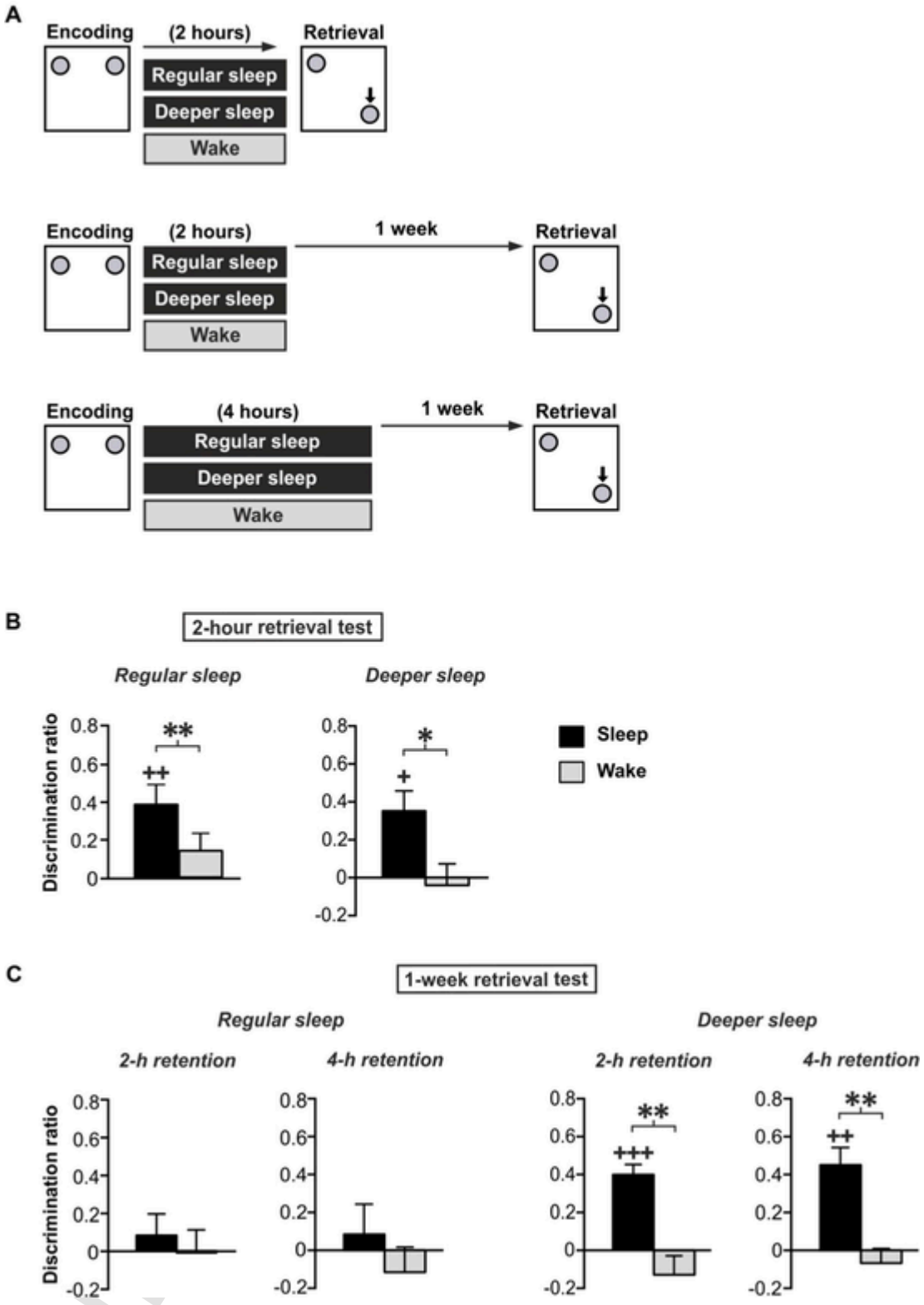

Fig. 1. Deeper sleep after encoding enhances consolidation of remote OPR memory. (A) Study design: During the Encoding phase of the OPR task, the rats explored two identical objects in an open field for 10 min. Then, an experimental period of either 2 h (upper panels) or 4 h (lower panel) followed, during which the rats slept in an "deeper sleep" or "regular sleep" condition, or the rats remained awake ("wake" control condition). To generally deepen sleep in the "deeper sleep" condition, rats in this condition underwent an extended prior habituation to the sleep environment, 3 × 4 h/day compared to 3 × 2 h/day in the rats of the "regular sleep" conditions. The retrieval was tested either 2 h after encoding (recent test) or 1 week later (remote test). In the retrieval test, the rats explored the same objects in the arena for 5 min. One of the two objects was displaced (relative to its location at the encoding phase, arrow). The preferential exploration of the displaced object as compared to the stationary object (i.e., the discrimination ratio) represents memory for the place. (B) At the (recent) 2-hour retrieval test, OPR memory significantly benefited from both regular (n = 15 rats) and deeper sleep (n = 11 rats), in comparison with the respective wake control conditions, but was comparable between the regular sleep and deeper sleep conditions (p = 0.214). (C) OPR performance at the remote 1-week retrieval test did not reveal any significant memory in

the regular sleep or wake conditions (left panels), independently of whether the post-encoding period covered a 2-hour (n = 10 rats) or 4-hour interval (n = 10 rats). By contrast, remote OPR memory was distinctly enhanced after deeper sleep, in comparison with the wake control condition (right panels), with the enhancement being comparable for the 2-hour (n = 11 rats) and 4-hour retention intervals (n = 10 rats, p = 0.630), overall indicating that sleep depth rather than duration benefits the formation of long-term OPR memory. $+++p < 0.001$, $++p < 0.01$, $+p < 0.05$ for one-sample t tests against chance level; $^{**}p < 0.01$, $^{*}p < 0.05$ for pairwise t tests (two-sided) between sleep (black bars) and wake (white bars) conditions.

during the animal's rest phase (between 8:00 and 13:00 h with lights on).

2.4. Analysis of memory performance

Exploration behaviors were defined by the rat being within 2 cm of an object, directing its nose towards the object and engaging in active exploration behaviors such as sniffing. A discrimination ratio was calculated according to the general formula: (time spent at displaced object – time spent at stationary object)/(time spent at displaced object + time spent at stationary object). Preferential exploration of the displaced object, i.e., a positive value of the discrimination ratio, indicates memory for the familiar object configuration (of the encoding phase), whereas a value of zero indicates no exploration preference. The total time of object exploration (across both objects), distance travelled and mean speed were also assessed as indicators of locomotion and motivation.

2.5. EEG and hippocampal LFP recordings, and histology

The surgical implantation of electrodes for EEG and LFP recordings was performed under general anaesthesia (induction: 1–2%, maintenance: 0.8–1.2% in 0.35 l/min O_2). Preoperatively, fentanyl (0.005 mg/kg), midazolam (2 mg/kg) and medetomidine (0.15 mg/kg) were administered intraperitoneally. Rats were placed in the stereotaxic frame and the skull was exposed. For EEG recordings, four screw electrodes were implanted: two frontal electrodes (AP: +2.6 mm, ML: ±1.8 mm, relative to bregma) and two occipital electrodes (AP: −10.0 mm, ML: ±1.8 mm), serving as reference and ground electrode, respectively. For additional LFP recordings in the dorsal hippocampi, two platinum electrodes were implanted (AP: −4.3 mm, ML: ±2.8 mm, DV: −2.3 mm, relative to bregma). For EMG recordings, two stainless steel wire electrodes were also implanted bilaterally in the neck muscles. All electrodes were connected to a Mill-Max pedestal (Mill-Max Mfg. Corp., New York, USA) and fixed to the skull with cold polymerizing dental resin. After the surgery, carprofen (5 mg/kg) was injected subcutaneously, and the rats were allowed to recover for at least 8 days. For recordings, the electrodes were connected through a preamplifier headstage (HS-18MM, Neuralynx, Dublin, Ireland) to a Digital Lynx SX acquisition system (Neuralynx), amplified, filtered (EEG: 0.1–50.0 Hz; EMG: 30.0–300.0 Hz), and sampled at a rate of 1,000 Hz.

After completion of the experiments, histological verification of the placement of hippocampal LFP electrodes was performed. The rats were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde (PFA). After decapitation, the brains were removed and immersed in the 4% PFA for at least two days. Coronal sections of 50–70 μm were cut on a vibratome, stained with toluidine blue and examined under a light microscope. For all rats (n = 11), the tips of LFP electrodes were located in the dorsal hippocampus.

2.6. Analysis of sleep, EEG, and hippocampal LFP recordings

In the main experiment, sleep was assessed using video recordings according to standard visual procedures (Pack et al., 2007; Sawangjit, Kelemen, Born, & Inostroza, 2017; Van Twyver, Webb, Dube, & Zackheim, 1973). In brief, sleep was scored whenever the rat showed a typical sleep posture and stayed immobile for at least 5 s. If brief movements interrupted sleep epochs by <5 s, continuous sleep was scored. The agreement of the procedure with EEG-based scoring of sleep in the present (see below) and previous studies was >92% (Inostroza et al., 2013; Pack et al., 2007). In the experiments that aimed at characterizing deeper sleep, sleep was additionally assessed using EEG and EMG recordings. Sleep stage classification was performed offline by an experienced experimenter using 10-s epochs according to standard criteria (Neckelmann, Olsen, Fagerland, & Ursin, 1994). The stages identified were SWS, pre-rapid eye movement (PreREM) sleep, REM sleep, and wakefulness. The wake stage was characterized by predominant low-amplitude fast activity associated with increased EMG tonus. SWS was characterized by predominant high amplitude delta activity (<4.0 Hz) and reduced EMG activity, and REM sleep by predominant theta activity (4.0–8.0 Hz), phasic muscle twitches and minimum EMG activity. PreREM sleep was identified by a decrease in delta activity, a progressive increase of theta activity and the presence of sleep spindles (10.0–16.0 Hz).

Procedures for identification of slow oscillations (SO), spindles, and hippocampal ripples during SWS were the same as described in Sawangjit et al., 2018. In brief, the EEG signal during all SWS epochs of an animal was filtered between 0.3 and 4.5 Hz. A slow oscillation event was selected if the following criteria were fulfilled: *(i)* two consecutive negative-to-positive zero crossings of the signal occurred at an interval between 0.4 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. The algorithm resulted in the identification of SOs with negative peak amplitudes exceeding − 80 μV and peak-to-peak amplitudes exceeding 120 μV. For spindle detection, the Hilbert transform was calculated for the filtered EEG signal (10.0–16.0 Hz). The signal was 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. To identify ripples in the hippocampal LFP recordings, the Hilbert transform was calculated for the filtered EEG signal (150.0–250.0 Hz) and smoothed with a moving average (window size 200 ms). A ripple was identified when the transformed signal exceeded 2.5 standard deviations from the mean signal during an animal's SWS epochs, for at least 25 ms (including at least 3 cycles) and for not more than 500 ms.

2.7. Statistical analyses

Statistical analyses were performed using SPSS 21.0. To investigate the effect of sleep manipulation on memory performance (discrimination ratios), we used Analyses of Variance (ANOVA) including group factors for the sleep depth (Deeper/Regular sleep) and the length of the post-encoding period (2 h/4 h), and a repeated-measures factor representing the post-encoding Sleep/Wake conditions. ANOVA were separately applied to the recent (2-hour) and remote (1 week) recall data. Discrimination ratios were also compared with chance level performance (zero) using one-sample t-tests. For analysis of sleep data, the sleep duration for subsequent 1-hour time bins was included as repeated-measures factor (Hour). To examine the relationship between post-encoding sleep and memory performance, Pearson product–moment correlation coefficients were calculated. In EEG and LFP recordings from the additional experiment performed to compare Deeper vs Regular sleep during a 2-hour post-encoding period were likewise analysed base on ANOVA including a Deeper/Regular sleep group factor and a repeated measures factor Hour (1st vs. 2nd hour). Generally, ANOVA indicating significance for main or interaction effects of interest were followed by post hoc t-tests (two-sided). A $P < 0.05$ was con-

sidered significant. Results are reported as the mean ± SEM. In addition, estimates of effect size, i.e., Cohen's d and partial eta squared ($\eta 2$), respectively, were provided for significant terms.

3. Results

3.1. Stronger remote but not recent OPR memory after "deeper" compared with "regular" sleep

Fig. 1A summarizes the design of the experiments. At the recent recall test (following the 2-hour retention interval), OPR memory was enhanced in both the regular and deeper sleep conditions, in comparison with the wake condition ($F(1,24) = 15.932$, $p = 0.001$, partial $\eta^2 = 0.399$, for Sleep/Wake ANOVA main effect, see Fig. 1B, for pairwise comparisons). In both sleep conditions exploration discrimination ratios significantly differed from chance level confirming OPR memory (regular sleep: $t(14) = 3.865$, $p = 0.002$, $d = 1.414$; deeper sleep: $t(10) = 3.134$, $p = 0.011$, $d = 1.337$ one-sample t test), with no difference between the conditions ($p = 0.214$. for pairwise comparison).

Strikingly, at the 1-week (remote) recall test, significant OPR memory was only found when the post-encoding sleep period was filled with deeper sleep, regardless of whether the post-encoding period lasted 4 h (deeper sleep: $t(9) = 4.947$, $p = 0.001$, $d = 2.194$, wake control: $t(9) = -0.870$, $p = 0.407$, one-sample t test, $t(9) = 4.011$, $p = 0.003$, $d = 1.268$ for pairwise comparison between conditions), or only 2 h (deeper sleep: $t(10) = 6.993$, $p < 0.001$, $d = 2.977$, wake control: $t(10) = -1.248$, $p = 0.240$, one-sample t test, $t(10) = 4.654$, $p = 0.001$, $d = 1.403$ for pairwise comparison between conditions, $F(1,37) = 6.508$, $p = 0.015$, partial $\eta^2 = 0.150$, for Deeper/Regular × Sleep/Wake interaction in global ANOVA, Fig. 1C). In fact, OPR memory at the 1 week test was closely comparable for the rats with deeper sleep covering a 2-hour and 4-hour post-encoding period ($p = 0.630$, for pairwise comparison between conditions). For the 2-hour period, OPR memory after deeper sleep at the 1-week retrieval test was also superior to that after regular sleep ($p = 0.025$, $d = 1.082$, for pairwise comparison).

In contrast, in the regular sleep condition, like in the wake control condition, rats did not anymore show significant OPR memory at the 1-week (remote) retrieval test, i.e., discrimination ratios did not differ from chance level, regardless of whether the post-encoding interval covered a 2-hour period (regular sleep: $t(9) = 0.695$, $p = 0.505$, wake control: $t(9) = -0.065$, $p = 0.950$, one-sample t test, $p = 0.591$ for pairwise comparison between conditions), or a 4-hour period (regular sleep: $t(9) = 0.537$, $p = 0.604$, wake control: $t(9) = -0.880$, $p = 0.401$, one-sample t test, $p = 0.296$, for pairwise comparison between conditions, Fig. 1C). Taken together, these results indicate that the consolidation of both recent and remote OPR memory requires post-encoding sleep but, to form more persistent remote OPR memory post-encoding sleep needs to be deeper than for the formation of recent OPR memory.

Total object exploration, total distance travelled and mean speed at the encoding and retrieval phases were comparable between experimental groups (all $p > 0.177$, for relevant ANOVA main and interaction effects, Table 1), confirming that the differences in OPR memory were not influenced by nonspecific changes, for example, in locomotion or motivation.

3.2. Characteristics of "deeper sleep"

To probe the efficacy of our manipulation to selectively deepen sleep we recorded, in a separate group of rats, the EEG and hippocampal LFPs during a 2-hour post-encoding period filled either with deeper or regular sleep. Deeper sleep during the 2-hour period was associated with a selectively increased time spent in SWS, but not in preREM or REM sleep, when compared with the regular sleep group ($t(9) = 4.493$, $p = 0.002$, $d = 2.720$, Fig. 2). Also, the average duration of an SWS epoch was enhanced during deeper sleep ($t(9) = 2.762$, $p = 0.022$, $d = 1.672$). Interestingly, the enhancement in SWS duration occurred exclusively in the second hour of post-encoding sleep (first hour: $t(9) = 1.027$, $p = 0.331$, second hour $t(9) = 5.579$, $p = 0.0003$, $d = 3.378$, $F(1,9) = 20.187$, $p = 0.002$, partial $\eta^2 = 0.692$, for Deeper/Regular × Hour interaction; Fig. 2D). There were also differences in SWS oscillations between the deeper and regular sleep condition that occurred exclusively in the second hour of the post-encoding period: Number of slow oscillations (SOs) and spindles, spindle duration, and the number of hippocampal ripples were all higher during deeper than regular sleep ($t(9) = 3.260$, 2.967, 2.864, 7.436, $p < 0.019$, $d > 1.736$, $F(1,9) > 5.187$, $p < 0.049$, partial $\eta^2 > 0.366$, for respective Deeper/Regular × Hour interactions, Fig. 2E–H).

Consonant with these findings, (video-based) analyses of sleep in our behavioural study revealed that sleep duration was increased in the deeper versus the regular sleep condition for the 2-hour post-encoding period (from 34.60 ± 3.00 min to 45.86 ± 3.81 min; $p = 0.034$, $d = 0.999$, for pairwise comparison) as well as for the 4-hour post-encoding period (from 109.00 ± 6.18 min to 149.04 ± 11.46 min, $p = 0.007$, $d = 1.375$, for pairwise comparison; Fig. 3A). Interestingly, an analysis on subsequent 1-hour intervals indicated that the increase in sleep duration in the deeper sleep condition was focussed on the second hour of post-encoding sleep in both the 2-hour and 4-hour retention conditions ($p = 0.015$, $d = 1.166$ and $p = 0.017$, $d = 1.174$, for pairwise comparisons, $F(1,19) = 5.061$ and $F(1,18) = 7.178$, $p < 0.037$, partial $\eta^2 > 0.210$, for respective Deeper/Regular × Hour interactions; Fig. 3B). Notably, correlation analyses revealed that in the deeper sleep condition with a 2-hour post-encoding period, remote OPR memory (at the 1-week recall test) was strongly correlated with the total sleep time ($r = 0.805$, $p = 0.003$, Pearson's correlation) as well as with the sleep duration during the second hour of post-encoding sleep ($r = 0.697$, $p = 0.017$, Fig. 3C). Similar associations were not observed for the conditions with a 4-hour post-encoding period ($r = -0.085$, $p = 0.816$, and $r = -0.346$, $p = 0.328$), overall suggesting that sleep depth during the first 2 h after encoding is crucial for the consolidation of remote OPR memory.

4. Discussion

Using a classical object-place recognition (OPR) task in rats, the present study confirms that sleep, in comparison with post-encoding wakefulness, enhances the consolidation of hippocampus-dependent memory. Previous studies demonstrated a beneficial effect of sleep for OPR memory tested up to 24 h after learning (Bett et al., 2013; Binder et al., 2012; Howard & Hunter, 2019; Inostroza et al., 2013; Ishikawa, Yamada, Pavlides, & Ichitani, 2014; Oyanedel et al., 2014; Ozawa, Yamada, & Ichitani, 2011). By systematically varying the duration and depth of post-encoding sleep, the present study goes beyond those previous findings indicating that sleep can strengthen OPR memory such that it is even maintained over one week. However, such persisting long-term OPR memories emerge only when the post-encoding sleep is of deeper quality. On the other side, we did not find evidence that the mere duration of sleep plays an essential role in producing persisting OPR memory, but the relevant consolidation processes appear to be associated with the first 2 h – particularly with the second hour – after the encoding session.

Our findings seem to diverge from previous human study suggesting that enhanced time spent in sleep per se is linked to building stronger memories (e.g., Diekelmann et al., 2012). However, such study manipulated sleep duration within shorter intervals (e.g., between 40 and 90 min), whereas the present study compared a 2-hour and 4-hour post-encoding periods of sleep. Thus, the findings can be reconciled by assuming a minimum amount of sleep (of about 2 h) that provides optimal consolidation and with no additional benefits when sleep duration

Table 1
Total exploration time, distance travelled, and mean speed during the Encoding and Retrieval phases.

Encoding phase	2-Hour OPR test				1-Week OPR test							
	2-Hour retention interval				2-Hour retention interval				4-Hour retention interval			
	Regular sleep	Wake control	Deeper sleep	Wake control	Regular sleep	Wake control	Deeper sleep	Wake control	Regular sleep	Wake control	Deeper sleep	Wake control
Total exploration (s)	8.70 ± 1.30	8.34 ± 0.94	9.65 ± 3.15	7.16 ± 0.87	13.61 ± 3.01	13.56 ± 3.48	11.55 ± 1.94	9.14 ± 0.99	11.02 ± 2.62	9.17 ± 2.19	10.09 ± 2.32	12.05 ± 1.63
Distance travelled (m)	53.84 ± 2.96	52.53 ± 4.15	48.15 ± 3.09	47.37 ± 2.43	42.03 ± 2.73	37.70 ± 3.24	60.46 ± 2.48	60.17 ± 3.59	58.28 ± 7.09	53.68 ± 7.05	49.76 ± 3.14	52.19 ± 3.67
Mean speed (m/s)	0.091 ± 0.005	0.088 ± 0.007	0.080 ± 0.005	0.079 ± 0.004	0.070 ± 0.005	0.076 ± 0.012	0.101 ± 0.004	0.100 ± 0.006	0.097 ± 0.012	0.089 ± 0.012	0.083 ± 0.005	0.087 ± 0.006
Retrieval phase	2-Hour OPR test				1-Week OPR test							
	2-Hour retention interval				2-Hour retention interval				4-Hour retention interval			
	Regular sleep	Wake control	Deeper sleep	Wake control	Regular sleep	Wake control	Deeper sleep	Wake control	Regular sleep	Wake control	Deeper sleep	Wake control
Total exploration (s)	5.59 ± 0.81	5.33 ± 0.72	3.22 ± 0.52	3.56 ± 0.75	8.89 ± 2.14	8.71 ± 1.28	6.15 ± 0.77	4.64 ± 0.70	6.04 ± 0.68	7.07 ± 1.58	8.75 ± 2.48	8.76 ± 0.95
Distance travelled (m)	24.36 ± 2.38	26.29 ± 2.67	26.37 ± 1.93	27.26 ± 1.93	27.09 ± 2.31	27.12 ± 2.06	38.22 ± 1.59	37.91 ± 2.25	34.21 ± 2.90	35.41 ± 3.50	31.77 ± 2.04	32.31 ± 2.41
Mean speed (m/s)	0.081 ± 0.008	0.088 ± 0.009	0.088 ± 0.006	0.091 ± 0.006	0.090 ± 0.008	0.090 ± 0.007	0.128 ± 0.005	0.126 ± 0.007	0.115 ± 0.009	0.118 ± 0.011	0.106 ± 0.007	0.109 ± 0.008

Values represent mean ± s.e.m. Result are from the main experiment as illustrated in Fig. 1.

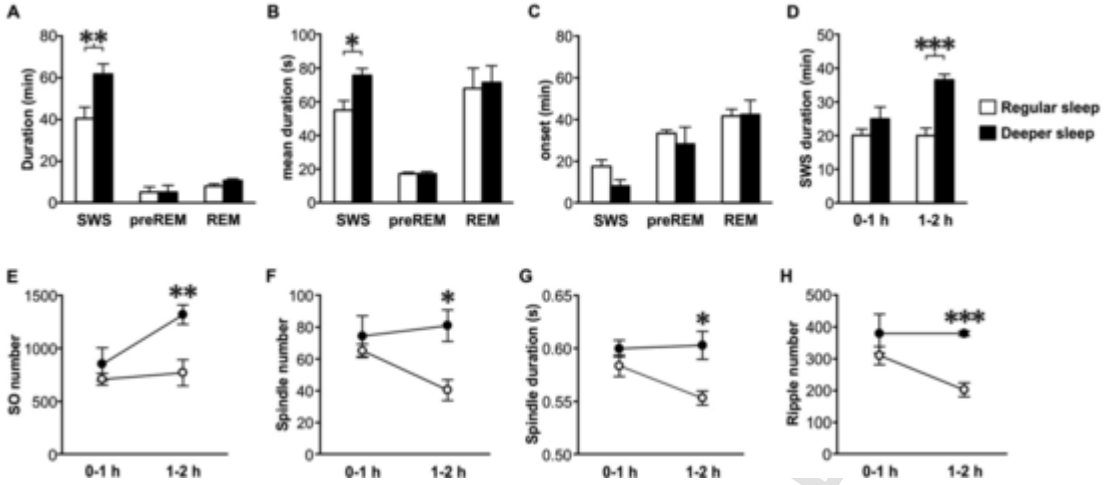

Fig. 2. "Deeper sleep" is characterized by a selectively enhanced SWS-related activity. EEGs and intrahippocampal LFPs were recorded in two additional groups of rats to characterize deeper sleep (n = 6 rats, black bars and circles) in comparison with regular sleep (n = 5 rats, open bars and circles) during a 2-hour interval following encoding of the OPR task. Experimental procedures were the same as described in Fig. 1A except that the rats did not perform the retrieval test. Compared with regular sleep, deeper sleep was associated with an increased (A) total SWS duration, and (B) mean duration of SWS epochs. (C) Sleep stage onsets were comparable in both conditions. (D) Enhanced SWS duration during the deeper sleep condition was observed exclusively in the second hour of post-encoding sleep. Numbers of slow oscillations (E), and spindles (F), spindle duration (G), and the number of hippocampal ripples (H) were selectively increased in the second hour of post-encoding sleep. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ for t-tests (two-sided) between deeper and regular sleep.

is further enhanced. This view also concurs with the present evidence that the enhancing effects of deeper sleep results from sleep changes focussing on the second hour after encoding.

The central finding of this study is that deeper sleep – irrespectively of whether it extended over a 2-hour or 4-hour post-encoding period – strengthens OPR memory such that it can be retrieved 1 week later. Previous studies showed that OPR memory can be maintained up to 1 week (Hardt, Migues, Hastings, Wong, & Nader, 2010; Migues et al., 2016). However, those studies used a more intense training protocol for encoding the task. We show here that, without repeated exposures to the same learning context, a single 10-min exposure to the task configuration at encoding is sufficient for forming a long-term memory when the rat has subsequently deeper sleep. Moreover, deeper sleep seems to specifically support memory for the hippocampus-dependent spatial task aspects. In a previous study using a protocol identical with the "regular sleep" condition of the present study, we showed that, following a 2-hour post-encoding period of regular sleep non-hippocampus-dependent novel-object recognition memory is preserved for at least 3 weeks (Sawangjit et al., 2018). By contrast, in the regular sleep condition of the present study, OPR memory had already completely faded at the 1-week recall which, against this backdrop, reflects the inability to specifically maintain spatial aspects of the encoded episode, rather than a failure to recognize the objects (Moscovitch, Cabeza, Winocur, & Nadel, 2016).

What defines "deeper" sleep? Our comparisons of EEG recordings during regular and deeper sleep conditions revealed a selectively increased time in SWS, while time in preREM and REM sleep remained unchanged. In addition, the oscillatory hallmarks of SWS, i.e., number of slow oscillations, spindle number and duration as well as hippocampal ripples were increased. Altogether, these changes underline the importance of SWS for forming long-term hippocampus-dependent memory (Klinzing et al., 2019; Marshall & Born, 2007). The increase in ripples appeared to be particularly robust. Ripples in hippocampal networks typically accompany replay of newly encoded spatial memory, and the suppression of ripples impairs spatial memory formation (Ego-Stengel & Wilson, 2010; Girardeau, Benchenane, Wiener, Buzsáki, & Zugaro, 2009). Ripple as well as spindle activity is found to be enhanced during sleep after encoding of hippocampus-dependent memory (Eschenko, Mölle, Born, & Sara, 2006, 2008). Against this backdrop, increases in ripple and spindle activity during deeper sleep after OPR encoding might partly reflect the increased processing of the newly encoded spatial information especially occurring in conditions of deeper sleep. Enhanced slow oscillatory and spindle activity during deeper sleep might prime ripple-coupled replay of the newly encoded spatial memory and the transmission of the replayed information to networks outside the hippocampus. Concurrently, these enhancements in slow oscillatory and spindle activity support synaptic plastic processes enabling the formation of long-term memory for the information in extrahippocampal spatial networks (Eichenbaum, 2017; Maviel, Durkin, Menzaghi, & Bontempi, 2004; Niethard et al., 2018; Seibt et al., 2017) and, consequently, the better retrievability of these memories at a remote recall.

Importantly, the changes in sleep and associated oscillatory signatures characterizing deeper sleep focussed on the second hour after encoding. Fittingly, only the increased time asleep in this second post-encoding hour was found to positively correlate with OPR performance at the remote 1-week recall test, and there was no similar correlation for time asleep in the later hours of the 4-hour post-encoding sleep period. These results suggest that SWS-rich sleep within 2 h after encoding effectively strengthens memory, with additional sleep providing no further benefit. Findings from other studies likewise point to a particular importance of SWS within the first two hours after encoding for memory formation: Reinforcing the coordination of spindle-ripple-SO events by ripple-triggered cortical stimulation applied approximately within this post-encoding time interval, distinctly improved OPR memory performance tested on the next day (Maingret, Girardeau, Todorova, Goutierre, & Zugaro, 2016). Moreover, sleep spindle and hippocampal sharp-wave ripple activity after encoding of a hippocampus-dependent odor-place association task were elevated for up to 2 h after post-encoding sleep onset (Eschenko et al., 2006, 2008). Also, the hippocampal replay of newly encoded memory appears to occur most frequently during this early post-encoding period (Giri, Miyawaki, Mizuseki, Cheng, & Diba, 2019; Kudrimoti, Barnes, & McNaughton, 1999; O'Neill, Pleydell-Bouverie, Dupret, & Csicsvari, 2010). Sleep deprivation in mice limited to a 3-hour window after learning impaired long-term potentiation (LTP) as well as OPR memory tested 24 h later (Prince et al., 2014). Indeed, within hippocampal networks, protein synthesis is required within 2 h after encoding for consolidating OPR memory (Ozawa, Yamada, & Ichitani, 2017), and also hippocampal NMDA receptors have been shown to be specifi-

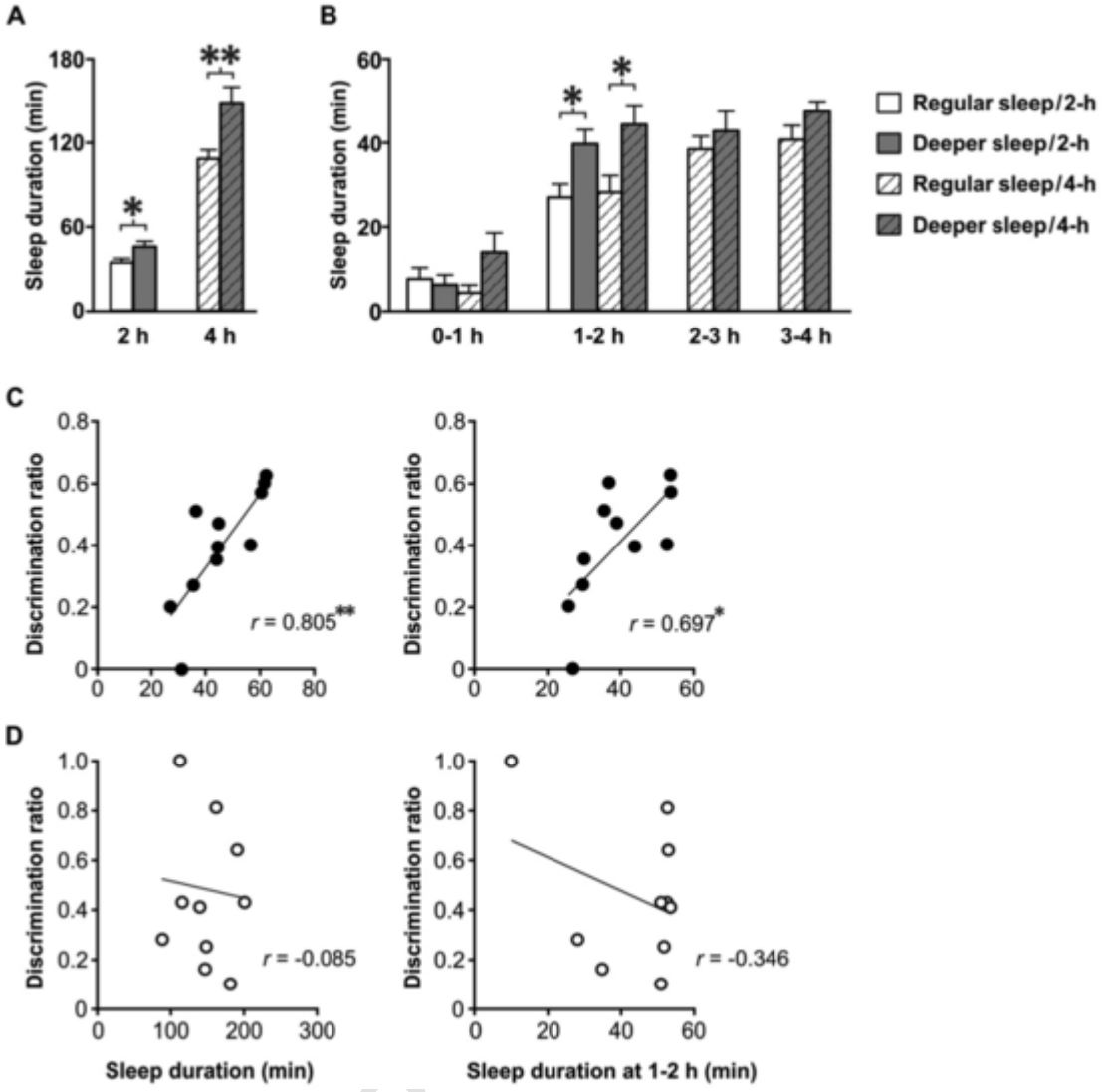

Fig. 3. "Deeper sleep" is characterized by enhanced sleep duration during the second hour of post-encoding sleep. (A) Total time spent asleep in the conditions of "deeper sleep" (grey bars,) and "regular sleep" (white bars) during post-encoding periods of either 2 h (plain) or 4 h (hatched). (B) Sleep duration (in min) during consecutive 1-hour bins in the same conditions (Deeper sleep/2-hour retention: $n = 11$; Deeper sleep/4-hour retention, $n = 10$; Regular sleep/2-hour retention, $n = 10$; Regular sleep/4-hour retention, $n = 10$). Note, increase in sleep duration during deeper sleep in the second hour of both 2-hour and 4-hour intervals. *$p < 0.05$ for t test between deeper vs regular sleep. (C) Correlation between OPR performance at the 1-week (remote) retrieval test and (left) the total sleep duration (in min) during a 2-hour post-encoding period of deeper sleep, and (right) sleep duration during only the second hour of this post-encoding period of deeper sleep ($n = 11$ rats, black circles). (D) The same as in C for deeper sleep during a 4-hour post-encoding period ($n = 10$ rats). Note, significant positive correlations in the 2-hour, but not in the 4-hour post-encoding period. **$p < 0.01$, *$p < 0.05$ for Pearson's correlations.

cally involved at this early stage of consolidation (Shimizu, Tang, Rampon, & Tsien, 2000; Yamada, Arai, Suenaga, & Ichitani, 2017). Deeper, i.e., SWS-rich sleep in the 2-hour post-encoding period might primarily support synaptic consolidation and a temporary stabilization of hippocampal OPR memory in this early phase, thereby setting the stage for an enhanced hippocampo-neocortical coordination underlying the more gradual emergence of long-term OPR memory in neocortical networks (Kitamura et al., 2017; Lesburguères et al., 2011).

However, any conclusions relating memory retrieval to EEG and LFP recordings remain tentative in the context of the present findings, because we did not measure OPR retrieval and sleep oscillations in the same animals, limiting any possible inferences as to the extent to which slow oscillations, spindles or ripples contributed to maintaining behavioural signs of OPR memory. Another limitation of our study to be mentioned here relates to the fact that our deeper sleep condition was associated with increased length of SWS bouts and also with a slight increased sleep duration, although this increase was much smaller than that observed after increasing the post-encoding retention period from 2 to 4 h. However, our EEG recordings also showed that the increases in the deeper sleep condition selectively pertained to the stage of SWS and its oscillatory hallmarks. Overall, this pattern indeed reflects that for SWS itself, effects of depth and duration are difficult to dissociate - unless highly artificial procedure such as selective auditory stimulation of slow oscillations are used (e.g., Ong et al., 2016; Papalambros et al., 2017; Ngo, Seibold, Boche, Mölle, & Born, 2019). Yet, rather than selectively deepening SWS the present study aimed at deepening sleep in terms of enhanced time in and signs of SWS, i.e., characteristics that were similarly used to define deeper sleep in previous human studies (Cordi, Schlarb, & Rasch, 2014).

Our experiments established the extended habituation to the sleep environment as an effective experimental procedure to deepen sleep and to specifically enhance SWS in rats. This habituation effect has been the focus of numerous studies in humans and, in this context, is explained by reduced activity of brainstem arousal systems that counteract sleep-promoting systems (e.g., Toussaint et al., 1997; Newell,

Mairesse, Verbanck, & Neu, 2012). The human studies confirm our findings in rats in consistently showing that increased habituation to the sleep lab over consecutive nights increases sleep efficiency and reduces time awake. However, in humans habituation also increased REM sleep, which we did not observe in our rats, possibly reflecting that the changes in sleep architecture vary depending on the actual degree of habituation achieved (Toussaint et al., 1997). If so, graded sleep habituation might be a promising tool for the study of effects of sleep depth with strong relevance for comparisons between species and for clinical populations. Whatever the case, here, we successfully used this procedure to show in rats that the effective formation of long-term OPR memory depends on the depth of post-encoding sleep, rather than on its duration.

CRediT authorship contribution statement

Anuck Sawangjit: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Carlos N. Oyanedel:** Software, Formal analysis. **Niels Niethard:** Software. **Jan Born:** Conceptualization, Writing - review & editing. **Marion Inostroza:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Significance statement

Comparing effects of the depth and duration of sleep after encoding spatial information, we show in a rat model that deeper sleep, i.e., a longer time spent specifically in slow wave sleep, is the essential factor mediating sleep-dependent long-term memory formation. Only with deeper sleep after encoding were remote spatial memories formed and successfully retrieved after one week. We identified a 2-hour window after encoding to be critical for sleep-dependent formation of remote memory.

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