Temporal associations between sleep slow oscillations, spindles and ripples

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Abstract
The systems consolidation of memory during slow-wave sleep (SWS) is thought to rely on a dialogue between hippocampus and neocortex that is regulated by an interaction between neocortical slow oscillations (SOs), thalamic spindles and hippocampal ripples. Here, we examined the occurrence rates of and the temporal relationships between these oscillatory events in rats, to identify the possible direction of interaction between these events under natural conditions. To facilitate comparisons with findings in humans, we combined frontal and parietal surface EEG with local field potential (LFP) recordings in medial prefrontal cortex (mPFC) and dorsal hippocampus (dHC). Consistent with a top-down driving influence, EEG SO upstates were associated with an increase in spindles and hippocampal ripples. This increase was missing in SO upstates identified in mPFC recordings. Ripples in dHC recordings always followed the onset of spindles consistent with spindles timing ripple occurrence. Comparing ripple activity during co-occurring SO-spindle events with that during isolated SOs or spindles, suggested that ripple dynamics during SO-spindle events are mainly determined by the spindle, with only the SO downstate providing a global inhibitory signal to both thalamus and hippocampus. As to bottom-up influences, we found an increase in hippocampal ripples ~200 ms before the SO downstate, but no similar increase of spindles preceding SO downstates. Overall, the temporal pattern is consistent with a loop-like scenario where, top-down, SOs can trigger thalamic spindles which, in turn, regulate the occurrence of hippocampal ripples. Ripples, bottom-up, and independent from thalamic spindles, can contribute to the emergence of neocortical SOs.

Abbreviations: ANOVA, analysis of variance; AP, anteroposterior; dHC, dorsal hippocampus; DV, dorsoventral; EEG, electroencephalography; EMG, electromyography; IS, intermediate stage; LFP, local field potential; ML, mediolateral; mPFC, medial prefrontal cortex; PFA, paraformaldehyde; REM, rapid eye movement; SD, standard deviations; SEM, standard error of the mean; SO, slow oscillation; SWS, slow-wave sleep.

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1 | INTRODUCTION

Sleep has been identified as a state that supports the systems consolidation of hippocampal memory (Diekelmann & Born, 2010; Dudai, Karni, & Born, 2015; Sawangjit et al., 2018). In particular, during slow-wave sleep (SWS), the hippocampus and neocortex establish a dialog where the depolarizing upstates of the slow oscillations (SOs) coordinate the occurrence of thalamic spindles in synchrony with hippocampal ripples, the latter accompanying the reactivations of hippocampal memory representations during SWS (Rasch & Born, 2013; Skelin, Kilianski, & McNaughton, 2019; Watson & Buzsáki, 2015). Ripples nesting in spindle oscillations have been proposed as a mechanism promoting the hippocampo-to-neocortical transmission of reactivated memory information and the more gradual redistribution of representations toward neocortical networks (Clemens et al., 2007, 2011; Latchoumane, Ngo, Born, & Shin, 2017; Siapas & Wilson, 1998; Sirotta, Csicsvari, Buhl, & Buzsáki, 2003; Staresina et al., 2015). The SO (~1 Hz) is a global and synchronized cortical phenomenon that preferentially originates in prefrontal cortex, substantially involving subcortical structures like the thalamus (Crunelli & Hughes, 2010; Hughes, Cope, BLETHYN, & Crunelli, 2002; NESKE, 2016; Steriade, Contreras, Contreras, CURRO DOSSI, & Nuñez, 1993), and typically travels toward posterior cortex, reaching also the hippocampus (MASSIMINI, Huber, Ferrarelli, Hill, & Tononi, 2004; STARESINA et al., 2015). The shorter downstate of the SO is associated with generalized hyperpolarization and reduced neuronal firing whereas the longer SO upstate goes along with synchronized membrane depolarization and increased neuronal firing and also drives the generation of thalamic spindles (DESTEXHE, Contreras, & Steriade, 1999; NESKE, 2016; NIETHARD, Ngo, EHRLICH, & Born, 2018; Steriade, MCCORMICK, & SEJNOWSKI, 1993b). Spindles (10–15 Hz), originating from GABAergic networks of the reticular thalamic nucleus, spread via thalamo-cortical fibers to the entire neocortex, but they also reach the hippocampus where they are phase-locking ripples (Clemens et al., 2007, 2011; KIM, Hwang, Lee, Sung, & Choi, 2015; Staresina et al., 2015; Steriade, Contreras, et al., 1993). Ripples are high-frequency bursts (100–200 Hz) that occur in hippocampal areas in conjunction with a CA3-generated sharp wave. But, they can also occur in associated regions outside the hippocampus (Buzsáki, 2015; Khodagholy, GELINAS, & Buzsáki, 2017; Mölle, Eschenko, Gais, Sara, & Born, 2009; Oliva, Fernández–Ruiz, Buzsáki, & Berényi, 2016). Ripples typically accompany the reactivation of neuronal ensembles that are activated and used for encoding the representation during prior wake phases (Diba & Buzsáki, 2007; Khodagholy et al., 2017).

Although there is a continuing controversy about the role SOs, spindles and ripples play in memory consolidation, for example Ackermann, Hartmann, Papassotiropoulos, de Quervain, and Rasch (2015); Kim, Gulati, and Ganguly (2019); Ngo and Born (2019), a large body of evidence supports the view that these oscillatory events are involved in memory formation (summarized in Inostroza & Born, 2013; Klinzing, Niethard, & Born, 2019; Skelin et al., 2018). Indeed, several studies consistently revealed an association of memory formation with an increased co-occurrence of these oscillatory events, where ripples tend to nest in the excitable phases of the spindle, and such spindle–ripples events tend to nest into the upstate of the SO (Helfrich, Mander, Jagust, Knight, & Walker, 2018; Latchoumane et al., 2017; MAINGRET, Girardeau, Todorova, Goutierre, & Zugaro, 2016; Mölle et al., 2009; Mölle, Yeshenko, Marshall, Sara, & Born, 2006; SIROTA et al., 2003; STARESINA et al., 2015).

In light of the strong implications of these findings for memory processing during SWS, here we aimed at a characterization of the dialogue between neocortex and hippocampus during SWS in terms of SO, spindle and ripple events, under natural conditions. Of special interest was the temporal relationship between these events—what comes first—which in connection with the spatial distribution of the events across the different brain regions allows to specify the direction of the interaction between these events. For this purpose, we recorded in rats EEG signals via skull electrodes over the frontal and parietal cortex and, local field potentials (LFPs) from medial prefrontal cortex (mPFC) and dHC. Our results are consistent with the view that top-down, the SO downstate provides a suppressive signal that synchronizes thalamic spindles and hippocampal ripples, whereas the SO upstate drives mainly thalamic spindles which, in turn, regulate hippocampal ripple occurrence. Hippocampal ripples might, bottom-up, contribute to the occurrence of neocortical SO events. Differing from previous findings, we do not find, under natural conditions, hints for a contribution of spindles to the generation of SO events.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Long Evans rats (Janvier, n = 5, 280–340 g, 14–18 weeks old) were used. The rats were kept in temperature (22 ± 2°C).
and humidity (45%–65%) controlled cages, on a 12-hr light/dark cycle with the lights off at 19:00 hr. Water and food were available ad libitum. All experimental procedures were approved by the University of Tübingen and the local institutions in charge of animal welfare (Regierungspräsidium Tübingen). The animals had been used in a previous study (Durán, Oyanedel, Niethard, Inostroza, & Born, 2018).

2.2 | Surgeries

Animals were anesthetized with an intraperitoneal injection of fentanyl (0.005 mg/kg of body weight), midazolam (2.0 mg/kg) and medetomidin (0.15 mg/kg). They were placed into a stereotaxic frame and were supplemented with isoflurane (0.5%) if necessary. The scalp was exposed and five holes were drilled into the skull. Three EEG screw electrodes were implanted: one frontal electrode (AP: +2.6 mm, ML: −1.5 mm, relative to Bregma), one parietal electrode (AP: −2.0 mm, ML: −2.5 mm, relative to Bregma) and one occipital reference electrode (AP: −10.0 mm, ML: 0.0 mm, relative to Bregma). Additionally, two platinum electrodes were implanted to record LFP signals (also referenced to the occipital skull electrode): one into the right medial prefrontal cortex (mPFC; AP: +3.0 mm, ML: +0.5 mm, DV: −3.6 mm) and one into the left dorsal hippocampus (dHC; AP: −3.1 mm, ML: +3.0 mm, DV: −3.6 mm). Electrode positions were confirmed by histological analysis (Figure S1). For EMG recordings, in all animals a stainless steel wire was implanted in the neck muscle. Electrodes were connected to a six-channel electrode pedestal (PlasticsOne) and fixed with cold polymerizing dental resin, and the wound was sutured. Rats had at least 5 days for recovery.

2.3 | Recordings

Rats were habituated to the recording box (dark gray PVC, 30 x 30 cm, height: 40 cm) for two days, twelve hours per day. On the third day, animals were recorded for 12 hr, during the light phase. The animal's behavior was continuously tracked using a video camera mounted on the recording box. EEG, LFP and EMG signals were continuously recorded and digitalized using a CED Power 1,401 converter and Spike2 software (Cambridge Electronic Design). During the recordings, the electrodes were connected through a swiveling commutator to an amplifier (Model 15A54, Grass Technologies). EEG signals were amplified and filtered between 0.1 and 300 Hz. LFP signals were amplified and filtered between 0.1 and 1,000 Hz. EMG signals filtered between 30 and 300 Hz. The signals were sampled at 1 kHz.

2.4 | Sleep stage determination

Sleep stages and wakefulness were determined offline based on EEG and EMG recordings, using standard visual scoring procedures for consecutive 10-s epochs as previously described (Durán et al., 2018; Neckelmann, Olsen, Fagerland, & Ursin, 1994) (Table 1). Three sleep stages were discriminated: slow-wave sleep (SWS), intermediate stage (IS) and REM sleep. Wakefulness was identified by mixed-frequency EEG and sustained EMG activity, SWS by the presence of high amplitude low activity (delta activity: <4.0 Hz) and reduced EMG tone, REM sleep by low-amplitude EEG activity with predominant theta activity (5.0–10.0 Hz), phasic muscle twitches and decrease of EMG tone. IS was identified by a decreased delta activity, progressive increase of theta activity and presence of sleep spindles. Recordings were scored by two experienced experimenters (intrater agreement >89.9%). Afterward, consensus was achieved for epochs with divergent scoring.

2.5 | Event detections

To identify SOs, standard procedures were used as described in detail previously (Mölle et al., 2006; Sawangjit et al., 2018). In brief, EEG and LFP signals were filtered between 0.3 and 4.5 Hz, and an SO event was selected in the EEG if the following criteria were fulfilled: (a) two consecutive negative-to-positive zero crossings of the signal occurred at an interval between 0.4 and 2.0 s, (b) of these events in an individual rat and channel, the 35% with the highest negative
peak amplitude between both zero crossings were selected and (c) of these events the 45% with the highest negative-to-positive peak-to-peak amplitude were selected. Because in the LFP the SO shows up in opposite polarity, LFP signals were inverted (multiplied by $-1$) before applying the detection algorithm. The criteria resulted in the detection of SOs with downstate peak amplitudes exceeding $-80 \ \mu V$ in the EEG and $110 \ \mu V$ in LFP recordings, and peak-to-peak

| TABLE 2 Absolute numbers and co-occurrence of oscillatory events – slow oscillations, spindles, ripples during slow-wave sleep |
|---------------------------------------------------------------|
| **Absolute numbers of events** | **Frontal EEG** | **Parietal EEG** | **mPFC LFP** | **dHC LFP** |
| SOs | $3,175.4 \pm 810.6$ | $3,507.6 \pm 920.8$ | $3,252.2 \pm 850.6$ | $3,128.6 \pm 809.8$ |
| Spindles | $399.6 \pm 109.2$ | $317.4 \pm 85.0$ | $176.2 \pm 43.2$ | $226.4 \pm 70.3$ |
| Ripples | $1,498.6 \pm 298.5$ | | | |
| **Event density (#/min)** | | | | |
| SOs | $20.9 \pm 0.5$ | $22.9 \pm 0.5$ | $21.0 \pm 0.7$ | $20.6 \pm 0.5$ |
| Spindles | $2.7 \pm 0.1$ | $2.0 \pm 0.1$ | $1.2 \pm 0.1$ | $1.5 \pm 0.2$ |
| Ripples | | | | $10.5 \pm 0.9$ |
| **Mean event duration (s)** | | | | |
| SOs | $0.666 \pm 0.007$ | $0.663 \pm 0.003$ | $0.832 \pm 0.014$ | $0.785 \pm 0.055$ |
| Spindles | $0.598 \pm 0.008$ | $0.565 \pm 0.008$ | $0.525 \pm 0.007$ | $0.519 \pm 0.008$ |
| Ripples | | | | $0.101 \pm 0.004$ |
| % SOs co-occurring with spindles | | | | |
| Frontal EEG | $14.80 \pm 1.06$ | $14.76 \pm 1.07$ | $11.44 \pm 0.68$ | $12.29 \pm 0.47$ |
| Parietal EEG | $11.46 \pm 0.80$ | $11.19 \pm 0.91$ | $8.53 \pm 0.78$ | $10.27 \pm 0.51$ |
| mPFC LFP | $7.04 \pm 0.46$ | $6.95 \pm 0.52$ | $6.14 \pm 0.34$ | $5.69 \pm 0.46$ |
| dHC LFP | $8.40 \pm 1.06$ | $8.75 \pm 1.06$ | $7.20 \pm 0.95$ | $7.68 \pm 1.45$ |
| % SOs co-occurring with ripples | | | | |
| dHC LFP | $39.27 \pm 2.28$ | $40.09 \pm 2.52$ | $36.71 \pm 2.63$ | $38.32 \pm 2.61$ |
| % Spindles co-occurring with SOs | | | | |
| Frontal EEG | $81.20 \pm 2.09$ | $80.05 \pm 1.94$ | $80.05 \pm 1.17$ | $78.05 \pm 2.14$ |
| Parietal EEG | $81.35 \pm 2.24$ | $80.80 \pm 2.42$ | $79.58 \pm 1.82$ | $81.11 \pm 1.30$ |
| mPFC LFP | $69.55 \pm 0.92$ | $66.70 \pm 3.37$ | $74.76 \pm 4.59$ | $72.38 \pm 1.75$ |
| dHC LFP | $75.01 \pm 1.55$ | $77.02 \pm 2.43$ | $68.12 \pm 4.13$ | $71.81 \pm 2.16$ |
| % Spindles co-occurring with ripples | | | | |
| dHC LFP | $47.69 \pm 4.40$ | $46.26 \pm 4.90$ | $44.99 \pm 3.98$ | $51.74 \pm 5.86$ |

*Table S1 provides comparisons of densities during versus outside of SO and spindle events, respectively.*
amplitudes exceeding 120 μV in the EEG and 160 μV in LFP recordings.

Spindle detection was also based on procedures described previously, for example (Mölle et al., 2009). The EEG signal was filtered between 10.0 and 16.0 Hz. Then, the envelope was extracted via the absolute value, that is, the instantaneous amplitude, of the Hilbert transform on the filtered signal, followed by an additional smoothing (moving average with 200-ms window size). A spindle was identified when the absolute value of the transformed signal exceeded 1.5 standard deviations (SD) of the mean signal in the respective channel, during the animal's SWS epochs, for at least 0.4 s and not more than 2.0 s. Spindle onset was defined by the time when the signal the first time exceeded the 1.5-SD threshold. The spindle power was calculated as the integral of the envelope of the Hilbert-transformed signal between spindle onset and end. For calculating Hilbert transformations, the MATLAB function Hilbert was used. The envelope was extracted using the MATLAB function abs, which returns the absolute value (modulus), that is, the “instantaneous amplitude” of the transformed signal.

Ripples were identified only in dorsal hippocampal (dHC) LFP recordings (as described in Mölle et al., 2009). The signal was filtered between 150.0 and 250.0 Hz. As for spindle detection, the Hilbert transform was calculated and the signal was smoothed using a moving average (window size 200 ms). A ripple event was identified when the smoothed Hilbert transform value exceeded a threshold of 2.5 SDs from the mean smoothed Hilbert transform of the filtered signal during an animal's SWS epochs, for at least 25 ms (including at least three cycles) and for not more than 500 ms.

2.6 Co-occurrence of events

For analyzing the temporal relationships between SOs, spindles and ripples, we calculated event correlation histograms, with one of the event types used as reference (e.g., SOs) and one of the respective other two event types (spindles or ripples) used as target event. For calculating event correlation histograms, only epochs were considered in which a target event occurred within a ±1.5- s window around the reference event. Table 2 summarizes the proportion of reference events co-occurring (in this interval) with one of the respective target events, separately for the three types of events of interest (SOs, spindles, ripples). To analyze the occurrence of spindle and ripple events with reference to the SO, the respective target events were time-locked to the SO downstate peak (t = 0 s) representing the most distinct and optimal time reference for scaling the SO cycle. The SO upstate peak is typically much flatter and more variable and has been proven to provide only a very imprecise reference for averaging and event time-locking (Buzsáki, 2006; Mölle, Marshall, Gais, & Born, 2002). For the analogous analyses with spindles and ripples as reference events, the spindle onset and the maximum trough of a ripple, respectively, were used for time-locking target events. Window sizes (around t = 0 s) were always 3 s (±1.5 s), and bin size was 100 ms. To calculate the event rate for SOs, the downside peaks of all detected events were taken. For spindle and ripple activity, all detected spindle and ripple peaks and troughs were taken (exploratory analyses on spindles revealed basically identical results when counting one event per spindle). The counts in every bin were divided by the number of the reference events (used for time-locking one of the respective other two event types) and then divided by the bin width to give event rate per second (Hz).

2.7 Phase-locking analyses

Supplementing event correlation histograms, we calculated the “preferred cycle phase”, for the temporal association of spindles and hippocampal ripples, respectively, with the SO, as well as for the temporal association of ripples with the spindle oscillation. For determining the temporal associations with the SO cycle, each detected SO associated with a spindle and ripple, respectively, was filtered between 0.3 and 4.5 Hz and the Hilbert transform was calculated. Then, the instantaneous phase of the SO at spindle onset and ripple maximum, respectively, was extracted. Correspondingly, for determining the temporal associations of ripples with the spindle cycle, each spindle that co-occurred with a ripple was first filtered between 10.0 and 16.0 Hz, and then, the Hilbert transform was calculated, and the instantaneous phase of the spindle at the time of a ripple was extracted. For calculating the average preferred phase, we used the function CircHist of the CircStat toolbox (Berens, 2009; Zittrell, 2019).

2.8 Power spectral analyses

In addition to event-based analyses, we calculated time–frequency plots of LFP power (in dHC recordings) to analyze the co-occurrence of SO-spindle events with ripples. For this purpose, time–frequency analysis was performed per SO and spindle event. The function mtmconvol of the FieldTrip toolbox (Oostenveld, Fries, Maris, & Schoffelen, 2011) was used for frequencies from 150.0 to 250.0 Hz in steps of 1 Hz using a sliding Hanning tapered window with a variable, frequency-dependent length that always comprised ten cycles. Time-locked time–frequency analysis of all events was normalized by dividing the values with the average power during the baseline between −2.0 and −1.0 s before the event (using the FieldTrip function ft_freqbaseline, baselinetype: “relative”) and then averaged across all events (using the FieldTrip function ft_freqgrandaverage).
2.9 | Statistical analyses

Kolmogorov–Smirnov tests were used to assure normality of the distribution for each parameter. Differences in SOs and spindles among the different recording sites were assessed using repeated measures analyses of variance (ANOVA) with “recording site” as factor (frontal EEG, mPFC LFP, parietal EEG, dHC LFP), followed by post hoc paired sample t tests, to specify significant differences between any two of the recording sites. For the evaluation of event correlation histograms, each bin was compared to a baseline interval which was the 1-s interval form −2.0 s to −1.0 s prior to the reference event at 0 s. For LFP recordings, these analyses were restricted to a ± 0.8-s interval around the reference event. Additionally, we tested the significance of the event correlation histograms against a randomized event distribution using procedures as described by Mölle et al., 2006. These analyses revealed essentially similar results and, hence, are not reported here. For statistical evaluation of ripple-related analyses revealed overall similar relationships to the SO for slow activity in an interval ± 1.5 s around the SO downstate peak, with a spindle was between 14.8 ± 1.1% in the frontal EEG and 6.1 ± 0.3% in mPFC LFP recordings (Table 2). Event correlation histograms of spindle events time-locked to the SO downstate peak confirmed a clear relationship in both frontal and parietal EEG recordings such that spindle occurrence was diminished for a more or less extended interval around the SO downstate peak, and distinctly increased during the subsequent SO upstate, reaching a maximum ~500 ms after the SO downstate peak (see Figure 3, also for statistical comparisons). The SO upstate-related increase in spindle occurrence was likewise demonstrated in phase-locking analyses (Figure 3, right panels). Surprisingly, there was no distinct temporal association between SOs and spindles in the mPFC LFP (Figure 3c) or dHC LFP (Figure 3d). Extended analyses showed that mPFC SOs also did not modulate spindle occurrence in the other recordings, except for a slight increase in spindles in the parietal EEG during the SO upstate (Figure S2).

A complementing pattern with an increase in the occurrence of SO downstates preceding spindle onset in the EEG was revealed when, conversely, spindles were taken as reference of events correlation histograms for SO events. However, with the alignment to spindle onset the temporal relationships between SOs and spindles generally appeared to be more variable (Figure S3). Additional exploratory analyses revealed overall similar relationships to the SO for slow spindles determined in the 7–10 Hz band.

3 | RESULTS

3.1 | Event detection during SWS in the EEG, and cortical and hippocampal LFP

We analyzed brain oscillations during all SWS epochs recorded for each rat in a 12-hr session during the light phase. Figure S1 shows examples of parietal EEG and dHC LFP recordings during these SWS epochs for individual rats. The rats spent on average 364.1 min in SWS (Table 1). Table 2 summarizes occurrence (absolute numbers, densities) of SO, spindle, and ripple events and their co-occurrence in the different recording channels. SO density was highest in the parietal EEG and distinctly lower in the frontal EEG and dHC LFP ($F(3,12) = 3.7, p = .043$, see Figure 1 for pairwise comparisons). SO duration was shorter in LFP than EEG recordings and shortest in the mPFC LFP ($F(3,12) = 26.9, p < .001$). SO amplitude was higher in the parietal than frontal EEG and higher in the dHC than mPFC LFP ($F(3,12) = 7.0, p = .006$, Figure 1).

The number of spindles identified ranged between 399.6 ± 109.2 in the frontal EEG and 176.2 ± 43.2 in the mPFC LFP (Table 2). Both spindle density and duration were higher in the frontal EEG than all other sites ($F(3,12) = 13.9, p < .001$ and $F(3,12) = 26.7, p < .001$, respectively, Figure 2). Spindle power was lowest in mPFC and highest and most variable in dHC LFP recordings ($F(3,12) = 6.0, p < .009$). Spindle frequency was generally higher in EEG than LFP recordings ($F(3,12) = 19.6, p < .001$). In dHC LFP recordings, we detected $1.498.6 ± 298.5$ ripples with an average density of $10.5 ± 0.9$ ripples per minute, duration of $101.4 ± 4.2$ ms and power of $1.1 ± 0.2$ mV²/s.

3.2 | Temporal association between SOs and spindles

The percentage (of the total number) of SOs that co-occurred, in an interval ± 1.5 s around the SO downstate peak, with a spindle was between 14.8 ± 1.1% in the frontal EEG and 6.1 ± 0.3% in mPFC LFP recordings (Table 2). Event correlation histograms of spindle events time-locked to the SO downstate peak confirmed a clear relationship in both frontal and parietal EEG recordings such that spindle occurrence was diminished for a more or less extended interval around the SO downstate peak, and distinctly increased during the subsequent SO upstate, reaching a maximum ~500 ms after the SO downstate peak (see Figure 3, also for statistical comparisons). The SO upstate-related increase in spindle occurrence was likewise demonstrated in phase-locking analyses (Figure 3, right panels). Surprisingly, there was no distinct temporal association between SOs and spindles in the mPFC LFP (Figure 3c) or dHC LFP (Figure 3d). Extended analyses showed that mPFC SOs also did not modulate spindle occurrence in the other recordings, except for a slight increase in spindles in the parietal EEG during the SO upstate (Figure S2).

Histology

After the last recording session, rats were terminally anesthetized with fentanyl (0.01 mg/kg of body weight), midazolam (4.0 mg/kg) and medetomidin (0.3 mg/kg). The electrodes positions were marked by electrolytic lesion (10 µA, 30 s; Figure S1). Rats were perfused with physiological saline (200–300 ml) followed by 4% paraformaldehyde (PFA, 200–300 ml). After decapitation, the brains were removed and post-fixed in 4% PFA for one day. Coronal sections of 60 µm were cut using a vibratome, stained with 0.5% toluidine blue and examined under a light microscope.
3.3 Temporal association between SOs and ripples

The percentage of SOs co-occurring with hippocampal ripples was comparable for all channels: 39.3 ± 2.3% in frontal EEG, 40.1 ± 2.5% in parietal EEG, 36.7 ± 2.6% in mPFC LFP and 38.3 ± 2.6% in dHC LFP recordings (Table 2). Event correlation histograms of ripple events, referenced to the SO downstate peak, indicated a suppression of hippocampal ripples around the downstate peak of SOs in the frontal and parietal EEG, followed by an increased ripple occurrence during the SO upstate (Figure 4a). These upstate-related increases in ripple occurrence were also revealed in phase-locking analyses of SO-ripple co-occurrence (Figure 4a, right panels). The parallel downstate-related decrease and upstate-related increase in ripples in mPFC recordings did not reach significance. Instead, there was a slight but significant increase in ripples preceding (by ~400 ms) the SO downstate in mPFC recordings.

SOs identified in dHC recordings displayed a distinct dynamic of accompanying ripple activity (Figure 4a). While showing the typical upstate-related increase in ripple events, hippocampal SOs were accompanied by a second increase in ripples that preceded the SO downstate peak and was even more pronounced than the upstate-related increase. This ripple increase preceding the SO did not reflect an upstate-related ripple increase of a foregoing SO, because a comparison of isolated SOs with SOs occurring in a train of several succeeding SOs revealed the ripple increase preceding the SO downstate to be even more distinct for SOs occurring in isolation (Figure S4). Moreover, the number of SOs with ripples preceding and following the downstate was significantly lower than the number of SOs with either a preceding ripple or a following upstate-related ripple (Figure S4B), indicating that the two types of ripples were independently occurring during the hippocampal SO cycle.

Event correlation histograms of SO events referenced to dHC ripples confirmed that ripples were preceded by an increase in SO events as defined by the downstate peak, in the frontal and parietal EEG and dHC LFP, and there was also a suppression of such SO events in the EEG and mPFC LFP during an ongoing ripple (Figure 4b). In addition, in these
FIGURE 2 Characterization of spindles. (a) Time-frequency plots of power in the 0.1–20.0 Hz frequency band and grand mean (±SEM) spindles in the unfiltered signal from all recording sites time-locked to the maximum trough of a spindle (for n see Table 2). Power is color-coded and given as normalized value, that is, divided by the average power during a baseline interval (b) Top left, spindle density (events/min), that is, the number of spindles in each channel divided by the time in SWS. Top right, spindle duration (in s), that is, time between onset and end of a spindle. Bottom left, spindle power (in mV²/s), that is, the integral of the Hilbert-transformed signal between spindle onset and end. Bottom right, spindle frequency (in Hz). Box-whisker plots indicate median, upper (top) and lower (bottom) quartiles. *p < .05; **p < .01; ***p < .001 for pairwise comparison, n = 5

Histograms, hippocampal ripples were followed, with a delay of 200–500 ms, by an increase in SO events in the frontal and parietal EEG consistent with a bottom-up influence of ripples on SO occurrence.

3.4 | Temporal dynamic between spindles and ripples

The percentage of spindles co-occurring (±1.5-s around spindle onset) with hippocampal ripples averaged between 45.0 ± 4.0% (mPFC LFP) and 51.7 ± 5.9% (dHC LFP, Table 2). Conversely, the percentage of ripples in dHC co-occurring with spindles averaged between 7.2 ± 0.6% (mPFC LFP) and 17.4 ± 2.6% (frontal EEG). Figure 5a shows event correlation histograms for ripple events time-locked to (the onset of) spindles identified in the four different recordings. A distinct relationship was observed only for spindles in the parietal EEG such that here spindle onsets were followed, with a delay of ~300 ms, by an increased occurrence of ripples. There was a parallel increase in ripples for spindles in the dHC LFP which approached significance. No consistent patterns occurred in frontal EEG and mPFC LFP recordings. A supplementary phase-coupling analysis confirmed in 4 of the 5 rats significant spindle-ripple nesting such that the occurrence of ripples concentrated on the excitable phase of the spindle oscillation, particularly for spindles identified in dHC recordings (Latchoumane et al., 2017; Staresina et al., 2015). Histograms of spindle occurrence time-locked to ripples confirmed that ripples were preceded by an increase in spindle events starting 300–100 ms before, in all the recordings (Figure 5b). The pattern is overall consistent with a driving influence of spindles on ripple occurrence in hippocampal networks.

3.5 | Triple co-occurrence of slow oscillations, spindles and hippocampal ripples

We finally examined the co-occurrence of SOs with spindles and hippocampal ripples which has been proposed as a mechanism regulating information flow during the systems consolidation of memories (Latchoumane et al., 2017; Staresina et al., 2015). Spindles, in these analyses, were
detected in dHC LFP recordings, because analyses accounting for spindles detected in other channels did not reveal channel-specific differences in associated ripple activity, and because evidence from foregoing studies suggested that coupling between spindles and hippocampal ripples is strongest for spindles detected in the hippocampus, in comparison with spindles identified in cortical LFP or EEG recordings, for example, Clemens et al., 2011; Latchoumane et al., 2017. An event-based analysis indicated that the number of SO events co-occurring with spindle and ripple events was overall low, reaching a maximum of 3.6 ± 0.4% in dHC recordings, and thus did not provide sufficient statistical power for a fine-grained analysis of temporal relationships. Given that the determination of the three kinds of events of interest was based on more or less arbitrary amplitude criteria, we therefore decided, with regard to hippocampal ripples, to shift the focus of analysis to the signal power in the respective 150–250 Hz frequency band.

In a first analysis focussing on the role of SOs, we compared average power spectra of the dHC LFP in a ±0.3 s interval around the maximum trough of the spindle (identified in the dHC), between spindles that did and did not co-occur with an SO event. With respect to SOs, analyses were performed collapsed across events identified in all four channels. The spectra indicated an increase in 150–250 Hz ripple power oscillating around the maximum trough of the spindle (p < .01) which did not differ between spindles occurring in isolation and spindles co-occurring with an SO (Figure 6), suggesting that the presence of an SO does not substantially add to the spindle-related modulation of ripple power. Spindles co-occurring with SOs and isolated spindles did not differ in terms of duration, frequency or power (all p > .1).

In a second analysis concentrating on the role of spindles, we compared average power spectra of the dHC LFP in a ±0.8 s interval around the SO downstate peak, between SOs that did and did not co-occur with a spindle (identified in the dHC LFP). Figure 7 summarizes results of this analysis (see Figure S7 for an exploratory analysis where ripple activity was related to spindle activity in the respective channel of SO detection). The spectra indicated a suppression of ripple power around the SO downstate peak that was most distinct for isolated SOs (Figure 7). Importantly, ripple power was distinctly higher during SOs that co-occurred with spindles than during SOs occurring...
in isolation, with this difference being restricted \((p < .05)\) to a 100-ms bin around the downstate peak in the analyses across all channels, as well as in a separate analysis of SOs identified in the dHC LFP (Figure 7). SOs co-occurring with spindles and isolated SOs did not consistently differ in terms of amplitude and duration \((p > .1)\). Together, these findings go beyond our event-based approach (above) in indicating that the spindle oscillation is the primary factor driving hippocampal ripple activity even in the presence of a SO upstate, whereas the direct hippocampal influence of the SO appears to be restricted to its suppression of ripple activity during the hyperpolarizing downstate.

4 | DISCUSSION

We examined the communication between neocortex and hippocampus as established during SWS in rats through the interaction of neocortical SOs, spindles and hippocampal ripples. Combining concurrent LFP recordings from mPFC and
dHC and EEG recordings from frontal and parietal sites, we aimed at an integrative assessment of the oscillatory events of interest and their temporal relationships in natural conditions. As to top-down modulations, we found that SO downstates in the EEG are associated with a parallel decrease in spindle and hippocampal ripple activity whereas the SO upstate was associated with increases in spindle and ripple activity. Notably, this dynamic was not obtained in mPFC LFP recordings. Spindle onsets were followed by an increase in hippocampal ripple activity with, this increase not depending on whether or not the spindle co-occurred with a SO suggesting that, once a spindle is released and reaches the hippocampus, it dominates the regulation of hippocampal ripple activity. As to bottom-up influences, an increase in hippocampal ripple activity preceding (~200 ms) the SO downstate, whereas no similar increase preceding SO downstates was found for spindles, which in combination suggests that ripples directly contribute to the occurrence of neocortical SOs. Overall, in conjunction with findings from foregoing studies (e.g., Karimi Abadchi et al., 2020; Rothschild, Eban, & Frank, 2017), our approach revealed a rather differentiated picture of the temporal relationships between the three oscillatory events of interest, that is, a picture suggesting a loop-like scenario where top-down, the SO downstate sets the frame for a global time window for processing memory information (Figure 8). In this window, the transition into the SO upstate drives thalamic spindles which, in turn, the occurrence of ripples and associated replay of memory information in hippocampal networks. Bottom-up, ripples might contribute to the emergence of a neocortical SO.

By determining the precise temporal relationships, we aimed to reveal hints about the direction of information flow between neocortex and hippocampus during memory processing in SWS. Focussing on the oscillatory configuration under natural conditions, we refrained from experimentally manipulating one of the oscillations. This approach comes with the price that our data do not allow for strictly causal inferences between the rhythms, although the identified temporal relationships allow to exclude certain causal interactions. Importantly, we here deliberately supplemented our LFP recordings by surface EEG recordings, in order to support the translation of our findings to the conditions in healthy humans only allowing for EEG recordings. Indeed, relevant electrophysiological results from rodent sleep studies are often ignored in human research simply because of the lack of precise knowledge about how an intracortical LFP event appears in the surface EEG recording. Generally, the comparison of EEG signals, for example, over frontal cortex, with LFP signals from mPFC in the present study revealed that SOs and spindles as picked up in the EEG are not necessarily related to corresponding oscillations of the LFP in underlying cortex. Thus, LFP recordings reflect the much more localized generation of these oscillations, particularly of spindles, which agrees with previous work (Andrillon et al., 2011; Ayoub, Mölle, Preissl, & Born, 2012; Nir et al., 2011). Indeed, also human studies revealed that many sleep spindles have an extremely small spatial extent and are thus picked up only by methods with high spatial resolution, like MEG and intracortical LFP recordings (Deghani, Cash, & Halgren, 2011; Deghani, Cash, Rossetti, Chen, & Halgren, 2010; Hagler et al., 2018; Ujma, Hajnal, Bódizs, Gombos, & Erőss, 2019).

In keeping with the majority of studies in the field, we concentrated on an event-based analysis of SOs, spindles and ripples, with the numbers of events detected during SWS closely comparable to those in previous studies (Mölle et al., 2006; Rasch & Born, 2013; Siapas & Wilson, 1998; Sirotia et al., 2003). Of note, whereas the proportion of spindles co-occurring with an SO was generally >65%, conversely, the proportion of SOs co-occurring with a spindle was generally rather low (<15%; Table 2) which may be taken to question the concept of a strong driving influence of SOs on the thalamic generation of spindles. However, spindle-generating mechanisms undergo fast refractoriness which prevents that each SO can trigger a spindle event (Destexhe, Contreras, & Steriade, 1998; Ngo et al., 2015).

![FIGURE 4](image-url) Temporal association between slow oscillations (SOs) and hippocampal ripples. (a) Left panels: Event correlation histograms of ripple events time-locked to the SO downstate peak (0 s, vertical dashed lines) in (top left) frontal EEG, (top right) parietal EEG, (bottom left) mPFC LFP and (bottom right) dHC LFP signals. Event rate (in Hz) refers to ripple events quantified by all ripple troughs and peaks. Mean (±SEM) rates across all SO epochs with co-occurring ripples from five rats are shown. Graphs above the histograms show means (±SEM) for the respective reference SOs, time-locked to the SO downstate peak. Right panels: Results from complementary phase-locking analyses. Circular histogram of preferred phase for ripple occurrence during SO cycle (12 bins, 30° each, SO downstate peak is at 180° in EEG and at 0° in LFP recordings). Red dashed line and red range of the circle represent average phase and 95% confidence interval. **p < .01; ***p < .001 for Rayleigh test of deviance from an overall uniform phase distribution of ripples. Note, event correlation histograms and phase-locking analyses indicate a decrease in ripple occurrence around the SO downstate peak followed by an increase in ripple activity, for SOs in both EEG channels. Also, note increase in ripple activity before the SO downstate peak in both LFP channels. (b) Event correlation histograms of SO events time-locked to (the maximum trough) of hippocampal ripples (0 s, vertical dashed lines). SO events were identified in (top left) frontal EEG, (top right) parietal EEG, (bottom left) mPFC LFP and (bottom right) dHC LFP signals. Event rate (in Hz) refers to SO events quantified by their downstate peak. Mean (±SEM) rates across all ripple epochs with co-occurring SOs from five rats are shown. Graphs above the histograms show mean (±SEM) for the respective reference ripples, time-locked to the maximum ripple troughs. Bin size for event correlation histograms: 100 ms. Significant increases (red) and decreases (blue) in event rates are indicated (thin lines: p < .05; and thick lines: p < .01, for pairwise comparison with a 1-s baseline interval (−2.0 to −1.0 s))
methodological factors play a role: The localized nature of spindle events might have prevented detection of events co-occurring with an SO and, also, events might have been missed due to too high detection criteria. In the case of spindles, the commonly used detection procedures have indeed been found to lack convergent validity and to differ in how they extract the EEG events contributing to spectral peaks (Bódizs, Körmendi, Rigó, & Lázár, 2009; Cox, Schapiro, Manoach, & Stickgold, 2017; Ujma et al., 2015). Event detection criteria mainly based on amplitude–thresholds, are thus arbitrary to a certain extent and, in addition, difficult to compare between event types like SOs and spindles. Implicating an all-or-none conceptualization of the event of interest, such event detection approach may not sufficiently reflect that SO and spindle generation can capture and synchronize more or less extended networks resulting in LFP and EEG oscillations of smaller or greater amplitude. Generally, for these reasons, it seems justified to supplement an event-based analysis by power spectral analyses, which we did here to examine the triple co-occurrence of SOs, spindles and ripples.

**FIGURE 5** Temporal association between spindles and hippocampal ripples. (a) Event correlation histograms of ripple events time-locked to the onset of spindles (0 s, vertical dashed lines) identified in (top left) frontal EEG, (top right) parietal EEG, (bottom left) mPFC LFP and (bottom right) dHC LFP signals. Event rate (in Hz) refers to ripple events quantified by all ripple troughs and peaks. Mean (±SEM) rates across all spindle epochs with co-occurring ripples from five rats are shown. Graphs above the histograms show mean (±SEM) root mean square amplitude of the respective reference spindles, time-locked to the spindle onset. (b) Event correlation histograms of spindle events time-locked to the maximum trough of ripples identified in dHC recordings (0 s, vertical dashed lines). Spindle events were identified in (top left) frontal EEG, (top right) parietal EEG, (bottom left) mPFC LFP and (bottom right) dHC LFP signals. Event rate (in Hz) refers to spindle events quantified by all spindle troughs and peaks. Mean (±SEM) event rates across all ripple epochs with co-occurring spindle events from five rats are shown. Graphs above the histograms show dHC LFP grand averages (±SEM) time-locked to the maximum ripple troughs. Bin size for histograms is 100 ms. Significant increases (red) and decreases (blue) in event rates are indicated (t: p < .1; thin lines; p < .05; and thick lines: p < .001, for pairwise comparison with a 1-s baseline interval (−2.0 to −1.0 s))
Our EEG recordings confirmed previous findings of a robust increase in spindle activity accompanying the early upstate of SOs (Mölle, Bergmann, Marshall, & Born, 2011; Mölle et al., 2009; Nir et al., 2011) which supports the view that membrane depolarization of cortico-thalamic projections during the SO upstate are driving the generation of spindle activity in thalamic networks (Steriade, Contreras, et al., 1993; Steriade, McCormick, et al., 1993). A clear coupling of spindles to SO upstates was not observed in hippocampal LFP recordings, suggesting that such SO-spindle coupling is specific to cortico-thalamic feedback loops. SO and spindles in hippocampal LFP recordings likely represent traveling waves that reach these networks via thalamic and cortical projections (Varela, Kumar, Yang, & Wilson, 2014; Vertes, Hoover, Szigeti-Buck, & Leranth, 2007; Wolansky, Clement,
Peters, Palczak, & Dickson, 2006). The hippocampus itself is not capable of generating SOs (Isomura et al., 2006).

Interestingly, a coupling of spindles to SO upstates was also entirely absent in mPFC recordings. This finding might surprise at first glance, as the majority of SOs typically arise from prefrontal cortical networks (Massimini et al., 2004). Accordingly, here, the number of SOs identified in the mPFC LFP was distinctly higher than that in parietal EEG or hippocampal LFP signals, which also confirms the sensitivity of our mPFC recordings. The absent SO-spindle coupling in mPFC recordings, however, well agrees with intracranial recordings in humans where such coupling was similarly weakened or even completely absent specifically in recordings from prefrontal regions (Andrillon et al., 2011). It might reflect anatomical conditions with only weak cortico-thalamic projections conveying frontal depolarization to thalamic spindle generators (Carman, Cowan, & Powell, 1964). SOs arising from medial prefrontal cortex may primarily propagate intracortically toward posterior areas, which is consistent with our observation that SO upstates in mPFC recordings were associated with an increased spindle activity in the parietal EEG (Tatsuno et al., 2020).

Not only spindles but also hippocampal ripples nested into the SO upstates, with this upstate-related increase being preceded by a dip in ripple activity during the prior SO downstate. Ripple occurrence distinctly increased also following the onset of spindles in the parietal EEG and hippocampal LFP, and hippocampal ripples were preceded by increased spindle activity in all channels. Moreover, hippocampal ripple power was increased during spindles regardless of whether the spindles co-occurred with an SO or not. On the other side, ripple activity was significantly higher when a spindle identified in hippocampal recordings co-occurred with a SO than during an isolated SO. Altogether these observations suggest that spindles reaching the hippocampus are the primary regulator of ripple activity in these networks, even in the presence of an SO. The influence of the SO, in this constellation, appears to be mainly restricted to a downstate-related suppression of ripples, indicating that the downstates of these global SOs also effectively inactivate hippocampal circuitry (Behrens, Van Den Boom, De Hoz, Friedman, & Heinemann, 2005). The view of spindles as the primary regulator of hippocampal ripple activity agrees with findings of a previous study, where optogenetically induced spindles identified in hippocampal LFP recordings synchronized hippocampal ripple activity regardless of whether or not the spindle was induced during an SO upstate (Latchoumane et al., 2017). The pathways mediating hippocampal spindle effects on ripple activity are unclear, but likely involve the nucleus reuniens of the thalamus projecting mainly to the CA1 subfield (Cassel et al., 2013; Varela et al., 2014; Vertes, Hoover, Do Valle, Sherman, & Rodriguez, 2006).

A related question arising here pertains to the precise site of the ripples regulated by spindles, since our hippocampal LFP recording sites in the different animals were not restricted to CA1 but also covered neighboring regions. In fact, recordings and positioning of electrodes in our study did not aim to reflect the layer-specific location of ripple generation in CA1, but to also capture volume conducted ripple signals as they can be recorded also from neighboring structures (e.g., Buzsáki, 2006; Mölle et al., 2009; Oliva et al., 2016). Given that frequency, amplitude and temporal criteria for our ripple detection adhered to common standards (e.g., Diba & Buzsáki, 2007; Maingret et al., 2016; Remondes & Wilson, 2015; Varela & Wilson, 2020), it is likely that the identified high-frequency oscillatory bursts represented ripples. Moreover, visual inspection did not reveal any differences in spindle–ripple relationships between rats with LFP recordings inside versus outside CA1, suggesting that ripples in recordings outside CA1, to a large part, reflect volume conducted signal from this area, although this is in need of further study.

Our data also provide cues about possible bottom-up contributions of hippocampal ripples to neocortical SOs.
Hippocampal ripples were consistently followed by an increased occurrence of SO downstates. This relationship was likewise evidenced when ripples were aligned to SO downstates in dHC recordings, and such increase in ripples also preceded the SO downstates identified in mPFC recordings. These findings concur with previous studies suggesting that hippocampal ripples can directly prime the occurrence of cortical downstates by activating inhibitory cortical networks, especially in prefrontal cortex (Logothetis et al., 2012; Maingret et al., 2016; Xia et al., 2017). Interestingly, immediately during a hippocampal ripple the occurrence of cortical SO downstates was suppressed, suggesting a rebound mechanism that produces the increase in SOs with a delay of about 200 ms. Such mechanism would also be consistent with findings indicating that during the SO downstate, cortical inhibitory interneurons themselves are inactive (Niethard et al., 2018).

Surprisingly, we did not find clear hints at increases in spindle events that precede increases in cortical SO events. In previous studies, the stimulation of thalamic spindle activity consistently induced neocortical SOs (Latchoumane et al., 2017; Lewis et al., 2015). In combination, these data suggest that thalamic spindles, in principle, can contribute to SO generation, although this rarely happens in natural “unstimulated” conditions as examined here. This conclusion fits with evidence that spindle-generating networks appear to go into refractoriness distinctly faster than SO-generating networks (Antony et al., 2018; Ngo et al., 2015). It highlights the importance to examine the oscillatory interactions of interest in natural conditions. In sum, the temporal relationships revealed here suggest the presence of a loop-like scenario with a top-down global inactivation of the loop during the SO downstate, followed by a spindle regulated increase in ripples (and associated memory processing) in hippocampal circuitry during the SO upstate (Figure 8). Bottom-up, hippocampal ripples might trigger SOs and this influence appears to bypass spindle-generating thalamic networks. Thus, the temporal relationships described here provide a framework for the probing of causal relationships between these oscillatory events to be performed in future studies.

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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION
C.N.O., E.D., M.I. and J.B. planned and designed the experiments. C.N.O. and E.D. performed the experiments and performed the histology. C.N.O. E.D. and N.N. analyzed the data. C.N.O., E.D. and J.B. wrote the manuscript. All authors approved the final version of the paper.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from corresponding authors on reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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