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Auditory Closed-Loop Stimulation of the Sleep Slow Oscillation Enhances Memory

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SUMMARY

Brain rhythms regulate information processing in different states to enable learning and memory formation. The <1 Hz sleep slow oscillation hallmarks slow-wave sleep and is critical to memory consolidation. Here we show in sleeping humans that auditory stimulation in phase with the ongoing rhythmic occurrence of slow oscillation up states profoundly enhances the slow oscillation rhythm, phase-coupled spindle activity, and, consequently, the consolidation of declarative memory. Stimulation out of phase with the ongoing slow oscillation rhythm remained ineffective. Closed-loop in-phase stimulation provides a straight-forward tool to enhance sleep rhythms and their functional efficacy.

INTRODUCTION

Brain activity oscillates at different frequencies, reflecting synchronized activity that organizes information processing and communication in neuronal cortical networks in a state-dependent manner (Buzsáki and Draguhn, 2004; Varela et al., 2001). The <1 Hz slow oscillation (SO) represents the most distinct of these oscillations that hallmark the electroencephalogram (EEG) during slow-wave sleep (SWS) (Steriade, 2006; Timofeev, 2011). The SO is generated in cortical and thalamic networks and reflects global synchronous neural activity alternating between up states of membrane depolarization and increased excitability and down states of hyperpolarization and widespread neuronal quiescence, which spreads across the neocortex, also capturing subcortical structures like the hippocampus (Isomura et al., 2006; Massimini et al., 2004). Importantly, the SO critically contributes to information processing during sleep: apart from an involvement in synaptic downscaling and homeostasis (Tononi and Cirelli, 2006), SOs play a causal role for the consolidation of memory (Chauvette et al., 2012; Diekelmann and Born, 2010; Marshall et al., 2006). For this consolidating function, the synchronization of fast-spindle activity (12–15 Hz) together with hippocampal ripples to the depolarizing up state appears to be critical (Mölle et al., 2011; Mölle and Born, 2011).

The obvious functional importance has stimulated attempts to induce synchronized cortical SO activity through external stimulation, mainly by rhythmic electrical, transmagnetic, and auditory stimulation in humans and rats (Marshall et al., 2006; Massimini et al., 2007; Tononi et al., 2010; Vyazovskiy et al., 2009). Importantly, such studies imposed rhythms on the brain disregarding the phase of ongoing endogenous oscillating activity, which might explain the overall limited functional enhancement in memory retention accompanying SO induction. Here, we utilized the ongoing oscillatory EEG activity to apply, in a closed-loop feedback system, auditory stimulation in synchrony with the brain's own rhythm, thereby enhancing and extending trains of SOs during sleep.

RESULTS

Auditory In-Phase Stimulation Induces SO Activity and Enhances Memory Consolidation

Subjects (n = 11) were tested on two experimental nights, balanced in order. In the Stimulation condition, upon online detection of an SO negative half-wave peak during nonrapid eye movement (non-REM) sleep, two auditory stimuli (50 ms, pink noise) were delivered such that they concurred in time with the predicted up phases of the detected and the succeeding SOs (Figure 1A). The stimulation started with onset of consolidated non-REM sleep and was discontinued after 210 min. During the Sham condition, stimulation time points were marked but no stimulation was applied. The detection routine was resumed 2.5 s after presentation of the second auditory stimulus.

Averaging the EEG time locked to the first auditory stimulus revealed a clear increase in slow oscillatory activity, in comparison with the Sham condition (Figure 1B). Whereas in the Sham condition an individual SO cycle occurred, the two auditory stimuli in phase with the predicted SO up states formed a sequence of three succeeding SO cycles (in the following referred to as an "SO train"). This suggests a resonating response of the network induced by the in-phase stimulation. The decrease in SO amplitude across these trains might reflect that the second auditory stimulus did not always hit the optimal SO up state phase (due to jitter in the SO rhythm) or some kind of network refractoriness.

Spectral analysis performed on SWS epochs during the stimulation period showed that in-phase stimulation increased power

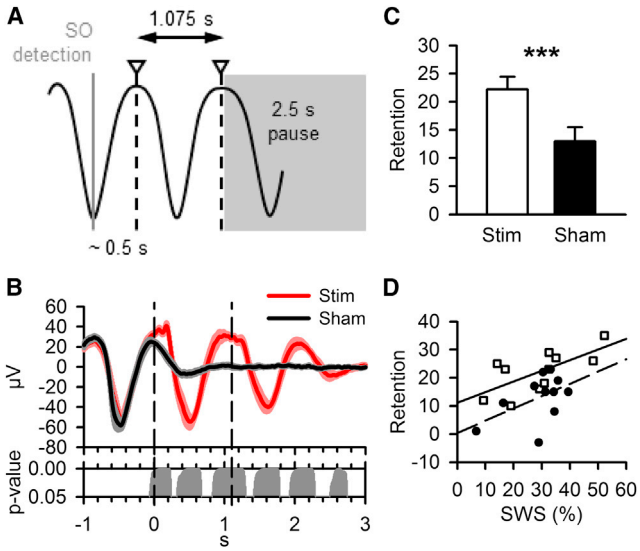

Figure 1. Closed-Loop Auditory Stimulation In-Phase with SO Up States Induces Trains of SOs and Enhances Declarative Memory

(A) Stimulation procedure. The detection of an SO negative half-wave peak triggered two auditory stimuli (vertical dashed lines), with the first occurring during the predicted up state of the detected SO and the second after a 1.075 s interval corresponding to the SO period length. During the Sham condition, time points of stimulation were marked, but no stimuli were presented. The detection routine was resumed 2.5 s after the second stimulus.

(B) Mean (±SEM) EEG signal (at Cz) averaged (across subjects) time locked to the first auditory stimulus (t = 0 s) for the Stimulation (red line) and Sham (black) conditions. Bottom panel indicates significant differences between conditions. Note that criterion electrode site used for online detection of negative half-wave peaks was Fpz, which explains that depicted negative half-wave amplitudes in the Stimulation condition, which were recorded from more posterior sites (i.e., Cz), are smaller than the −80 μV threshold used for online detection.

(C) Mean (± SEM) retention of word pairs across sleep for the Stimulation (Stim) and Sham conditions (***p < 0.001).

(D) Correlation between retention of word pairs and SWS percentage during the stimulation period separately for the Stimulation (empty squares, solid line, r = 0.68, p = 0.022) and Sham (filled circles, dashed line, r = 0.50, not significant) conditions.

Table 1. Properties of Slow Oscillation Cycles during In-Phase Auditory Stimulation

	Stim	Sham	p Value
Number of SO cycles (stimulation period)	781.3 ± 89.66	801.7 ± 89.8	0.839
Number of SO cycles (entire night)	1079.9 ± 118.7	983.6 ± 106.1	0.333
Amplitude (negative peak to positive peak)	133.6 ± 11.4 μV	122.6 ± 10.2 μV	0.009
Slope	351.0 ± 33.3 μV/s	322.3 ± 28.9 μV/s	0.008
Duration	0.98 ± 0.01 s	0.97 ± 0.01 s	0.086

Mean (±SEM) number of SO cycles identified (offline) in SWS epochs during the 210 min stimulation period and during the entire night, their negative-to-positive peak amplitude, slope, and duration (i.e., time between the two succeeding positive-to-negative zero crossings) during the stimulation period for the in-phase auditory Stimulation condition (Stim) and the Sham condition. p values are indicated for pairwise comparisons between conditions.

in the SO band (0.5–1 Hz) by about 9% (area-under-curve, $F_{1,10}$ = 20.4, p < 0.001, Figure S1, available online), i.e., in the frequency band matching the 1,075 ms interval between the two auditory stimuli. Of note, whereas examination of the entire slow-wave range (0.5–4 Hz) revealed no significant difference ($F_{1,10}$ = 0.04, p = 0.849), power in the neighboring delta band (1–4 Hz) was concurrently decreased by about 5% ($F_{1,10}$ = 8.8, p = 0.014). Together these findings suggest that in-phase stimulation entrained ongoing slow rhythms to the external frequency.

To assess effects of stimulation on overnight memory consolidation, subjects performed a paired-associates learning task (120 word pairs) before sleep. Generally, the subjects recalled more paired associates after sleep than at the immediate testing before sleep, which enabled re-encoding as feedback of the correct response word was provided. Strikingly, in the Stimulation condition, the retention rate, defined by the difference in recall performance after sleep minus immediate recall performance before sleep, was distinctly higher than in the Sham condition (22.2 ± 2.3 versus 13.0 ± 2.5 words, p < 0.001, paired t test, Figure 1C). In both the Stimulation and Sham conditions, overnight retention of word pairs was positively correlated with the percentage of SWS during the stimulation period, which was significant for the Stimulation condition (r = 0.68, p = 0.022) but not for the Sham condition (r = 0.50, p = 0.115, Figure 1D). Overall, this pattern suggests that entraining of slow-wave rhythms during SWS to the SO frequency through external stimulation is critical to the retention of the word-pair memories.

In-Phase Stimulation Modulates SOs and Phase-Locked Spindle Activity

In a more fine-grained analysis of oscillatory activity, we identified offline SO events during non-REM sleep (Table 1 summarizes SO characteristics). Subsequent averaging limited to the stimulation period revealed that the in-phase stimulation increased both the amplitude of the hyperpolarization down phase and the amplitude of the depolarization up phases of the SO ($F_{1,10}$ = 23.1, p = 0.004 and $F_{1,10}$ > 13.4, p < 0.004, respectively, Figure 2A).

Event correlation histograms of SOs, with reference to the auditory stimulation, confirmed that the stimulation was indeed capable of inducing trains of SOs, as indicated by an increased probability that one or two SO cycles followed the endogenous SO used for triggering the stimulation (p < 0.001, compared with the Sham condition, paired t test; Figure 2B). Also, probability for a third SO cycle was significantly elevated (p = 0.021, paired t test), pointing out that the auditory stimulation elicits a damped oscillation. Interestingly, the increased occurrence of SO trains after stimulation did not translate into an overall increased number of identified SOs in SWS (Table 1). In fact, there were no significant differences in sleep architecture between the Stimulation and Sham conditions for the stimulation period (Table 2 and Table S1), underlining that the in-phase stimulation chiefly entrained SO activity, leaving the processes initializing SOs unaffected.

In parallel, stimulation boosted phase-locked spindle activity during the SO cycle, i.e., the increase in fast-spindle activity

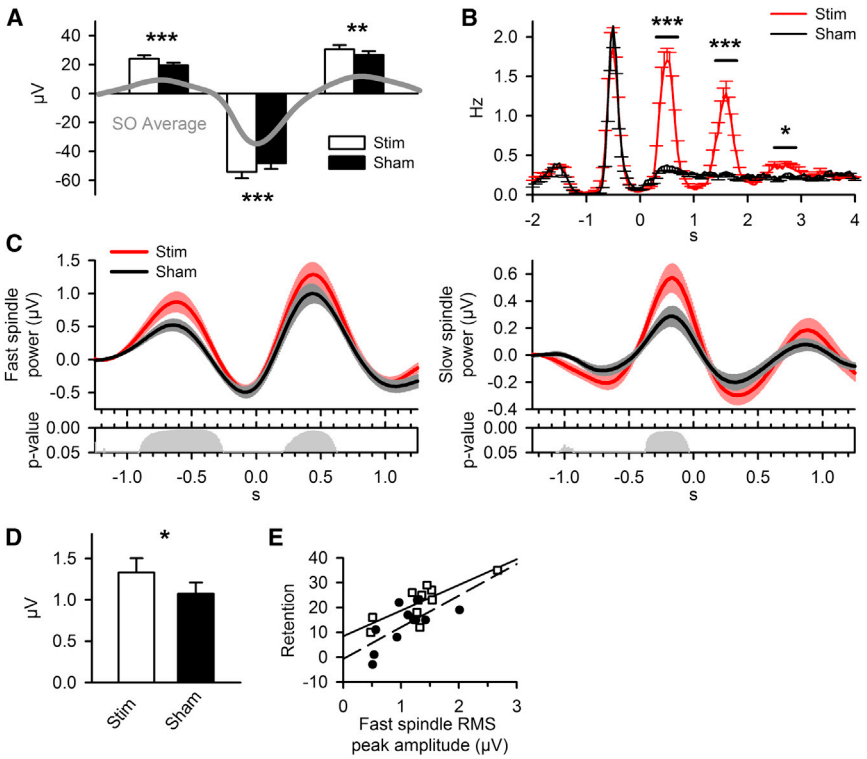

Figure 2. In-Phase Auditory Stimulation Enhances SOs and Spindle Activity

(A) EEG amplitude (averaged across all recording channels) determined for two succeeding depolarizing up states and the intermittent hyperpolarizing down state of offline detected SOs during SWS epochs of the stimulation period in the Stimulation (empty bars) and Sham (black bars) conditions; gray line illustrates average potential curve. Error bars denote SEM.

(B) Event-correlation histogram indicating occurrence of SO events during SWS for an interval between −2 s and +4 s around the first auditory stimulus (t = 0 s), for the Stimulation (red line) and Sham (black line) conditions. Data are means (±SEM) across n = 11 subjects.

(C) Fast- (12–15 Hz, left) and slow- (9–12 Hz, right) spindle band activity (root-mean-squared ± SEM, at Cz) averaged time locked to the negative half-wave peak of all offline detected SO cycles (t = 0 s) during SWS epochs of the stimulation period for the Stimulation (red line) and Sham (black line) conditions. Bottom panels indicate significant differences between conditions.

(D) Mean (±SEM) across all subjects of the rms peak amplitude determined from the fast-spindle activity phase locked to the SO up state after the negative half-wave for the Stimulation (empty bar) and Sham (black bar) conditions.

(E) Correlation between retention of word pairs and fast-spindle rms peak amplitude during the stimulation period separately for the Stimulation (empty squares, solid line, r = 0.798, p = 0.004) and Sham (filled circles, dashed line, r = 0.69, p = 0.018) conditions.

*p < 0.05, **p < 0.01, ***p < 0.001, for comparisons between Stimulation and Sham conditions.

(12–15 Hz) typically accompanying the SO up phase, and the increase in slow-spindle activity (9–12 Hz) occurring at the up-to-down transition of the SO cycle (Figure 2C, see Figure S2 for the evolvement of spindle activity across the induced SO train) (Andrillon et al., 2011; Mölle et al., 2011). Remarkably, this significant increase in fast-spindle activity (Stim: 1.3 ± 0.2 μV, Sham: 1.0 ± 0.1 μV, p = 0.013, for the peak root-mean-square [rms] amplitude derived during the up state after the negative half-wave, Figure 2D) revealed for both the Stimulation (r = 0.79. p = 0.004) and Sham (r = 0.69, p = 0.018) conditions a strong positive correlation with the overnight retention of word pairs (Figure 2E). As spindle power averaged across SWS of the entire stimulation period did not differ between conditions (Figure S1), these observations indicate that in-phase stimulation chiefly enhanced the synchronization of spindles to the SO and corroborates findings showing that the synchrony with the SO cycle rather than the amount of fast-spindle activity is critical for memory consolidation (Cox et al., 2012; Ruch et al., 2012; Timofeev et al., 2002).

Induced SOs Do Not Differ from Spontaneous Slow Oscillations

Does the induced SO represent a true SO? To answer this question, we more closely examined topography, slope, and traveling of SO cycles during in-phase stimulation. In both the Stimulation and Sham conditions, the SO showed the typical maximum over frontocentral cortical regions (Figure 3A). Analyses of variance indicated a significant effect of topography ($F_{1,18}$ = 193.6, p < 0.001) but no difference between the Stimulation and Sham conditions in the topographical distribution ($F_{18,180}$ = 1.294, p > 0.295, for Condition × Topography interaction). Also, the decrease in SO slope across sleep time, which is considered to reflect a global downscaling of cortical synapses (Riedner et al., 2007; Tononi and Cirelli, 2006), reached comparable values in the late-night half (Stimulation: $F_{1,9}$ = 31.2, p < 0.001, Sham: $F_{1,9}$ = 6.2, p = 0.034, Figure 3B). However, in-phase auditory stimulation acutely increased the SO slope so that the overnight decrease was greater than in the Sham condition ($F_{1,9}$ = 6.8, p = 0.028, for Condition × Night period interaction). Corresponding analyses based on SO amplitude, defined by the negative-to-positive peak amplitude, yielded essentially the same results, except that downscaling for the Sham condition did not reach significance (Stimulation: $F_{1,9}$ = 7.2, p = 0.025, Sham: $F_{1,9}$ = 2.6, p = 0.144 and $F_{1,9}$ = 6.8, p = 0.029 for Condition × Night period interaction; Figure 3B).

Analyses of SO traveling revealed in both conditions a preferential origin of SOs from anterior cortical sites (Figure 3C) and, depending on the point of origin, i.e., frontal, central, or parietal, a traveling mainly to anterior or posterior areas, respectively (Figure S3A). There were no differences in topographical or temporal features of traveling between the Stimulation and Sham conditions ($F_{18,180}$ < 1.3, p > 0.300, for all Condition × Topography interactions). Interestingly, an analysis of traveled path lengths indicated a reduced number of SOs that stayed local but more

Table 2. Sleep during the 210 Min Stimulation Periods

Parameter	Stim	Sham	p Value
In-phase stimulation			
W	6.3% ± 1.6%	7.3% ± 1.1%	0.545
S1	8.2% ± 1.0%	7.5% ± 1.4%	0.662
S2	48.6% ± 2.7%	47.3 ± 2.2	0.676
SWS	29.1% ± 4.1%	28.7% ± 2.8%	0.905
REM	7.7% ± 1.6%	9.2% ± 1.8%	0.148
MA	6.7% ± 0.4%	6.7% ± 1.0%	0.883
Out-of-phase stimulation (control)			
W	5.9% ± 1.5%	8.4% ± 1.7%	0.339
S1	3.5% ± 0.8%	5.7% ± 1.9%	0.236
S2	44.9% ± 5.8%	46.8% ± 4.2%	0.661
SWS	39.3% ± 7.0%	30.8% ± 5.2%	0.062
REM	6.4% ± 1.8%	8.3% ± 2.2%	0.094
MA	5.2% ± 0.7%	5.3% ± 0.8%	0.898

Mean (±SEM) percentage of time spent in different sleep stages during the 210 min stimulation period for auditory stimulation (Stim) in phase with SO up states and respective Sham condition and for a control study with auditory stimulation (Stim) out of phase with predicted SOs and respective Sham condition. p values are indicated for pairwise comparisons between Stimulation and respective Sham conditions. W, wake; S1 and S2, non-REM sleep stages 1 and 2; SWS, slow-wave sleep; REM, rapid eye movement sleep; MA, movement arousals.

SOs traveling longer paths in the Stimulation condition ($p = 0.011$ for local and traveling SOs, respectively, paired t test; Figure S3B) (Nir et al., 2011). However, additional analyses did not reveal any significant correlation between traveled path length or travel speed (assessed by the average delay in SO negative peak latency along the traveled path) and overnight retention of word pairs. (Significance for the maximum correlation of $r = -0.626$ observed, pointing toward an association of traveling speed with word retention, specifically for induced SO cycles originating from central sites, did not survive correction for multiple testing.) In combination, these analyses showed that in-phase auditory stimulation acutely amplifies SOs in terms of amplitude, slope, and spreading. However, the basic SO features of topography, morphology, and temporal dynamics remained essentially the same as under unstimulated conditions.

Out-of-Phase Stimulation Disrupts SO Activity and Does Not Affect Memory Consolidation

Is phase locking of the stimulation to the rhythm of SO up states crucial for the enhancing effect on SO activity? To test this, we modified the stimulation protocol. Not surprisingly, maintaining the interstimulus interval of 1,075 ms but merely shifting the auditory stimulation forward into the down state did not effectively disrupt endogenous SO activity. The first stimulus delayed occurrence of the succeeding SO cycle such that the second stimulus tended to fall into the depolarizing phase of an SO, thereby promoting rather than suppressing consecutive SOs (Figure S4A). However, additionally reducing the interstimulus interval to about half of the SO period effectively interfered with the development of SO trains (Figure 4A). With this protocol, the second stimulus tended to coincide with the hyperpolarization induced by the first stimulus (Figure 4B). Indeed, spectral analysis confirmed a decrease in power for frequencies <1 Hz during intervals of acute out-of-phase stimulation ($F_{1,6} = 14.6$,

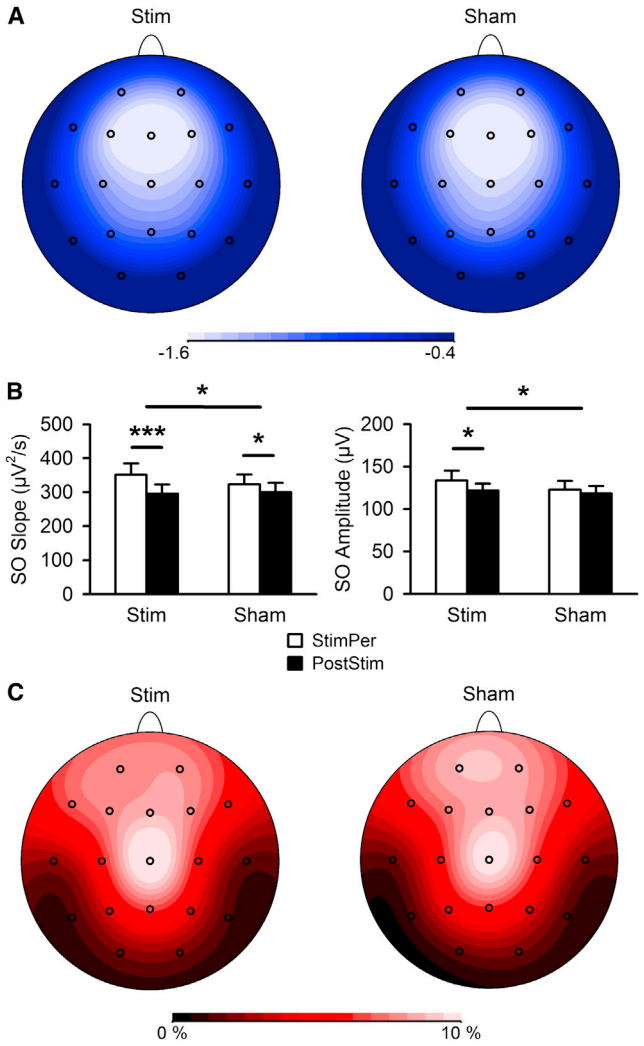

Figure 3. Induced and Spontaneous SOs Exhibit Identical Topography, Downscaling, and Traveling Characteristics

(A) Topography of offline detected SOs for the in-phase Stimulation (left) and Sham (right) conditions. The topographical distribution was determined with respect to the negative half-wave peak amplitude for all SOs detected during the stimulation period. Color code depicts the peak amplitude averaged across all subjects. Before averaging, individual amplitude values were normalized by the subject's absolute mean across all EEG channels for the respective condition.

(B) Downscaling of SO slope (left) and peak-to-peak amplitude (right) across the sleep period. Mean (±SEM) slope and amplitude of offline detected SOs averaged across all EEG channels for the 210 min stimulation period (StimPer, empty bars) and for the remaining sleep period during the late-night half (PostStim, black bars) for the Stimulation (Stim) and Sham conditions. n = 10, data from one subject were discarded because he did not enter SWS during the late-night half. *p < 0.05, ***p < 0.001, for pairwise comparisons.

(C) SOs originate preferentially from frontocentral sites. Topographical distribution of SO origins for the in-phase Stim (left) and Sham (right) conditions, expressed (color coded) as percentage of all traveling SOs detected during the stimulation period. Maps indicate means across all subjects.

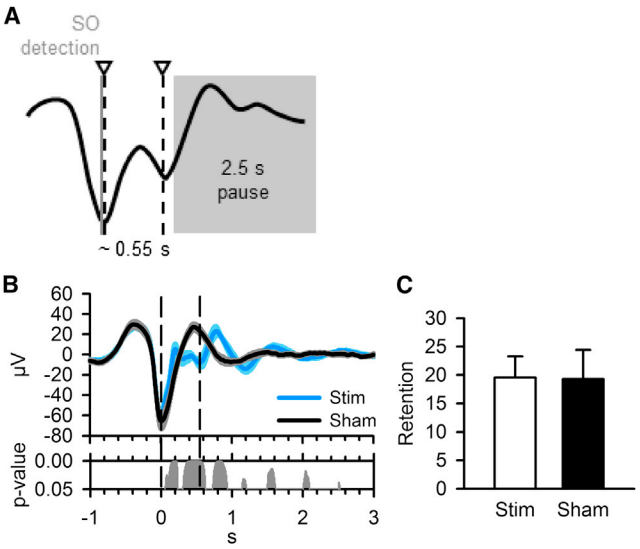

Figure 4. Closed-Loop Auditory Stimulation Out of Phase with the SO Up State Disrupts SO Activity and Does Not Improve Memory Consolidation

(A) Stimulation procedure. Upon detection of an SO negative half-wave peak, two auditory stimuli (vertical dashed lines) were applied, the first immediately and the second after a delay of about half the SO period length and individually adjusted to the hyperpolarization induced by the first stimulus.

(B) Mean (±SEM) EEG signal (at Cz) averaged (across subjects) in a 4 s interval time locked to the first auditory stimulus (t = 0 s) for the Stimulation (blue line) and Sham (black line) conditions. Bottom panel indicates significant differences between conditions.

(C) Mean (±SEM) retention of word pairs across sleep for the Stimulation (Stim) and Sham conditions. p = 0.93 for the difference between Stimulation and Sham conditions.

p = 0.009, Figures S4B and S4D). However, power in this frequency band recovered normal values as soon as acute stimulation stopped, reflecting the strong drive of cortical networks to oscillate in the natural SO frequency. Accordingly, power in the <1 Hz band did not differ between conditions for the entire time in SWS during the stimulation period ($F_{1,6}$ = 3.0, p = 0.136; Figures S4C and S4D). Importantly, in the absence of any SO enhancement, out-of-phase stimulation, unlike in-phase stimulation, did not produce any improvement in retention of word pairs (p = 0.925, paired t test; Figure 4C). These data underline that phase synchrony of the stimulation to the SO up state is essential to the enhancement of memory consolidation.

Separation of Auditory-Evoked Responses from Spontaneous Ongoing SO Activity

To separate the potential response evoked by the auditory stimulus during both the in-phase stimulation (main experiment) and out-of-phase stimulation (control experiment) from ongoing spontaneous SO activity, we subtracted the EEG averaged time locked to the onset of the first stimulus for the Sham condition from that obtained for the Stimulation condition. The evoked potential responses revealed this way comprised an early positive component with two overlapping peaks at about 75 and 180 ms poststimulus onset, followed by a pronounced negative component with a maximum at approximately 500 ms (Figure 5).

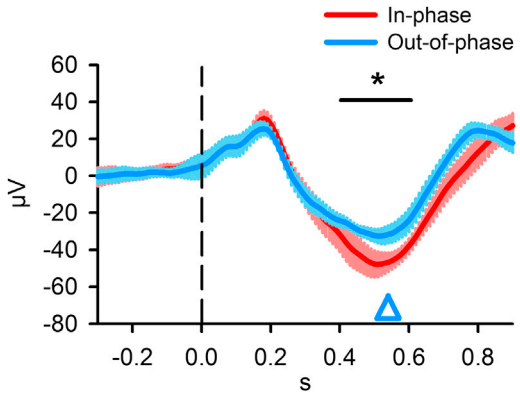

Figure 5. Auditory-Evoked Response to the First Stimulus during In-Phase and Out-of-Phase Stimulation

Mean (±SEM) evoked potential response (at Cz) for the in-phase (red, n = 11) and out-of-phase (blue, n = 7) stimulation derived by subtracting the average response time locked to the first stimulus of the Sham condition from that of the Stimulation condition. Vertical dashed line, stimulus onset; an 800 ms poststimulus interval is depicted; note that occurrence of second stimulus during out-of-phase stimulation at 550 ms (blue triangle) could confound the evoked response in this condition during later portions. *p < 0.05 for difference in amplitude between in-phase and out-of-phase stimulation. Note that whether and to what extent the auditory-evoked response revealed by the subtraction reflects potential activity elicited by the stimulus itself or changes to ongoing oscillations cannot be discriminated.

Early components did not differ between stimulation conditions, arguing against the view that SO down and up states are linked to a gating of ascending information, although the scalp-recorded EEG might not be sufficiently sensitive to reveal such effects (Rosanova and Timofeev, 2005). Amplitude of the late negative component was slightly smaller for the out-of-phase than in-phase stimulation (p = 0.019 for comparison at Cz), which probably reflects reduced neocortical excitability when the stimulus is presented during the SO down state (Bergmann et al., 2012). Latency of these components did not differ between in-phase and out-of-phase stimulation (p > 0.347).

DISCUSSION

Our data indicate that an enhancement of SO activity and overnight memory consolidation by auditory stimulation depends essentially on a timing in phase with SO up states. Utilizing closed-loop control, we took a novel approach to fine-tune auditory stimulation to the SO phase, thereby inducing resonant slow oscillatory activity. Although similar resonance in neocortical networks might be invoked by other kinds of stimulation, e.g., transcranial electrical or magnetic stimulation (Marshall et al., 2006; Massimini et al., 2007), auditory stimulation at low intensity is quite simple to apply and, more importantly, auditory stimuli during non-REM sleep are known to often evoke a K-complex-like response that shares basic characteristics with the SO in both morphology and generating mechanisms (Amzica, 2010; Cash et al., 2009; Colrain, 2005; Rosanova and Timofeev, 2005). The response comprises a marked frontocentral hyperpolarization at ~500 ms poststimulus followed by a broader depolarization highest at ~900 ms, often with spindles nesting

into the transition toward depolarization. The K-complex originates from the same corticothalamo circuitry that underlies the generation of SO, suggesting that this circuitry also mediates the resonant SO activity observed here after in-phase auditory stimulation (Amzica and Steriade, 2002; Rosanova and Timofeev, 2005). However, K-complexes occur as singular events, rather than in a rhythm.

Our analyses aiming to separate evoked auditory activity from spontaneous ongoing SO activity likewise indicated that the observed enhancement in the SO activity with in-phase stimulation cannot be simply reduced to a change in the response evoked by the auditory stimulus. In fact, responses to the (first) auditory stimulus during in-phase and out-of-phase stimulation showed quite similar waveforms with a comparable temporal component structure, although the amplitude of the late component slightly differed, which is a pattern consistent overall with the view that the primary impact of the phase-dependent administration of the stimuli was on ongoing SO rhythm generation. Whether and to what extent the evoked response obtained by subtracting spontaneous ongoing oscillatory activity reflects potential activity elicited by the stimulus itself or evoked changes to ongoing oscillatory activity (including SO activity) cannot be answered (e.g., Makeig et al., 2002).

Notably, in-phase stimulation did not produce an increase of SO events per se but rather caused an enhancement in SO amplitude, probably reflecting increased synchrony of up and down state transitions, and prolonged sequences of SO cycles, together with an increased alignment of fast- and slow-spindle activity to the SO cycle. Specifically, fast-spindle activity accumulated in the up phase and slow-spindle activity in the up-to-down transition within the SO cycle. Changes in slow oscillatory activity and nested fast-spindle activity have been consistently associated with a consolidating effect on memory (Huber et al., 2004; Mölle and Born, 2011; Ruch et al., 2012; Sejnowski and Destexhe, 2000; Timofeev et al., 2002; Wilhelm et al., 2011). In addition, spindle activity synchronized to the SO cycle might favor offline memory processing by gating external sensory input to the neocortex (Dang-Vu et al., 2011; Schabus et al., 2012).

In conclusion, while closed-loop stimulation has been successfully used to suppress pathological EEG rhythms in rats (Berényi et al., 2012), we demonstrated here that auditory closed-loop stimulation can be administered in humans to enhance normal brain rhythms, specifically of sleep SOs and their functional efficacy in memory consolidation. Indeed, closed-loop in-phase auditory stimulation at low intensity might be a promising tool to generally ameliorate efficacy of sleep rhythms, also in pathological conditions such as insomnia (Riemann et al., 2011).

EXPERIMENTAL PROCEDURES

Subjects

Eleven volunteers (eight women, three men; mean ± SEM age: 24.2 ± 0.9 years) participated in the main experiments. They were native German speakers, free of medication, and nonsmokers. Routine examination ensured that they had no history of neurological or psychiatric disease, including any sleep disorder. Subjects had followed a normal sleep-wake rhythm, i.e., no shift work, for at least 4 weeks before the experiments. Prior to the experiments, subjects were accustomed to sleeping under laboratory conditions during an adaptation night, including the attachment of electrodes for polysomnographic recordings. On experimental days, subjects were required to get up at 7:00 a.m. and not to take any naps during these days. Moreover, on these days, they were not allowed to consume alcohol or, after 3:00 p.m., caffeine-containing drinks. The study was approved by the ethics committee of the University of Lübeck, and all subjects gave written informed consent prior to participation.

Design and Procedure

Each subject was tested in two experimental conditions, a Stimulation condition and a Sham condition, with the order of conditions balanced across subjects. The subject's two experimental conditions were separated by an interval of at least 1 week. On experimental days, subjects arrived at the laboratory at 8:00 p.m. After preparation for EEG and polysomnographic recordings, subjects performed on a declarative memory task (word-pair associates) between 9:00 and 10:30 p.m. (learning phase) and then went to bed. Polysomnographic and EEG recordings started at 11:00 p.m. (lights off). Auditory stimulation started ~5 min after the subject displayed stable sleep stage 2 (or deeper) for the first time after sleep onset and was discontinued 210 min later. Subjects were awakened after 7 hr of sleep (~6:00 a.m.) the next morning, whenever they had entered light non-REM sleep, i.e., stages 1 or 2. About 30 min after awakening, recall of memories was examined (retrieval phase).

Memory Task

To assess declarative memory, a paired-associates learning task was used that had proven sensitive to the effect of sleep previously (Marshall et al., 2004; Plihal and Born, 1997). The task consisted of the sequential presentations of 120 pairs of nouns on a monitor, each for 4 s and with an interstimulus interval of 1 s. The words of each pair were moderately semantically related (e.g., brain, consciousness and solution, problem). Two different word lists were used for the subject's two experimental conditions, with the order of word lists balanced across subjects and conditions. At learning before sleep, presentation of the list was followed by an immediate cued recall test, in which the subject had to respond by naming the second word on presentation of the first word of each pair, with the word pairs presented in random order. The subject had unlimited time to recall the appropriate response word. Immediately after the response, the correct answer was revealed on the screen. At retrieval testing in the morning after sleep, cued recall was tested in the same manner as immediately after learning, except that no feedback was presented after the subject's response. Overnight memory retention was determined by the difference in the number of recalled words between morning retrieval testing after sleep and immediate recall performance at learning before sleep. To exclude confounds by general changes in executive function (including capabilities to retrieve information), subjects after memory testing performed on the psychomotor vigilance test (PVT) and rated sleepiness on the Stanford Sleepiness Scale (SSS), both of which did not reveal any difference between Stimulation and Sham conditions in the respective experiments, i.e., no effects of stimulation (PVT, in-phase stimulation: $p = 0.958$; out-of-phase stimulation: $p = 0.630$; SSS: $p = 0.341$ and $p = 0.356$). Also, rated sleep quality ($p = 0.831$ and $p = 0.618$) and the feeling of being well-rested at retrieval testing ($p = 0.893$ and $p = 0.844$) was comparable between Stimulation and Sham conditions in the two experiments.

Sleep Recordings and Auditory Stimulation

EEG Recordings and Polysomnography

The EEG was recorded continuously with a BrainAmp DC amplifier (Brain Products) from 19 channels (international 10–20 system, Fp1, Fp2, F7, F3, Fz, F4, F8, T3, C3, Cz, C4, T4, T5, P3, Pz, P4, T6, O1, O2) referenced to the average potential from electrodes attached to the mastoids (M1, M2). Ag-AgCl electrodes were used, and impedances were always kept below 5 kΩ. Signals were filtered between 0.03–150 Hz, sampled at 500 Hz, and stored for later offline analysis on a PC together with the stimulation triggers. Vertical and horizontal eye movements (VEOG, HEOG) as well as electromyogram (EMG) from the chin were obtained for standard polysomnography and artifact detection.

Online Detection of Slow Oscillations and In-Phase Auditory Stimulation

An additional EEG recording system, consisting of a "Digitimer D360" EEG amplifier (Digitimer) and a "Power1401 mk 2" high-performance data acquisition interface (Cambridge Electronic Design) connected to a separate PC, was used to accomplish the online detection of SOs and the auditory stimulation. With this set-up, the prefrontal EEG was recorded from an electrode at Fpz referenced to the average potential from linked electrodes attached to the earlobes. The EEG was filtered between 0.25 and 4 Hz and sampled with 200 Hz. A custom-made script running under Spike2 software version 7 (Cambridge Electronic Design) together with a sequencer in the Power1401 mk 2 enabled responding to the incoming prefrontal EEG data in real time. Basically, each time the EEG signal crossed an adaptive threshold toward larger negative values, the auditory stimulation was triggered. On default, the threshold was set to −80 μV. Every 2 s, it was updated to the minimal (i.e., largest negative) instantaneous EEG amplitude within the preceding 5 s interval, however, only if this value exceeded (in negativity) −80 μV. This algorithm ensured a reliable way to continuously detect SOs of increasing and decreasing amplitude within a sleep cycle by its negative half-wave peak.

For each subject, the online SO detection algorithm was also applied to the first SWS epoch of the adaptation night (without auditory stimulation), in order to determine the subject's individual delay time between the detected negative half-wave peak and the succeeding depolarizing up state, i.e., the mean time between the SO negative peak and the following positive peak. This time (averaging 508.2 ± 18.3 ms across subjects) was used to individually adapt the stimulation such that the auditory stimuli were most likely to occur in phase with the SO up states. Upon detection of an SO, the first stimulus was delivered after the subject's individual delay time. The second stimulus then followed after a fixed interval of 1,075 ms. Thereafter, stimulation was discontinued for 2.5 s. We focused on the stimulation of only two succeeding SO cycles based on previous observations (Mölle et al., 2011), indicating that spontaneous SO typically occurs in trains of two to three SO cycles, the rate of which we aimed to increase with the stimulation protocol. The detection algorithm was applied throughout the stimulation period of 210 min but halted whenever the subject left non-REM sleep stage 2 or SWS or arousals occurred. Triggers were sent to the BrainAmp to mark the starting points of the first and second stimulus in the EEG. During the Sham condition, SO detection was performed in the same way, and the respective time points were marked in the EEG, but no auditory stimuli were delivered. SO detection, auditory stimulation, and presentation of trigger to the EEG recording system were all controlled by the Power 1401 system and required a constant time interval of 2.4 ms, i.e., the equipment enabled precise timing of stimulus delivery.

Auditory Stimulation in Control Experiments

To evaluate to what extent changes in SO activity and memory performance depended on the in-phase presentation of auditory stimuli, we performed two control experiments that aimed to disrupt the SO rhythm (1) by changing the phase of stimulation within the SO cycle and (2) by changing additionally the interstimulus interval such that it did not correspond to the natural SO period length.

Changing only the phase of the stimulation from the up state to the down state did not effectively suppress SO activity. In these experiments (n = 4, 4 women, age: 22.0 ± 0.4 years), the two auditory stimuli were presented with the same interstimulus interval of 1,075 ms (corresponding to the SO period length), but presentation was shifted forward in time by ∼500 ms, i.e., the first auditory stimulus was presented immediately upon detection of the down state. As shown in Figure S4A, in this case the first stimulus delayed emergence of the succeeding depolarization such that the second stimulus coincided already with this emergent depolarization, thereby promoting, rather than disrupting, a further SO.

In the second control study (n = 7, 5 women, 2 men, age 21.1 ± 0.7 years), out-of-phase stimulation was generated by reducing the interstimulus interval to about half of the SO period (in addition to presenting the first stimulus immediately upon detection of the negative half-wave peak). The exact length of the interstimulus interval was individually fine-tuned to the timing of the hyperpolarization phase after the first auditory stimulus, based on the online assessment of the response to the first five stimulation trials. Across subjects, the interstimulus interval averaged 550.0 ± 19.7 ms. With this out-of-phase stimulation, the second stimulus indeed tended to coincide with emerging hyperpolarization that effectively suppressed the development of SO trains (Figure 4). Study design and analyses were otherwise identical to those of the main experiments.

Auditory Stimuli

The stimuli were bursts of pink 1/*f* noise of 50 ms duration with a 5 ms rising and falling time, respectively. Sound volume was calibrated to 55 dB SPL using a Voltcraft sound level meter SL-400 (Conrad Electronic SE). Stimuli were uploaded as a digital sequence into the memory of the Power1401 mk 2 and played back over a digital analog port. They were presented binaurally via MDR-EX35 in-ear headphones (Sony Deutschland). Eight of the 11 subjects of the in-phase stimulation protocol and four of the seven subjects receiving the out-of-phase stimulation protocol reported (in the morning after retrieval testing) that they had noticed the auditory stimuli during the night. This might reflect that the presence of sleep stage 2 (and SWS) does not completely preclude any awareness of stimulation or that during stimulation sudden transient arousals occurred before stimulation stopped, which happened in rare cases.

Analyses of Sleep Measures and EEG

Analyses were performed with Spike2, Brain Vision Analyzer 2 (Brain Products), and SPSS Statistics (SPSS). First, the EEG and EOG signals were digitally filtered with a band pass between 0.3–30 Hz and the EMG with a high pass of 5 Hz. Sleep stages were determined visually by two raters, who were blinded with regard to the experimental condition, using EEG recordings from C3, C4, EOG, and EMG for subsequent 30 s epochs according to standard criteria (Rechtschaffen and Kales, 1968). Total sleep time (TST), time spent in different sleep stages (wake; sleep stages 1, 2, 3, 4; slow-wave sleep, i.e., the sum of sleep stage 3 and 4; REM sleep), and movement arousals were determined for the whole night. The time in the different sleep stages was also determined separately for the 210 min stimulation period and the remaining sleep time during the late night. Because SOs are most pronounced during SWS, analyses of SOs as well as accompanying EEG analyses reported here concentrated on SWS epochs, although results did not essentially change with additional inclusion of stage 2 sleep epochs. All EEG analyses were based on generic scripts that treated every data set equally.

Offline Analyses of Slow Oscillations

SOs were identified offline according to an algorithm described in detail in Mölle et al. (2002). This algorithm was based on a virtual channel representing the mean EEG signal recorded from F3, Fz, F4, C3, Cz, C4, P3, Pz, and P4. In brief, the EEG was first low-pass filtered at 30 Hz and down sampled to 100 Hz. For the identification of large SOs, a low-pass filter of 3.5 Hz was applied. Then negative and positive peak potentials were derived from all intervals between consecutive positive-to-negative zero crossings (i.e., one negative and one positive peak for every interval). Only intervals with durations of 0.9–2 s (corresponding to a frequency of 0.5–1.11 Hz) were included. The mean values of the negative and positive peak potentials were calculated across the two conditions and those intervals were marked as SO epochs whose negative peak amplitude was lower than 1.25 times the mean negative peak and whose amplitude difference (positive peak minus negative peak) was larger than 1.25 times the mean amplitude difference. Negative half-waves were used for the identification of SO cycles because they typically show more discrete peaks in the EEG compared to positive half-waves, which have a broader and more variable shape. Averages of original EEG potentials were calculated in a 2.5 s window ± 1.25 s around the peak of the negative half-wave of all identified SOs. Statistical comparison between Stimulation and Sham conditions was performed on succeeding 250 ms intervals. To examine whether the auditory stimulation induced SOs, we calculated event correlation histograms using 6 s intervals and a bin size of 0.05 s. The histograms were referenced to the first of the two auditory stimuli with an offset of 2 s. Testing of significant differences between Stimulation and Sham conditions was performed on succeeding 300 ms intervals.

Additionally, slow- and fast-spindle activity was analyzed by filtering the original EEG between 9–12 and 12–15 Hz, respectively, and subsequent calculating of the rms of the filtered signal. Averages of the rms signals were calculated in a ±1.25 s window around the negative half-wave peak of all identified SOs. Rms activity was then expressed as difference from a baseline value, defined by the average rms value during the first 100 ms of the window. The

peak amplitude of fast-spindle rms phase locked to the up state, which succeeded the identified negative half-wave peak, was determined for each individual subject and correlated with the overnight retention of word pairs.

To examine topography, morphology, and traveling of SOs, we identified SOs in each individual EEG channel, applying basically the same algorithm as described above to the filtered (0.3–3.5 Hz) EEG signal (rather than to a virtual channel). To compare the topography of SOs between the Stimulation and Sham conditions, we normalized negative SO half-wave peak amplitudes for each subject by dividing amplitude values in each channel by the absolute value of the mean across all channels (Landsness et al., 2009). The slope of individual SO cycles was determined in the down-to-up state transition as the ratio between the absolute value of the negative half-wave peak and the time delay to the next zero crossing (Riedner et al., 2007). For analyses of SO traveling, single SO cycles were assessed using the timing of the negative half-wave peak in each EEG channel with respect to the channel revealing the earliest peak, with the latter considered to reflect the origin of the traveling SO (time $t = 0$). Topographical maps of SO origins and their delays were generated with regard to the SO negative half-wave peaks (subdivided into SOs emerging in frontal, central, and parietal areas) by third-order spherical spline interpolation (Massimini et al., 2004). Finally, the path length of SO traveling was evaluated in an undirected network constructed by connecting neighboring electrode positions, i.e., for each SO cycle and beginning with the SO origin, the neighboring nodes with the smallest (forward) delay were iteratively selected until a break in the gradient occurred. The path length refers to the number of traversed nodes in each SO cycle. For statistical comparisons, SOs were categorized into "local" SOs with a path length equal to zero and "traveling" SOs showing a path length greater than zero.

EEG Analyses

To assess immediate effects of auditory stimulation, we averaged the EEG signal across all stimulations during SWS epochs per night within a 4 s window time locked to the first of the two auditory stimuli and a prestimulus onset time of 1 s. In the Sham condition, the EEG was averaged across corresponding periods, time locked to the marked time points. The number of windows averaged in the Stimulation and Sham conditions were n = 245.6 ± 38.1 and n = 306.3 ± 42.4, respectively, for the in-phase auditory stimulation protocol and n = 457.7 ± 90.9 and n = 366.9 ± 69.4, for the out-of-phase stimulation protocol. These averages were further used to separate evoked auditory responses from ongoing spontaneous SO activity by subtracting the average signal obtained for the Sham condition from that of the Stimulation condition. Prestimulus baseline-to-peak amplitude and latency of these evoked responses were determined for two positive peaks (occurring between 50 and 100 ms and between 150 and 200 ms poststimulus) and a negative component (300–800 ms) for each subject. For the negative component, the mean amplitude was additionally calculated for a 200 ms interval centered on the peak.

Power spectra were determined by Fast Fourier Transformations (FFTs) using a Hanning window with 4,096 data points (~8.2 s), resulting in a frequency resolution of ~0.122 Hz. The power spectra were averaged across all 8.2 s windows and subsequently smoothed with a three-point moving average. For the out-of-phase control experiments, additional spectral analyses were performed separately on intervals of acute stimulation and on immediately succeeding intervals when stimulation was discontinued, using a Hanning window of 2,048 data points (~4.1 s). To account for individual variability, we normalized the power spectra for each EEG channel by its cumulative power up to 30 Hz.

Statistical Analyses

Unless stated otherwise, data are presented as mean ± SEM. Statistical analysis was generally based on repeated-measures ANOVA, including a within-subject factor condition (in-phase or out-of-phase Stimulation versus Sham condition). Additional repeated-measures factors were topography (representing the different recording channels), acute/discont (representing intervals of acute stimulation versus intervals when stimulation was discontinued during the 210 min stimulation period) in the analyses of SO amplitudes, and slopes across the night and night period (representing the 210 min stimulation period and the late part of the night, respectively). For assessing differences in the auditory-evoked potential between in-phase and out-of-phase stimulation, an additional between-subject factor Stimulation protocol was introduced. The Greenhouse-Geisser correction of degrees of freedom was applied where appropriate. Additionally, where indicated, paired t tests were employed. A p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.03.006.

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