PSYCHOPHYSIOLOGY

Psychophysiology, 54 (2017), 496–507. Wiley Periodicals, Inc. Printed in the USA.

DOI: 10.1111/psyp.12807

The elicitation of steady-state visual evoked potentials during sleep

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Abstract

This study confirmed the hypothesis that it is possible to elicit SSVEPs through closed eyelids during NREM sleep. To test this hypothesis, SSVEP amplitudes were measured in eight subjects across two conditions of stimulation (stimulation on and stimulation off) and three brain states (waking, light sleep, and deep sleep). Results showed a significant interaction between stimulation and brain state. In particular, EEG activity at the frequency of stimulation was higher during both light sleep and deep sleep in the stimulation on condition than in the stimulation off condition. The fact that it is possible to elicit SSVEPs during sleep may provide a new way to study how SSVEPs are generated in the brain—one that might help resolve open questions such as identifying the SSVEP activation sequence or deciding if SSVEPs derive from evoked or oscillatory neural processes.

Descriptors: SSVEP, EEG, ERP, Sleep, NREM, Visual evoked potential

For more than 50 years, steady-state visual evoked potentials (SSVEPs) have provided a tool for the study of visual information processing, the clinical assessment of visual function, and the development of brain-computer interfaces (BCIs; Norcia, Appelbaum, Ales, Cottereau, & Rossion, 2015; Vialatte, Maurice, Dauwels, & Cichocki, 2010). Typically measured using EEG, SSVEPs were discovered by Adrian and Matthews (1934), just 5 years after Hans Berger's (1929) initial description of the alpha rhythm. In the course of confirming Berger's discovery, Adrian and Matthews (1934) demonstrated that a repetitively flickering visual stimulus elicits EEG activity at the same frequency as the stimulus, a phenomenon now known as the SSVEP. Thirty years later, David Regan studied SSVEPs more extensively, introduced them as a method for studying visual information processing (Regan, 1968), and clearly described several advantages of SSVEPs over transient visual evoked potentials (VEPs; Regan, 1989). Compared with VEPs, SSVEPs (a) are easier to quantify (Luck, 2014; Regan, 1989), (b) provide high signal-to-noise ratio (SNR) signals in less time (Regan, 1989), and (c) are less prone to several common sources of noise (Regan, 1989) and artifacts (Gray, Kemp, Silberstein, & Nathan, 2003; Perlstein et al., 2003).

Despite the widespread use of SSVEPs, it is still not completely understood how they are generated in the brain. For example, consider that neither the activation sequence nor the neural processes that lead to the generation of SSVEPs is known.

SSVEPs reflect the combined electrical activity from multiple neural sources within the brain (Di Russo et al., 2007; Fawcett, Barnes, Hillebrand, & Singh, 2004; Pastor, Valencia, Artieda, Alegre, & Masdeu, 2007). It is believed that these individual sources are activated sequentially. The order in which these neural sources are activated, or the SSVEP activation sequence, remains an open question in the literature (Di Russo et al., 2007; Regan, 1989). To try to determine this activation sequence, Di Russo and colleagues (2007) used a combination of source localization and phase analysis techniques. Based on these methods, they proposed that the activation sequence from earliest to latest was V1, V5/MT, V3A, and then V4/V8. Di Russo et al. (2007) specifically caution, however, that the overlapping nature of the SSVEP response precludes the exact determination of the neural activation sequence using phase analysis, beyond the usual problems associated with source localization (Luck, 2014).

Another open question, which Norcia et al. (2015) label the "nature of the underlying neural mechanism," is whether SSVEPs are generated through evoked (Shah et al., 2004) or oscillatory (Makeig et al., 2002) neural processes. Some researchers hypothesize that SSVEPs are the result of time-locked activity in the cortex that is evoked by the stimulus (Shah et al., 2004), while others hypothesize that they are the result of a "phase-resetting" of an ongoing neural oscillation (Moratti, Clementz, Gao, Ortiz, & Keil, 2007; Notbohm, Kurths, & Herrmann, 2016). Some evidence for the oscillatory hypothesis comes from the existence of resonance frequencies, frequencies at which the response amplitude of SSVEPs is naturally larger (Herrmann, 2001). However, these

This work was supported by NSF grants No. 0955088 and 0903622. The authors would like to thank Bonnie Chen, Randy Lefkowitz, Siyuan Wu, and the ECE445 team for their efforts in the development of the stimulation hardware. Thanks to Joost Rommers, Kara Federmeier, Alex Iordan, Jessica Mullins, and Claudia Lutz for their helpful feedback and incredible insight. The authors would also like to thank the Christie Clinic Sleep Center for their insights on polysomnography and sleep scoring. SU is currently with DoveMed, Champaign, IL.

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resonance frequencies can also be explained by the temporal superposition of waves as predicted by the evoked activity hypothesis (Capilla, Pazo-Alvarez, Darriba, Campo, & Gross, 2011). Capilla et al. (2011) provide evidence for the evoked hypothesis by showing that SSVEPs are well modeled by the superposition of time-jittered VEPs presented at the same average frequency as SSVEPs. However, the rapid presentation of these VEPs may engage fundamentally different neural processes than those presented at much lower rates (Norcia et al., 2015). It is also possible that SSVEPs arise as the result of a combination of these two types of activity (Colon, Legrain, & Mouraux, 2012).

The motivation for this paper is to provide a new method for investigating how SSVEPs are generated in the brain—one that might help resolve open questions such as the two we just described. If these questions could be resolved, researchers may be able to improve the interpretation of visual information processing experiments using SSVEPs (Di Russo et al., 2007), more precisely define their utility in the clinical assessment of visual function (Di Russo et al., 2007), and increase the performance of SSVEP-based BCIs (Vialatte et al., 2010). The new method we propose is based on the elicitation of SSVEPs during sleep. We believe that eliciting SSVEPs during sleep would provide new insight into how these signals are generated in the brain, for the following reasons:

First, research over the course of 60 years—often using EEG—has established that information processing still occurs in the brain during sleep (Hobson, 2005), despite a reduction in overt responsiveness to external stimuli. For instance, the auditory N1 and P2 ERPs can be elicited during both waking and sleep. These ERPs, however, are altered as a function of the participant's brain state (Colrain & Campbell, 2007). For example, the amplitude of the auditory N1 is reduced to baseline levels during NREM (nonrapid eye movement) sleep but is apparent during REM sleep. The amplitude of the P2, on the other hand, increases during NREM sleep and is visible during REM sleep (Colrain & Campbell, 2007).

Second, altered information processing during sleep offers the possibility of comparative studies to dissect SSVEP activity in new ways. Recent evidence suggests that the differences between ERPs elicited during waking and ERPs elicited during sleep are due to a reduction in cortical connectivity. Using a combination of transcranial magnetic stimulation (TMS) and EEG, Massimini et al. (2005) demonstrated that TMS stimulation during sleep caused a reduction in the response of areas that were cortically connected to the stimulation site. Furthermore, the amplitude of this reduction was correlated with the depth of sleep.

Given that information processing still occurs during sleep, but cortical connectivity is reduced, it may be possible to infer the SSVEP activation sequence without resorting to the phase analysis techniques of Di Russo et al. (2007). As sleep deepens, neural sources that occur later in the activation sequence become cortically disconnected from earlier sources. This reduction in cortical connectivity may lead to a decrease in response amplitude from these later sources as compared to earlier sources. One would then predict that the SSVEP activation sequence could be determined by comparing the response amplitudes of different individual sources measured during sleep with the response amplitudes from those same sources measured during waking.

With respect to the neural processes that lead to the generation of SSVEPs, it is reasonable to assume that the reduction in cortical connectivity will have an effect on the oscillatory activity occurring in the brain. If so, one would predict that the existence of SSVEP resonance frequencies would dissipate, or at least be attenuated, during sleep. Even though the mechanisms underlying visual steady-state responses and other sensory modalities may be fundamentally different, some evidence for this can be found in the auditory modality. In a study comparing steady-state auditory potentials elicited by modulated tones during waking and sleep, Cohen, Rickards, and Clark (1991) found that the response at resonance frequencies was more attenuated during sleep than at other stimulation frequencies.

The question then, which is the one we answer in this paper, is can SSVEPs be elicited during sleep? Effectively, this requires that it be possible to elicit an SSVEP through a participant's closed eyelids, elicit and record an SSVEP during sleep without waking the participant, and measure the response using time-frequency analysis of the data.

It has been previously established that SSVEPs can be elicited through closed eyelids during waking. While the eyelids completely cover the eyes, they do not perfectly filter all light. In fact, along the visual spectrum, the eyelid acts as a red-pass filter. Up to 10% of red light (above 600 nm) passes through the eyelid as well as 1–2% of light in the remaining visual spectrum (430 nm–600 nm) (Moseley, Bayliss, & Fielder, 1988; Robinson, Bayliss, & Fielder, 1991). This property is what allows visual stimuli to be perceived through closed eyelids, a fact that is well demonstrated by Lim and colleagues (Lim, Hwang, Han, Jung, & Im, 2013) in their paper on the "eyes closed" SSVEP BCI.

It is already known that VEPs can be elicited during sleep without waking the participant. For instance, a magnetoencephalography (MEG) study of visual evoked fields (VEFs)—VEFs are the MEG analog of the VEP—during sleep by Kakigi et al. (2003) found that VEFs elicited during sleep are simpler than those elicited during waking. Their data, in support of Massimini et al.'s (2005) hypothesis that sleep reduces cortical connectivity, showed that VEFs elicited during sleep exhibit a reduction in later stage components as compared with VEFs elicited during waking. Other studies have reported that it is possible to elicit VEPs in sleeping infants (Apkarian, Mirmiran, & Tijssen, 1991; Shepherd, Saunders, & McCulloch, 1999). The results from these studies suggest that, similarly to adults, brain state has an effect on the VEPs elicited from infants.

It remains unclear whether SSVEPs can be elicited during sleep—indeed, only a few studies have investigated repetitive visual stimulation during sleep. A study by Born et al. in 2002 found that repetitive visual stimulations (which generate SSVEPs in EEG) cause cortical deactivation as measured using fMRI and positron emission tomography (PET), but did not report the presence of SSVEPs in the EEG. The only other previous reports of the elicitation of SSVEPs during sleep were conducted in the context of epilepsy research. Rodin, Daly, and Bickford (1955) reported that repetitive stimulation during sleep elicited entrained EEG responses in the time domain; the experimenters did not specifically analyze the frequencies of the elicited responses, nor did they analyze SSVEPs across the sleep stages. In addition, they used a stimulation intensity of 250,000 foot candles (2,500,000 lux), roughly 20 times brighter than the brightest sunlight (Baertschi et al., 2013). A few studies have since followed up on the work of Rodin (Leschey & Hall, 1977; Meier-Ewert & Broughton, 1967; Sato, Dreifuss, & Penry, 1975; Yamamoto, Furuya, Wakamatsu, & Hishikawa, 1971), but they all investigated clinical populations with the goal of determining whether seizures can be induced during sleep. These researchers also continued to use very bright strobes as stimuli and analyzed the results in the time domain.

In our study, we examined whether SSVEPs could be elicited during sleep by investigating the EEG activity resulting from the

presentation of a repetitively flickering stimulus (with a brightness of less than 1.5 lux) during waking and sleep. We hypothesized that the presentation of a visual stimulus would elicit an increase in EEG activity at the same frequency as the stimulus during both waking and NREM sleep. To test this hypothesis, we invited participants to sleep in our laboratory while we recorded their EEG. A head-mounted stimulator was then used to elicit SSVEPs during waking and sleep. Results from eight participants confirmed the hypothesis that visual stimulation during sleep significantly increases EEG activity at the frequency of stimulation.

Method

Participants

Eight volunteers (five males, three females, 20–32 years old) participated in the study. All participants reported having no history of seizures, frequent or severe migraines, motor impairments, or sleep disorders. The experiments were approved by the University of Illinois Institutional Review Board. Each participant was informed about the procedure and signed an informed consent before the experiment.

Recording Parameters

Twenty-one channels of physiological data were recorded from each participant using solid tin electrodes at impedances of less than 10 kΩ. Sixteen channels of EEG data (online supporting information Figure S1) were recorded from the following International 10-10 sites (Sharbrough et al., 1991): right mastoid, FPz, F3, F4, FCz, C3, C4, CPz, PO7, PO3, POz, PO4, PO8, O1, Oz, and O2. Two channels of electrooculogram (EOG) were recorded to monitor for eye movements; one EOG electrode was placed approximately 1 cm lateral to and 1 cm below the outer canthus of the left eye, while the other EOG electrode was placed approximately 1 cm lateral to and 1 cm above the right eye (Rechtschaffen & Kales, 1968). All EEG and EOG electrodes were referenced to the left mastoid. Two channels of electromyogram (EMG) were recorded to monitor for muscle activity. These channels were placed equidistant from the midline of the chin approximately 3 cm apart and were bipolar referenced with one electrode placed on the mentalis muscle and the other electrode placed on the submentalis muscle. One bipolar channel of electrocardiogram (ECG) was recorded to measure heart rate and placed on the chest. A single ground electrode was situated on the dorsum of the nose.

A James Long EEG amplifier (model TCP-128BA) was used to amplify the EEG and EOG signals (10,000 times). All EEG and EOG data were analog filtered from 0.3 Hz to 30 Hz. The EMG and ECG channels were recorded using the same amplifier, but at a lower gain (2,000 times) using different analog filter settings (1–300 Hz). The data were then digitized at 1000 Hz using a National Instruments data acquisition unit (DAQ; Model NI PCI-6225).

Stimulation Hardware

The visual stimulation system (Figure 1) was created using a pair of glasses, two green light-emitting diodes (LEDs), and an Arduino Uno microcontroller (Arduino LLC, Somerville, MA). Each LED was placed at the lateral angle $\theta \approx 50°$ to the center of the participant's left and right eyes near the lateral canthus. A flicker frequency of approximately 7 Hz was chosen, because the fundamental frequency was less than the alpha range (8 Hz to 12 Hz). The

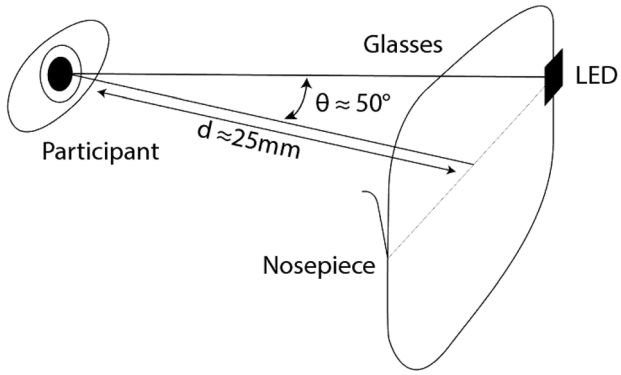

Figure 1. Diagram showing the setup of the visual stimulation system.

method used to program the Arduino resulted in an exact frequency of 7.03 Hz. The duty cycle of the LEDs was 50%. The intensity of the LEDs was measured using a photometer, emitting 1.5 lux at a distance of approximately 25 mm. The onset and offset of stimulation from the LED glasses were captured using a photodiode wired directly in the DAQ and sampled at 1000 Hz.

Experimental Procedure

On the evening of the experiment, the participant arrived at the laboratory 1 h before his or her habitual bedtime (between 2300 and 0300 hours). All experiments were conducted in a sound-attenuated, light-controlled, and air-conditioned room. Following informed consent, the participant performed his or her nightly routine. When each participant was ready for sleep, they positioned themselves in a comfortable reclining chair for the duration of the study. The recording electrodes and LED stimulator glasses were then placed on the participant. Following setup, all experiments were monitored from an adjacent room. A baseline, consisting of two 1-min periods of SSVEP stimulation, was then recorded from each participant. During this baseline recording, the participants were asked to close their eyes, to relax, and to ignore the stimuli. After the baseline, participants were permitted to fall asleep. Participants were given between 20–40 min to fall asleep before SSVEP stimulation was started. The exact amount of time was different for each participant and determined by the experimenters. During each sleep stimulation period, the SSVEP stimuli flickered for 5 min. Each stimulation period was followed by an interstimulus interval of 10 min. The length of this interstimulus interval was chosen ad hoc by the experimenters. Each sleep recording consisted of two to eight stimulation periods. The number of stimulation periods was determined by allowing the participant to sleep for up to 2 h or until he or she woke up, whichever came first. If a participant was awakened before the stimulation periods, or by the stimulation itself, they were given the option to try again or to end the experiment.

Data Analysis

All data were analyzed using MATLAB (The Mathworks Inc., Natick, MA).

Preprocessing. For each subject, two digital filters were first applied to each channel of the raw EEG data: (1) A 60 Hz notch filter with a 1 Hz bandwidth (−3 db) was applied to the data for the purpose of removing power line noise and implemented using the

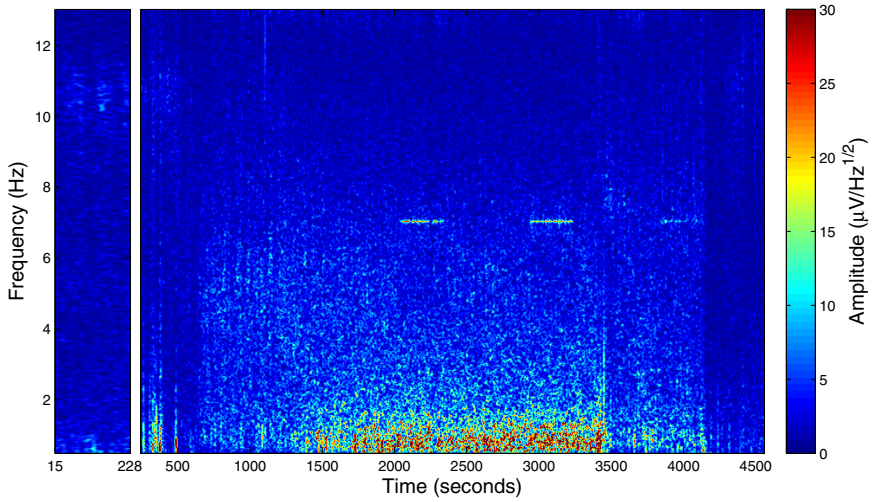

Figure 2. STFT showing sleep recording session for Subject s04, averaged across channels O1, Oz, and O2. The image was created using the spectrogram function in MATLAB with input parameters of a 30-s time window, a 29-s window overlap, and a Hanning taper to reduce spectral leakage. The three 5-min SSVEP stimulation periods can be seen at 7.03 Hz.

iirnotch function in MATLAB. (2) A second-order infinite impulse response band-pass filter with a pass-band of 0.5–30 Hz was applied to reduce noise and implemented using the butter function in MATLAB. Both filters were applied both forward and backward to prevent any phase shifts in the data. After filtering, each channel of the EEG data was rereferenced to the average of the left and right mastoid. The preprocessed data for Subject s04 averaged across channels O1, Oz, and O2 have been visualized in Figure 2 using the short-time Fourier transform (STFT).

Sleep scoring. The sleep stage of each subject was scored using the sleepSMG toolbox (S. Greer and J. M. Saletin, Walker Laboratory UC Berkeley; http://sleepsmg.sourceforge.net/), a visualization tool for MATLAB. Nine channels of data were used for sleep scoring, two chin EMG channels, two EOG channels, and five EEG channels (C3, C4, O1, Oz, O2). The continuous EEG data for each participant were first divided into 30-s epochs. Two raters then scored (Berry et al., 2012; Rechtschaffen & Kales, 1968) each EEG epoch independently as showing *waking, light sleep* (which we defined as stage N1 or N2; Genzel, Kroes, Dresler, & Battaglia, 2014; Miano et al., 2006; Penzel, Kantelhardt, Grote, Peter, & Bunde, 2003), or *deep sleep* (which we defined as stage N3). The two raters had an interrater reliability of 65%. Rater #2 agreed with Rater #1 for more than 80% of epochs labeled waking and 95% of the epochs labeled deep sleep. For light sleep, however, Rater #2 only agreed with Rater #1 48% of the time. The two raters also labeled epochs with artifacts for rejection from further analysis. Disagreements between these raters were resolved through consensus, and final labels were assigned to each epoch for further analysis.

Amplitude spectral density. To detect SSVEPs in the EEG data, we used amplitude spectral density (ASD). Since our sleep data were scored in 30-s epochs, we used the same 30-s epochs for calculation of the ASD. An estimate of the power spectral density was computed for each channel of EEG data within each epoch (including both the baseline and sleep data) using the pwelch function in MATLAB. The pwelch function, with a signal length equal to the window length, is equivalent to calculating the single-sided ASD using the Fourier transform. Given that the two methods are equivalent, pwelch is a single function call and was used to reduce the risk of coding errors. A Hanning window was first applied to each 30-s epoch to reduce spectral leakage. Each signal was zero-padded to four times the length of the epoch to improve visualization. Since each epoch was 30 s long, there was no overlap between successive windows. The square root of the data was then taken to convert the results from power spectral density to ASD. Figure 3 shows an overlay of the ASD values, sleep score, and stimulation periods for Subject s04.

Each estimate of the ASD was then binned into one of two stimulation conditions, stimulation off or stimulation on. Within each stimulation condition, the data were further subdivided into one of three brain states based on the results of the sleep scoring: waking, light sleep, or deep sleep. Note that the stimulation off waking condition and stimulation on waking condition included data from both the baseline session and data that were scored as waking during the sleep session. All estimates of the ASD within each bin were averaged across windows and channels O1, Oz, and O2 to create final estimates of the ASD for each subject in each condition (Pastor et al., 2007). Finally, the ASD value at the frequency of stimulation was extracted from each condition for further statistical analysis. This value, which we defined as the SSVEP amplitude, is similar to that of other researchers (Andersen & Müller, 2010; Pastor et al., 2007). The grand-averaged ASDs for each condition are shown in Figure 4.

Results

All eight subjects who participated in our study completed the experiments. Technical issues during Subject s03 resulted in the loss of half of the baseline data and half of the sleep data. Participant s08 reported difficulty falling asleep and was awoken by the stimulation, but did manage to sleep through one full stimulation period and one half of a second stimulation period.

Statistical analyses were conducted in SPSS Version 22 (IBM Corporation, Armonk, NY). The mean SSVEP amplitudes for each of the conditions and all eight subjects is reported in Table 1 and represented graphically in Figure 5. We performed an analysis comparing the difference in SSVEP amplitude using a two-way

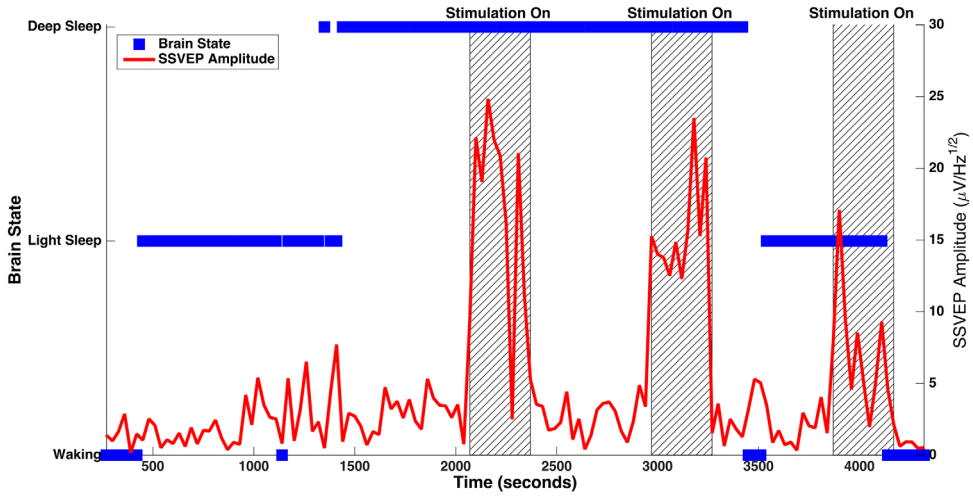

Figure 3. Plot that simultaneously overlays the scoring of sleep stage (floating bars), whether stimulation was on or off (shaded areas denote stimulation), and SSVEP amplitude (solid line) for Subject s04 during the sleep recording session. The increase in SSVEP amplitude can be seen to correspond exactly with the onset of stimulation.

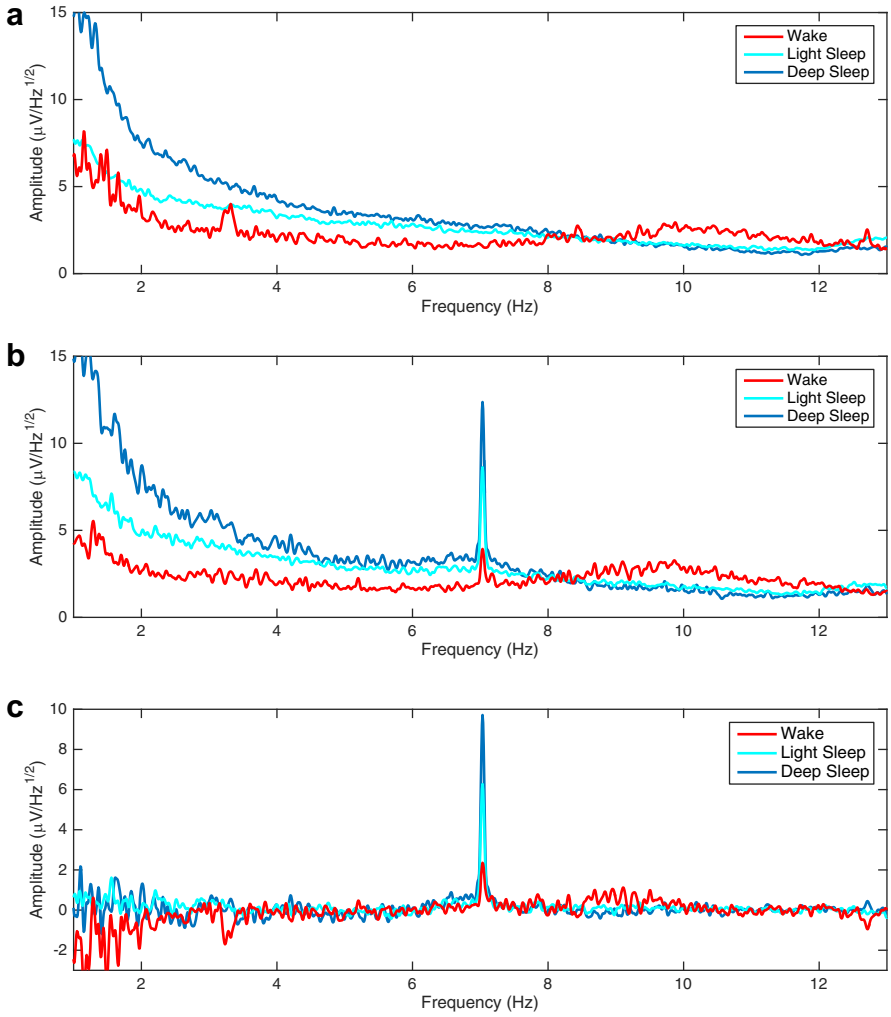

Figure 4. ASD values for frequencies between 1–13 Hz, averaged across all subjects, for each condition. a: Brain state (waking, light sleep, deep sleep) for the stimulation off condition. b: Brain state (waking, light sleep, deep sleep) for the stimulation on condition. c: Brain state (waking, light sleep, deep sleep) for the difference between the stimulation on condition and the stimulation off condition. The effect of stimulation is apparent at 7.03 Hz.

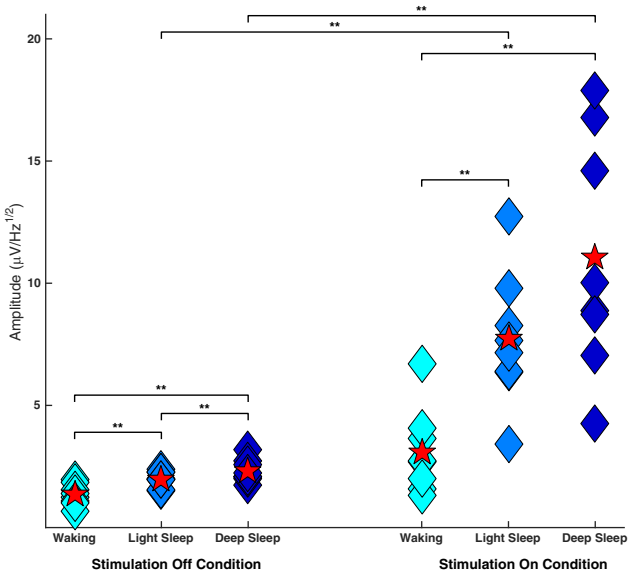

Figure 5. Scatter plot of SSVEP amplitudes for all subjects, brain states (waking, light sleep, deep sleep), and stimulation conditions (stimulation off, stimulation on). For each condition, mean amplitude across all subjects has been denoted with a star. **Statistically significant simple main effects, $p < .01$.

(brain state and stimulation) repeated measures analysis of variance (ANOVA) with three levels (waking, light sleep, and deep sleep).

We first assessed whether our data met the assumptions of the two-way ANOVA (no significant outliers, normally distributed data, and equal variances). Examination of the studentized residuals revealed no outliers for values fewer than −3 or greater than 3 standard deviations from the mean. To determine if the ASD values were normally distributed, a Shapiro-Wilk test was used. All conditions ($p > .05$) were normally distributed. Mauchly's test of sphericity was used to determine whether the ASD values from each of the conditions was of equal variance. The test revealed differences in variance across brain states, $\chi^2(2) = 8.01$, $p = .02$, and in the interaction between stimulation and brain state, $\chi^2(2) = 8.20$, $p = .02$. To account for these differences in variance, Greenhouse-Geisser correction was used. The corrected two-way repeated measures ANOVA on SSVEP amplitude revealed an interaction between stimulation and brain state, $F(1.15,8.02) = 16.51$, $p = .00$, $\eta_p^2 = .70$, uncorrected degrees of freedom were −2,14.

Simple main effect tests with Bonferroni-Holm correction revealed additional differences within the data. Simple main effect test for brain state showed that SSVEP amplitudes were higher for stimulation on ($M = 7.72$, $SD = 2.73$) compared to stimulation off ($M = 1.99$, $SD = 0.33$) during light sleep, $F(1,7) = 30.21$, $p = .01$, $\eta_p^2 = .81$, as well as stimulation on ($M = 11.04$, $SD = 4.86$) compared to stimulation off ($M = 2.33$, $SD = 0.47$) during deep sleep, $F(1,7) = 25.41$, $p = .01$, $\eta_p^2 = .78$. Simple main effect tests of stimulation showed that there was a difference, $F(2,14) = 26.77$, $p < .01$, $\eta_p^2 = .79$, between waking, light sleep, and deep sleep during stimulation off. Further pairwise comparisons showed that SSVEP amplitudes elicited during light sleep (mean difference = 0.64, $p = .01$) and deep sleep (mean difference = 0.98, $p = .01$) were higher than those during waking, and SSVEP amplitudes elicited during deep sleep (mean difference = 0.34, $p = .01$) were higher than those recorded during light sleep. During stimulation on, there was also a difference, $F(1.14,7.99) = 22.55$, $p = .01$, $\eta_p^2 = .76$, uncorrected degrees of freedom were −2,14, between waking, light sleep, and deep sleep. Pairwise comparisons revealed that SSVEP amplitudes elicited during light sleep (mean difference = 4.63, $p < .01$) and deep sleep (mean difference = 7.94, $p = .01$) were higher than those during waking. There were two marginal effects that did not meet the threshold of significance when corrected for multiple comparisons. During waking, SSVEP amplitude in the stimulation on ($M = 3.09$, $SD = 1.74$) condition was marginally higher (mean difference 1.75, uncorrected $p = .03$, corrected $p = .06$, $\eta_p^2 = .52$) than it was during the stimulation off ($M = 1.34$, $SD = 0.44$) condition. Finally, in the stimulation on condition, SSVEP amplitude was marginally higher (mean difference = 3.32, uncorrected $p = .04$) during deep sleep than it was during light sleep. In accordance with the ranking procedure of Bonferroni-Holm, no correction factor was applied to this last comparison, but it does not meet the threshold of significance.

There were differences in the number of time windows averaged together to obtain measurements of SSVEP amplitude from different subjects and conditions (Table 2). To determine if these differences had any effect on the results, we conducted two additional analyses: (1) a correlation analysis comparing the number of time windows with SSVEP amplitude, and (2) an analysis of the bootstrap confidence intervals of the individual subject data.

When not corrected for multiple comparisons, there was a moderate correlation ($r = -.30$, $p = .04$) between the number of time windows and the mean SSVEP amplitude (supporting information Figure S2). If the data were analyzed by stimulation condition, however, there was no significant correlation. The results for stimulation off ($r = -.14$ and $p = .50$) and stimulation on ($r = -.01$ and $p = .96$) are shown in Figure 6.

Table 1. *Mean SSVEP Amplitude (μV/Hz$^{1/2}$) Computed for Each Subject Binned by Stimulation Condition*

	Stimulation off			Stimulation on		
Subject	Waking	Light sleep	Deep sleep	Waking	Light sleep	Deep sleep
s01	1.23	2.40	3.17	2.70	6.36	7.06
s02	1.97	2.27	2.73	2.72	6.41	14.62
s03	1.85	1.99	2.08	3.65	8.25	8.89
s04	1.56	2.26	2.56	1.57	7.65	16.80
s05	0.66	1.49	1.74	4.08	9.79	10.02
s06	1.38	1.56	1.99	6.70	12.73	17.90
s07	1.09	1.95	2.08	1.30	3.40	4.27
s08	1.01	1.96	2.25	2.01	7.15	8.74
Mean	1.34	1.99	2.33	3.09	7.72	11.04
Median	1.31	1.98	2.17	2.71	7.40	9.46
Standard deviation	0.44	0.33	0.46	1.74	2.73	4.87

Table 2. *Number of Time Windows for Each Subject Binned into Each Condition of Stimulation and Brain State*

	Stimulation off			Stimulation on		
Subject	Waking	Light sleep	Deep sleep	Waking	Light sleep	Deep sleep
s01	21	72	70	4	41	22
s02	5	86	59	5	30	11
s03	18	15	12	13	9	1
s04	6	42	46	4	8	18
s05	3	67	21	4	13	14
s06	44	97	21	33	19	7
s07	84	87	46	8	32	19
s08	238	43	4	17	10	4
Mean	52.38	63.63	34.88	11.00	20.25	12.00
Median	19.50	69.50	33.50	6.50	16.00	12.50
Standard deviation	79.75	28.03	23.68	10.11	12.53	7.56

Bootstrap confidence intervals were used to test simple main effects within each subject following the procedures of Oruç et al. (2011). For each subject and comparison, the individual trials of the two conditions of interest were resampled with replacement. These two sets of resampled data were then averaged and subtracted from one another to create a new estimate of SSVEP amplitude. As an example, consider Subject s01 and a comparison of the light sleep stimulation off condition with the light sleep stimulation on condition. We first resampled the 72 light sleep stimulation off data trials (Table 2) and the 41 light sleep stimulation on data trials (Table 2) with replacement. Each of these resampled datasets was averaged, and the two resampled averages were subtracted from one another. This resulted in a measurement of the difference in SSVEP amplitude between the two conditions. This process was then repeated 1,000 times. After 1,000 iterations, the lowest 2.5% of values and the highest 2.5% of values were trimmed to create a 95% confidence interval. If the resulting confidence interval did not include zero, it was inferred that there was a significant difference between the means at the $p < .05$ level. The confidence intervals for light sleep stimulation off versus light sleep stimulation on and deep sleep stimulation off versus deep sleep stimulation on are shown in Figure 7. For both conditions, every single subject showed a significant increase in SSVEP amplitude during stimulation. For s03 (stimulation on deep sleep), calculation of a bootstrap confidence interval was not possible; SSVEP amplitude was estimated from a single trial. Bootstrap confidence intervals for differences in the other simple main effects are reported in supporting information (Figure S4–S10).

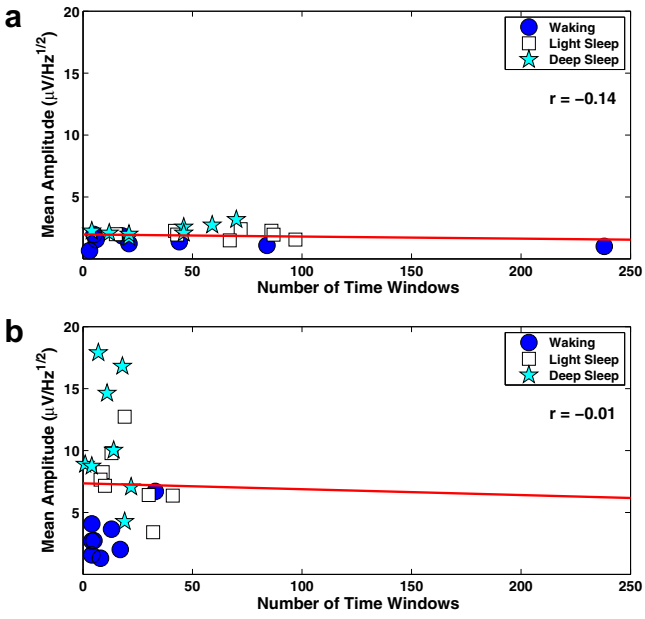

Figure 6. Scatter plot showing the number of time windows averaged together in a condition compared with the mean SSVEP amplitude for (a) stimulation off (all brain states), and (b) stimulation on (all brain states). A linear trend line has been added to each plot, and the correlation value is written in the upper right-hand corner.

Given the length of our stimulation periods (5 min), we examined the data for correlations between the length of time following stimulation onset and SSVEP amplitude. Using the data from the sleep session, the stimulation periods for each participant were extracted from the filtered EEG data. Then, each stimulation period was analyzed using the STFT. The input parameters for this STFT were a 30-s nonoverlapping window, Hanning taper, and no additional zero padding. The STFT returned 10 values, two for each minute of stimulation. These 10 values represent the SSVEP amplitude at a different length of time following stimulation onset. For example, the first value contained data from 0–30 s after stimulation onset. Following this, the data were averaged across all subjects and simulation periods (Figure S11). There appeared to be a moderate negative correlation ($r = -.48$) between SSVEP amplitude and time after stimulation onset, but it was not significant ($p = .16$).

Discussion

Our results confirmed the hypothesis that SSVEPs can be elicited during sleep. Recall that, in this paper, we define SSVEP amplitude as the amplitude of EEG activity at the frequency of a target stimulus averaged across channels O1, Oz, and O2 (see Data Analysis). Statistical analyses showed an increase in SSVEP amplitude during the stimulation on condition as compared to the stimulation off condition for both light sleep and deep sleep (Figure 5). This result is important because it directly enables the investigation of several open questions relating to how SSVEPs are generated in the brain. Specifically, sleep may provide a means to investigate the SSVEP activation sequence and the neural processes that lead to the generation of SSVEPs.

The data also show that the SSVEP amplitude is larger for the stimulation on condition during light sleep and deep sleep than it is

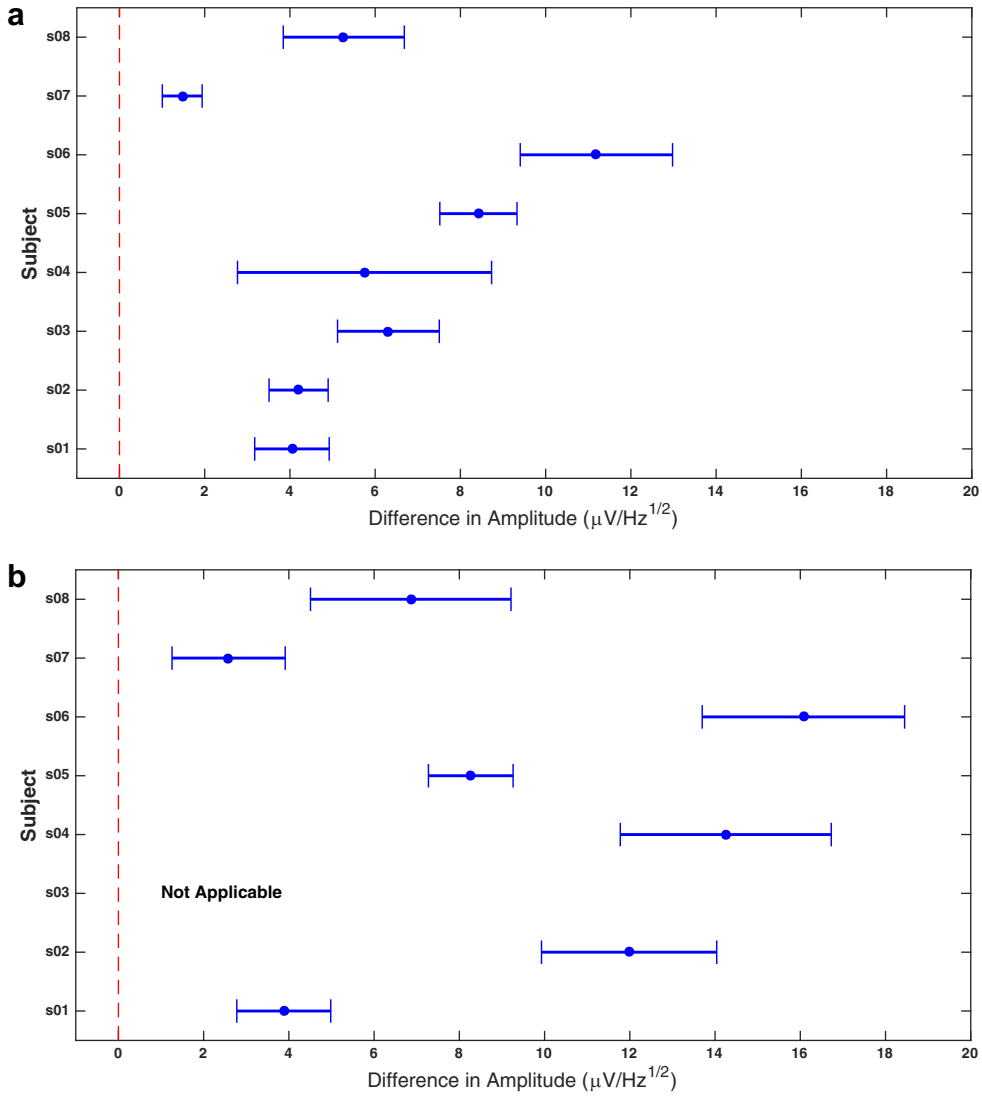

Figure 7. Bootstrap confidence intervals for the analysis of simple main effects within subjects. This figure shows the data for two conditions. a: Stimulation off (light sleep) versus stimulation on (light sleep). b: Stimulation off (deep sleep) versus stimulation on (deep sleep). Other comparisons are included in the supporting information. For conditions with a single trial, calculation of the confidence interval was not possible (Subject s03, stimulation on condition).

during waking (Figure 5). Evidence from our study and others suggest that the effect of brain state on steady-state and transient evoked potentials is dependent on the type of stimulation. For example, previous studies have found that sleep reduces the amplitude of steady-state responses using auditory stimuli (Cohen et al., 1991; Linden, Campbell, Hamel, & Picton, 1985). Massimini et al. (2005), however, showed that early EEG responses to TMS during sleep were larger in amplitude than they were during waking. Kakigi et al. (2003) reported that the middle latency components of VEFs were larger during sleep, and Shepherd et al. (1999) reported that the N1 and P2 amplitudes of VEPs elicited from infants increased during sleep. While our results are intriguing, they may have been affected by dark/light adaptation (Spafford & Lu, 1989). Even though the absolute light levels were the same during the baseline (used for most of the waking data) and the sleep experiments (used for all of the light sleep and deep sleep data), no time was given after the lights were turned off and before the baseline recording for the participant's eyes to adjust. To test the effect of light adaptation on our results, we conducted a pilot study (supporting information Analysis SII) with two participants. SSVEP amplitude, through closed eyelids, was more than twice as large after 30 min of light adaptation than it was at baseline (Figure S14). Another potential confound is that the majority of the waking data were from the baseline stimulations, which were only 1 min in length. The stimulation periods during the sleep sessions were five times longer. It is possible that the eyes adapted to the stimulations over the course of this time, although an analysis of SSVEP amplitude as a function of time after stimulation onset (Figure S11) showed no significant correlation. Given our current results, the differences between the existing literature on steady-state and transient evoked potentials during sleep, and the limitations of our current study, further investigation of the changes in SSVEP amplitude that occur across brain states is warranted.

In the stimulation on condition, SSVEP amplitudes were marginally higher during deep sleep than they were during light sleep (Figure 5). The data for these two conditions was collected more

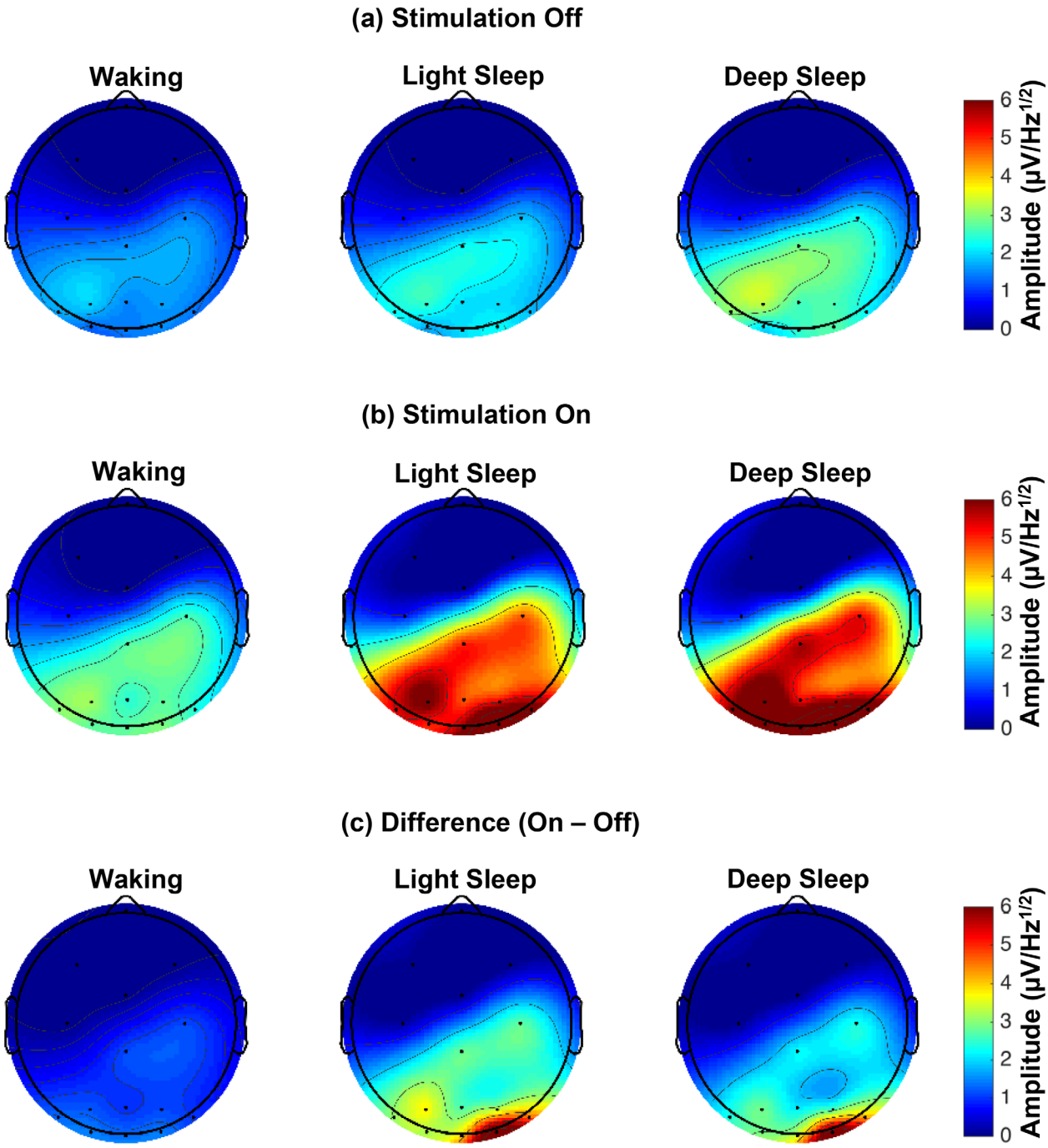

Figure 8. Topographic plot of the ASD values, averaged across all subjects, at the frequency of stimulation for the EEG channels. a: Brain state (waking, light sleep, deep sleep) for the stimulation off condition. b: Brain state (waking, light sleep, deep sleep) for the stimulation on condition. c: Brain state (waking, light sleep, deep sleep) for the difference between the stimulation on condition and the stimulation off condition.

than 20 min after the lights had been turned off (which were unlikely to have been affected by dark/light adaptation; Spafford & Lu, 1989) and provide further evidence that SSVEP amplitude increases from waking to light sleep to deep sleep. In addition, this is one of the first studies to report a difference in the amplitude of steady-state responses in light sleep versus deep sleep. Neither Linden et al. (1985) nor Cohen et al. (1991) reported differences in the amplitude of steady-state responses between light sleep and deep sleep. To improve future studies, we suggest that the stimulation procedure be changed to reduce the variance of SSVEP amplitudes recorded in the stimulation on condition during deep sleep (Table 1). This high variance may have been caused, in part, by the limited number of epochs recorded during deep sleep. The experiments did not selectively stimulate participants during specific stages of sleep. The stimulations were a fixed time apart, which was much easier to program and implement, skewing the number of samples within each condition for each subject (Table 2). While no significant correlation was found between the number of time

windows and the SSVEP amplitude for either the stimulation off or the stimulation on (Figure 6) conditions, implementation of an online sleep classification (Ebrahimi, Mikaeili, Estrada, & Nazeran, 2008) system would allow better control of stimulation during specific stages of sleep. In addition, a more thorough comparison of SSVEP amplitude across brain states should include all of the stages of NREM sleep (N1, N2, N3) as well as REM sleep (since this data were excluded from the present study).

SSVEP amplitude during waking (Figure 5) was only marginally higher in the stimulation on condition than it was in the stimulation off condition; we expected the difference between these two conditions to be larger. We attribute this result to five factors. First, the participants in our experiments were instructed to ignore (i.e., not attend to) the baseline stimuli. Unattended flickering stimuli elicit a much lower amplitude SSVEP than attended stimuli (Müller, Teder-Sälejärvi, & Hillyard, 1998). Second, the length of baseline stimulation (2 min total) was shorter than the stimulations used during sleep (5 min per stimulation period). The choice of two 1-min stimulation periods was made due to the worry that participants would fall asleep during the baseline. Although an analysis revealed no significant correlation between time following stimulation onset and SSVEP amplitude (Figure S11), there appears to be a negative correlation between these two variables. Third, the effect size was smaller than expected; if a smaller effect size had been predicted, and more data had been collected, this result may have been different. Fourth, we always recorded the baseline before the participant went to sleep, potentially causing ordering effects. In the future, the order of the baseline and experimental sessions should be randomized. Finally, since these experiments were conducted using sleeping participants in a dark room, dark/light adaptation may have impacted the results (Spafford & Lu, 1989; Figure S14).

For the stimulation off condition, there was an increase in SSVEP amplitude as brain state changed from waking to light sleep to deep sleep (Figure 5). These differences reached significance despite appearing to be much smaller in amplitude than the differences between the brain states in the stimulation on condition. We attribute this to two factors. First, SSVEP amplitude (Table 1) varied less during the stimulation off condition than during the stimulation on condition. Second, baseline EEG activity is known to change as a function of brain state. For example, theta activity (4–7 Hz) increases during sleep (Cote, 2002). The frequency of stimulation used in this study (7.03 Hz) was very close to the theta range. Figure 4 shows that ASD values for all frequencies between 4 Hz and 8 Hz are larger during light sleep and deep sleep (for both the stimulation off and the stimulation on conditions) than during waking.

The topography of ASD values at the frequency of stimulation are shown in Figure 8. During waking, the topography of the stimulation on condition appears to be similar to those previously reported by Herrmann (2001). As brain state changes from waking to light sleep to deep sleep, the ASD values measured during the stimulation on condition appear to grow larger, similar to the analysis of SSVEP amplitude. The distribution of these ASD values, however, appears to remain the same with the largest ASD values recorded from near electrode Oz. Further analysis of changes in topography is limited by the number of electrodes used during the experiments, the distribution of these electrodes, and the fact that we did not control for the subject's position (Rice, Rorden, Little, & Parra, 2013). Future work investigating changes in the neural sources of SSVEP across brain state should use more electrodes since this is known to improve EEG source localization (Lantz, de Peralta, Spinelli, Seeck, & Michel, 2003).

Finally, we acknowledge that many factors are known to affect SSVEP amplitude (Zhu, Bieger, Molina, & Aarts, 2010). These factors include:

- ***Spatial location of the stimulus.*** All of the changes in the SSVEP reported here were the result of LED stimulation near the lateral canthus of the eye (Figure 1). This location was chosen based on the previous work of Lim and colleagues (2013). As lateral stimuli have been previously shown to lateralize SSVEP topography (Skrandies, 2007), the choice of a lateral stimulus location may have altered the experimental results.
- ***Brightness/contrast of the stimulus***. We used a dim (approximately 1.5 lux) stimulus to demonstrate that SSVEPs could be elicited during sleep at brightness levels several orders of magnitudes less than those reported in the work of Rodin et al. (1955). Previous research has shown that the amplitude of SSVEPs is related to the suprathreshold contrast of the stimuli (Campbell & Kulikowski, 1972). Additionally, while the eyelid is known to act as a red-pass filter (Moseley et al., 1988), it is unlikely that the filter characteristics are the same across individuals.
- ***Color of the stimulus***. A green stimulus was chosen, ad hoc, for use in these experiments. Studies show that stimulus color affects SSVEP amplitude in waking participants (Regan, 1966). Duszyk et al. (2014) recently revisited the impact of color on SSVEP amplitude and found that blue stimuli elicit a smaller response than other colors. Future experiments investigating color would have to account for the filtering characteristics of an individual's eyelids (Moseley et al., 1988), but may provide additional insight into how SSVEPs elicited during sleep differ from those elicited during waking.
- ***Frequency of the stimulus.*** These experiments used a 7.03 Hz stimulus to avoid overlap with alpha activity (8–13 Hz) that spontaneously occurs during waking. Different flicker frequencies are known to elicit different SSVEP amplitudes with resonance peaks occurring at 10, 20, 40, and 80 Hz (Herrmann, 2001). These resonance peaks are evidence for the oscillatory hypothesis of SSVEP generation (Herrmann, 2001; Makeig et al., 2002). Since SSVEPs can be elicited during sleep, and the oscillatory dynamics of the brain change during sleep (Cote, 2002), a logical question to ask is whether these SSVEP resonance frequencies change or disappear during sleep.

Changing any of these factors may lead to a different set of results and represent possible directions of future work.

Conclusion

The experiments presented here show that SSVEPs can be elicited during sleep using a dim (approximately 1.5 lux) stimulus, through closed eyelids, without waking the participant. We have also provided evidence that there may be amplitude differences in SSVEPs elicited during sleep compared with those elicited during waking. As discussed in the introduction, there are at least two specific hypotheses that can be tested based on this result: (1) Given that SSVEPs can be elicited during sleep and that cortical connectivity decreases with sleep (Massimini et al., 2005), one could hypothesize that neural responses later in the SSVEP activation sequence will be attenuated more during sleep than earlier ones. (2) Given that SSVEP amplitude is dependent on frequency and that the

oscillatory dynamics of the brain change during sleep (Cote, 2002), one could hypothesize that SSVEP resonance frequencies would change or disappear during sleep. While future work might concentrate on confirming these hypotheses, they represent just two examples of the types of hypotheses that can be tested since SSVEPs can be elicited during sleep.

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(Received April 3, 2016; Accepted November 10, 2016)

Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1: Locations of electrodes used to record EEG during experiments.

Figure S2: Scatter plot showing mean amplitude versus number of time windows for all conditions.

Figure S3: Scatter plots showing mean amplitude versus number of time windows for the stimulation on condition (a) waking ($r = .71$, $p = .047$), (b) light sleep ($r = -.47$, $p = .242$), and (c) deep sleep ($r = -.20$, $p = .642$).

Figure S4: Bootstrap confidence intervals (95%) for comparison of stimulation off—waking versus light sleep.

Figure S5: Bootstrap confidence intervals (95%) for comparison of stimulation off—waking versus deep sleep.

Figure S6: Bootstrap confidence intervals (95%) for comparison of stimulation off—light sleep versus deep sleep.

Figure S7: Bootstrap confidence intervals (95%) for comparison of stimulation on—waking versus light sleep.

Figure S8: Bootstrap confidence intervals (95%) for comparison of stimulation on—waking versus deep sleep.

Figure S9: Bootstrap confidence intervals (95%) for comparison of stimulation on—light sleep versus deep sleep.

Figure S10: Bootstrap confidence intervals (95%) for comparison of waking—stimulation off versus stimulation on.

Figure S11: Mean SSVEP amplitude, averaged across all subjects and stimulation periods, elicited during sleep session as a function of time following stimulation onset.

Figure S12: The ASD value for the individual alpha frequency for s01 during waking and the stimulation off condition.

Figure S13: Scatter plots of the IAF for each subject during the stimulation off condition.

Figure S14: Scatter plot to assess the effect of light adaptation on the experimental setup for Subject 1 and Subject 2.

Analysis SI: Data analysis showing the correlation between individual alpha frequency (IAF) and the SSVEP amplitude from each subject.

Analysis SII: Effect of light adaptation on SSVEP amplitude.

Table S1: Individual alpha frequency by subject (stimulation off).

Table S2: Individual alpha frequency by subject (stimulation on).