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1 **Visually evoked neuronal ensembles reactivate during sleep**

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1 **Abstract**

2 Neuronal ensembles, defined as groups of coactive neurons, dominate cortical activity and are
3 causally related to perceptual states and behavior. Interestingly, ensembles occur spontaneously
4 in the absence of sensory stimulation. To better understand the function of ensembles in
5 spontaneous activity, we explored if ensembles also occur during different brain states, including
6 sleep, using two-photon calcium imaging from mouse primary visual cortex. We find that
7 ensembles are present during all wake and sleep states, with different characteristics depending
8 on the exact sleep stage. Moreover, visually evoked ensembles are reactivated during
9 subsequent slow wave sleep cycles. Our results are consistent with the hypothesis that repeated
10 sensory stimulation can reconfigure cortical circuits and imprint neuronal ensembles that are
11 reactivated during sleep for potential processing or memory consolidation.

12

13 **One-Sentence Summary:** Cortical neuronal ensembles are present across wake and sleep
14 states, and visually evoked ensembles are reactivated in subsequent slow-wave sleep.

15

16 **Keywords:**

17 Neuronal ensemble, sleep, brain state, memory processing, replay, reactivation, slow-wave
18 sleep, REM sleep, NREM sleep

19

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1 **Introduction**

2 Perceptual memory is believed to be encoded in organized ensembles formed by
3 synchronous neuronal firing by small groups of neurons within the cortex [1–3]. Neuronal ensembles
4 in the cortex can be activated by the exposure to a sensory stimulus but can also occur
5 spontaneously [4,5]. The function of the spontaneous activation of cortical ensembles is unknown.
6 Previous studies have shown that spontaneous ensembles are statistically identical to sensory
7 evoked ensembles [4,5]. This resemblance suggested the hypothesis that ensembles are
8 endogenous building blocks of cortical function that are recruited by sensory stimuli, from an
9 internal lexicon of patterns. However, another possibility is that sensory stimulus creates and
10 "burns in" ensembles into cortical activity, and that these ensembles are later reactivated
11 spontaneously during rest, as echoes of past sensory experience [6]. To discern these possibilities,
12 one potential clue comes from studies of sleep.
13
14 During sleep, the brain is placed in a quiescent state, disconnected from sensory input.
15 Cortical activity during wake and rapid eye movement sleep (REM) displays high frequency brain
16 waves [7]. In contrast, cortical activity during slow-wave sleep (SWS) is reduced to low frequency
17 oscillations [8], where the newly formed ensembles of neuronal activity are thought to
18 spontaneously reactivate [9,10]. Additionally, reports have found differential calcium activity between
19 wake, REM sleep and SWS [11–13]. In addition, REM and SWS are believed to play differential roles
20 in synaptic scaling with downscaling and upscaling occurring respectively [14,15]. Measures of
21 functional network architecture have been done at the mesoscopic scale or motor cortices across
22 wake and sleep [16,17], however the functional architecture underlying neuronal network activity
23 across wake and sleep in sensory cortices have not been fully examined.
24
25 Previous reports have shown neuronal sequential replay during slow-wave sleep in the
26 hippocampus and primary visual cortex (V1) during an exploration task [9,10]. Consistent with this,
27 neuronal activity has been shown to replay during wake, slow-wave sleep, and REM sleep [9,10,18,19].
28 Reactivation is thought to underlie memory consolidation, where the spontaneous activation of
29 newly formed neuronal ensembles has been posited to improve the stability of ensemble activity
30 and improve memory retention [20–22]. Further, the spontaneous reactivation of visually evoked
31 neuronal ensembles in the cortex across wake and sleep is understudied.
32
33 The past results on reactivation during sleep suggest that the reactivation of cortical
34 ensembles could also be linked to the consolidation of memories, or, more generally, to the further
35 processing of sensory information. To explore this possibility, and further investigate the relation
36 between visually evoked ensembles and ongoing cortical activity, we studied neuronal ensembles
37 during wake and sleep. First, we characterize the functional architecture of the primary visual
38 cortex across wake and sleep. Next, we evaluate neuronal ensembles across wake and sleep
39 brain stages. Finally, we assess the spontaneous reactivation of visually evoked neuronal
40 ensembles during the subsequent wake and sleep stages. Our results are consistent with the
41 hypothesis that sensory activity creates ensembles that are then reactivated spontaneously, in
42 both wake and sleep, for potential memory consolidation.

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Results

Conserved neuronal population activity in different wake and sleep states

To record neuronal ensembles across wake and sleep states, we measured the activity of neuronal populations using two-photon calcium imaging in parallel with electrocorticogram (ECoG) and electromyography (EMG) in head-restrained mice (Figure 1A-C). In addition to electrophysiological recordings, an infrared camera was used to monitor the mouse to confirm sleep status (Figure 1B). Mice were handled for a few days leading up to habituation on the head-restraint system that took 2-3 days of consistent runs. To aid sleep, the room was temperature controlled to stay above 25° C and a lavender scent was introduced. We implemented an automatic sleep rating algorithm to determine wake and sleep states of the animal while recording neuronal calcium activity in layer 2/3 of the primary visual cortex (Please see Materials and Methods: Figure 1C,D).

Following habituation to the experimental setup, mice were observed during different behaviors such as locomotion, non-locomotive quiet wake, REM sleep, light and deep slow-wave sleep (SWS). While there was a trend for neuronal frequency and neuronal synchrony to be reduced in SWS, we observed no significant difference in neuronal activity between these brain states (ANOVA; neuronal firing rate: $p = 0.43$; neuronal synchrony: $p = 0.91$). This demonstrates that neuronal activity, on average, is conserved across sleep states.

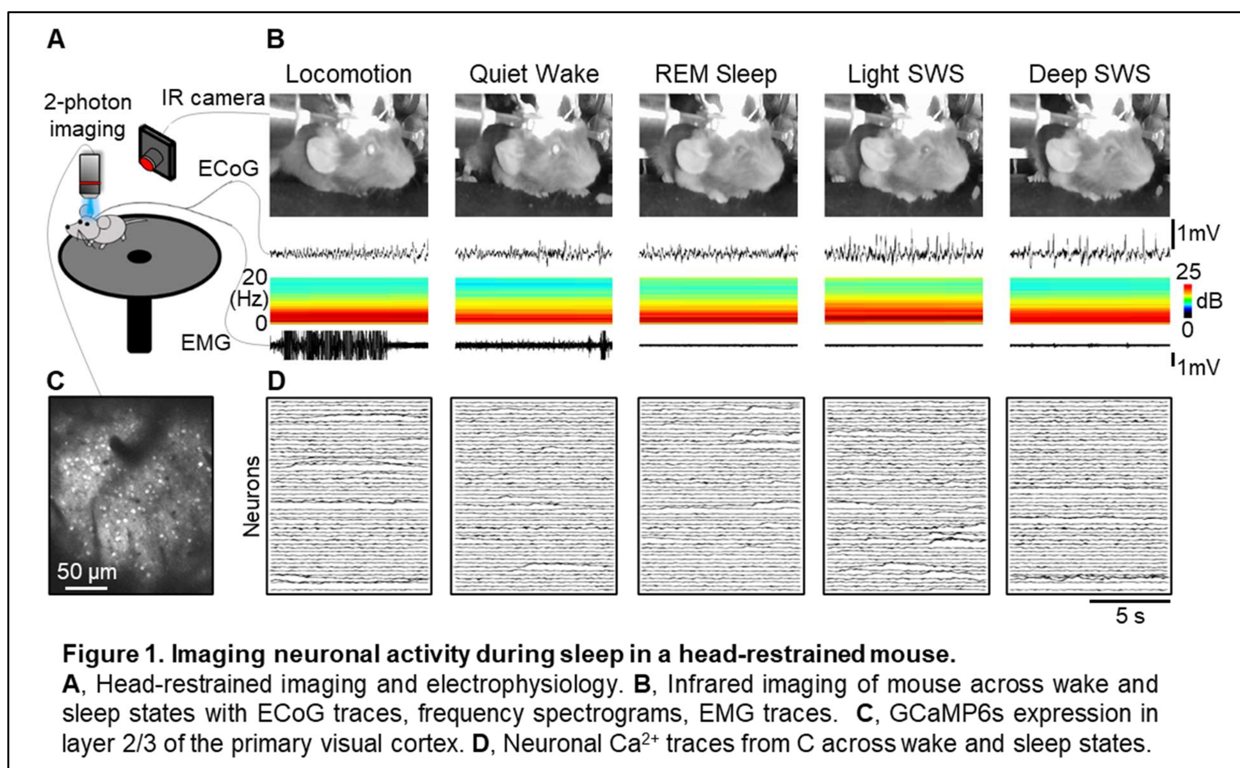

Figure 1. Imaging neuronal activity during sleep in a head-restrained mouse. **A**, Head-restrained imaging and electrophysiology. **B**, Infrared imaging of mouse across wake and sleep states with ECoG traces, frequency spectrograms, EMG traces. **C**, GCaMP6s expression in layer 2/3 of the primary visual cortex. **D**, Neuronal Ca^{2+} traces from C across wake and sleep states.

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Changes in cortical functional architecture across wake and sleep states

We next asked whether parameters of network functional architecture were altered across wake and sleep states. First, we determined the functional connectivity between neurons using cosine similarity of their rasterized traces. We found that the average functional connectivity was not significantly different across brain states (ANOVA: $p = 0.39$; 28 experiments, 7 animals). But, at the same time, when comparing neuron pairs with nonzero positive functional connectivity, network activity during REM sleep as well as light and deep SWS had an increased neuronal functional connectivity, when compared to activity during wake states (ANOVA: $p < 0.001$; 5.3 ± 0.8 % in wake vs. 6.6 ± 1.6 % in REM: $p < 0.001$; vs. 5.8 ± 1.2 % in light SWS: $p < 0.01$; vs. 6.2 ± 1.1 % in deep SWS: $p < 0.001$; 28 experiments, 7 animals: Figure 2A,B). These results demonstrate that neurons either fire more synchronously during sleep states or not at all and suggest potential differences in the underlying network architecture. To further investigate this, we next digitalized all positive entries as ones in the cosine similarity matrices to create adjacency matrices to determine the clustering coefficients of individual neurons within the network. We found that the average clustering coefficient was reduced in sleep compared to quite wake (ANOVA: $p < 0.001$; 0.97 ± 0.04 in wake vs. 0.83 ± 0.12 in REM: $p < 0.001$; vs. 0.89 ± 0.1 in light SWS: $p < 0.001$; vs. 0.83 ± 0.11 in deep SWS: $p < 0.001$; 28 experiments, 7 animals: Figure 2C,D). These results indicated that specific sleep states, although they have an overall similar amount of neuronal activity, have a distinct functional architecture.

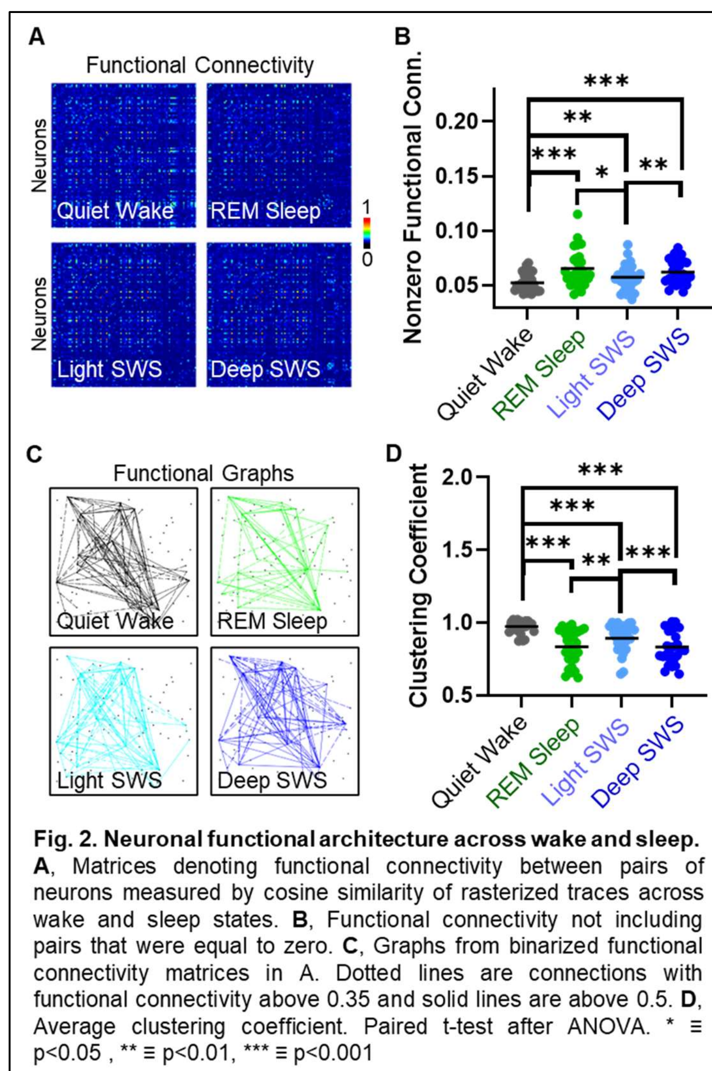

Fig. 2. Neuronal functional architecture across wake and sleep. **A**, Matrices denoting functional connectivity between pairs of neurons measured by cosine similarity of rasterized traces across wake and sleep states. **B**, Functional connectivity not including pairs that were equal to zero. **C**, Graphs from binarized functional connectivity matrices in A. Dotted lines are connections with functional connectivity above 0.35 and solid lines are above 0.5. **D**, Average clustering coefficient. Paired t-test after ANOVA. * ≡ $p<0.05$, ** ≡ $p<0.01$, *** ≡ $p<0.001$

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Neuronal ensembles occur during all wake and sleep states

Observing changes in the functional architecture of the cortical network suggested that encoding of sensory information by the network may be different between wake and sleep states. One way that cortical circuits encode sensory information occurs via the synchronous firing of neurons within ensembles. To explore this, we investigated the specific organization of neuronal ensembles in different wake and sleep states. We extracted neuronal ensembles from the raster of activity using Non-Negative Matrix Factorization (NMF) [23,24] (Figure 3A-D). We observed neuronal ensembles occurring in spontaneous activity across wake and sleep states (Figure 3E). At the same time, although neuronal ensembles were observed across all wake and sleep states, the frequency of neuronal ensembles were reduced in REM sleep, as compared to wake, light and deep SWS (ANOVA: $p < 0.0001$; 0.015 ± 0.009 Hz in REM sleep vs. 0.023 ± 0.008 Hz in wake: $p < 0.001$; vs. 0.024 ± 0.009 Hz in light SWS: $p < 0.0001$; vs. 0.025 ± 0.009 Hz in deep SWS: $p < 0.001$; 28 experiments, 7 animals: Figure 3F). Further, there was no significant differences between neuronal ensemble frequency during quiet wake and light ($p = 0.33$) or deep ($p = 0.09$) SWS. Next, we observed the percentage of neurons active within an ensemble, or neuronal ensemble expression, at every occurrence where over 35% of the ensemble neurons were active together, and found that the neuronal ensemble expression was decreased during REM sleep compared to wake, light and deep SWS (ANOVA: $p < 0.05$; 47.2 ± 3.1 % in REM sleep vs. 49.3 ± 3.0 % in wake: $p < 0.001$; vs. 49.2 ± 3.6 % in light SWS: $p < 0.001$; vs. 49.2 ± 2.7 % in deep SWS: $p < 0.001$; 28 experiments, 7 animals: Figure 3G). Again, we found no significant difference in neuronal ensemble expression between quiet wake and light ($p = 0.83$) or deep ($p = 0.72$) SWS. Together, these results demonstrate that, while ensembles appear altered during REM sleep, neuronal ensemble activity is overall similar to wake in both light and deep SWS.

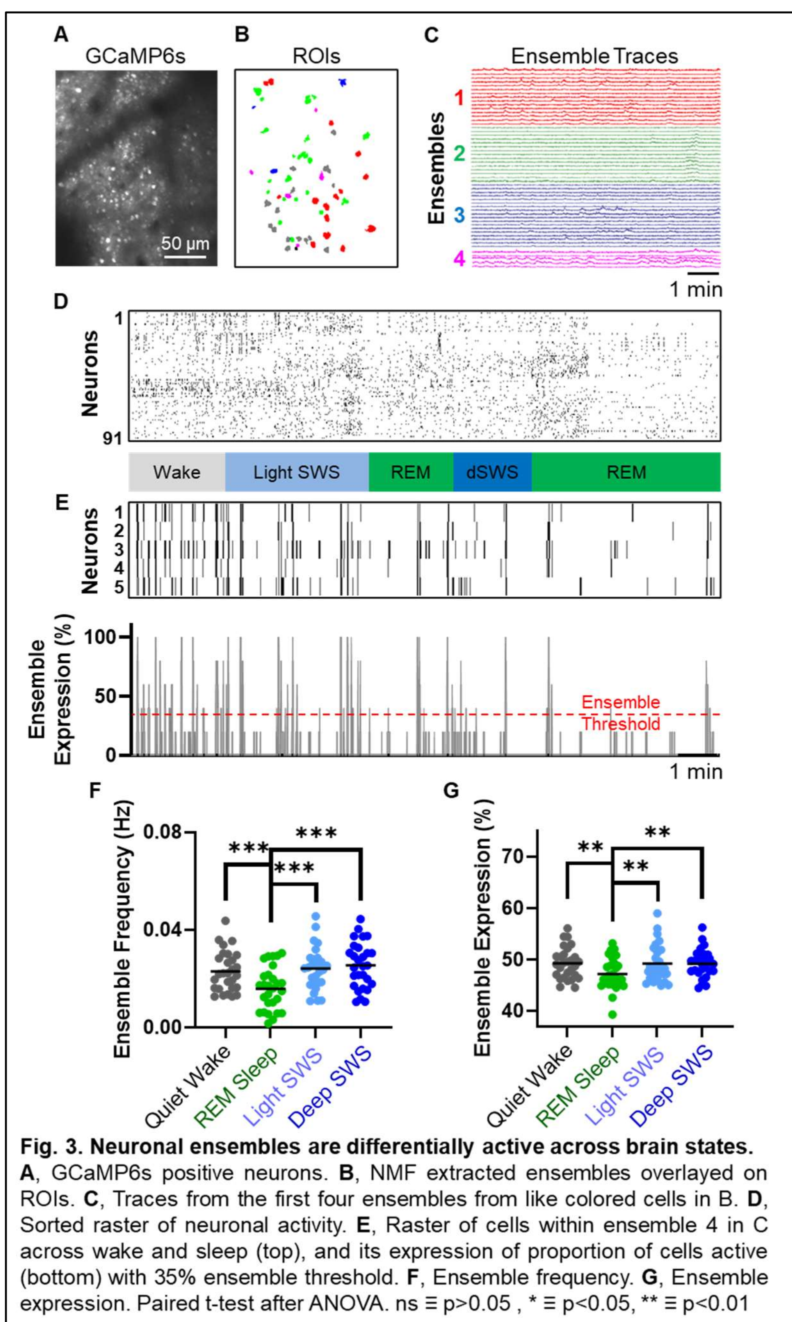

Fig. 3. Neuronal ensembles are differentially active across brain states. **A**, GCaMP6s positive neurons. **B**, NMF extracted ensembles overlayed on ROIs. **C**, Traces from the first four ensembles from like colored cells in B. **D**, Sorted raster of neuronal activity. **E**, Raster of cells within ensemble 4 in C across wake and sleep (top), and its expression of proportion of cells active (bottom) with 35% ensemble threshold. **F**, Ensemble frequency. **G**, Ensemble expression. Paired t-test after ANOVA. ns ≡ p>0.05 , * ≡ p<0.05, ** ≡ p<0.01

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Similar functional architecture during visual stimulation and spontaneous activity

The past results on spontaneous activity suggest that sleep states may implement distinct stages for processing of sensory information. To explore this in more detail, we evaluated neuronal activity in V1 across wake and sleep states before and after the presentation of novel visual gratings (Figure 4A-C). Drifting visual gratings were presented for 2 s with 3 s interstimulus interval repeated in a pseudorandom order in the cardinal directions over a ten-minute-long stimulation block. Rest periods were recorded 30 minutes before and after visual stimulus where animals could sleep. Similarly, to above, we monitored electrophysiology and neuronal calcium during visual stimulation (Figure 4A-C). We observed an increase in neuronal firing rate during light SWS (0.33 ± 0.05 Hz before stimulation vs. 0.36 ± 0.04 Hz after stimulation: $p < 0.05$; 18 experiments, 7 animals: Figure 4D,E). We did not observe any differences in quite wake, REM sleep or deep SWS (Figure S1A). This suggests that visual stimulation caused an increase in neuronal activity only during subsequent light SWS. We did not find any significant changes to functional connectivity or average clustering coefficient, suggesting that visual stimulation does not alter the functional architecture of wake and sleep states in the visual cortex.

Visually evoked ensembles reactivate during light slow-wave sleep

We then turned our attention to ensembles and investigated if the visual stimulation can alter the repertoire of ensembles present spontaneously in different wake and sleep states. To evaluate neuronal ensembles, we defined ensembles using NMF only on frames during the stimulation block, and then quantified the expression and frequency of these ensembles during the resting periods before and after visual stimulation (Figure 4F). We observed that neuronal ensembles following visual stimulation had no significant differences in expression during light SWS (47.1 ± 4.8 % before vs. 47.6 ± 5.5 % after: $p = 0.18$; 18 experiments, 7 animals: Figure 4G). However, the frequency of spontaneous neuronal ensembles was increased following visual stimulation during light SWS (0.018 ± 0.010 Hz before vs. 0.021 ± 0.011 Hz after; $p < 0.05$; 18 experiments, 7 animals: Figure 4H). At the same time, we did not observe changes in neuronal ensemble expression or frequency in quiet wake, REM sleep or deep SWS following visual stimulation (Figure S1B,C). Taken together, these results show that a novel visual stimulation increases the reactivation of neuronal and ensemble activity in subsequent SWS, and that this effect is specific to light SWS.

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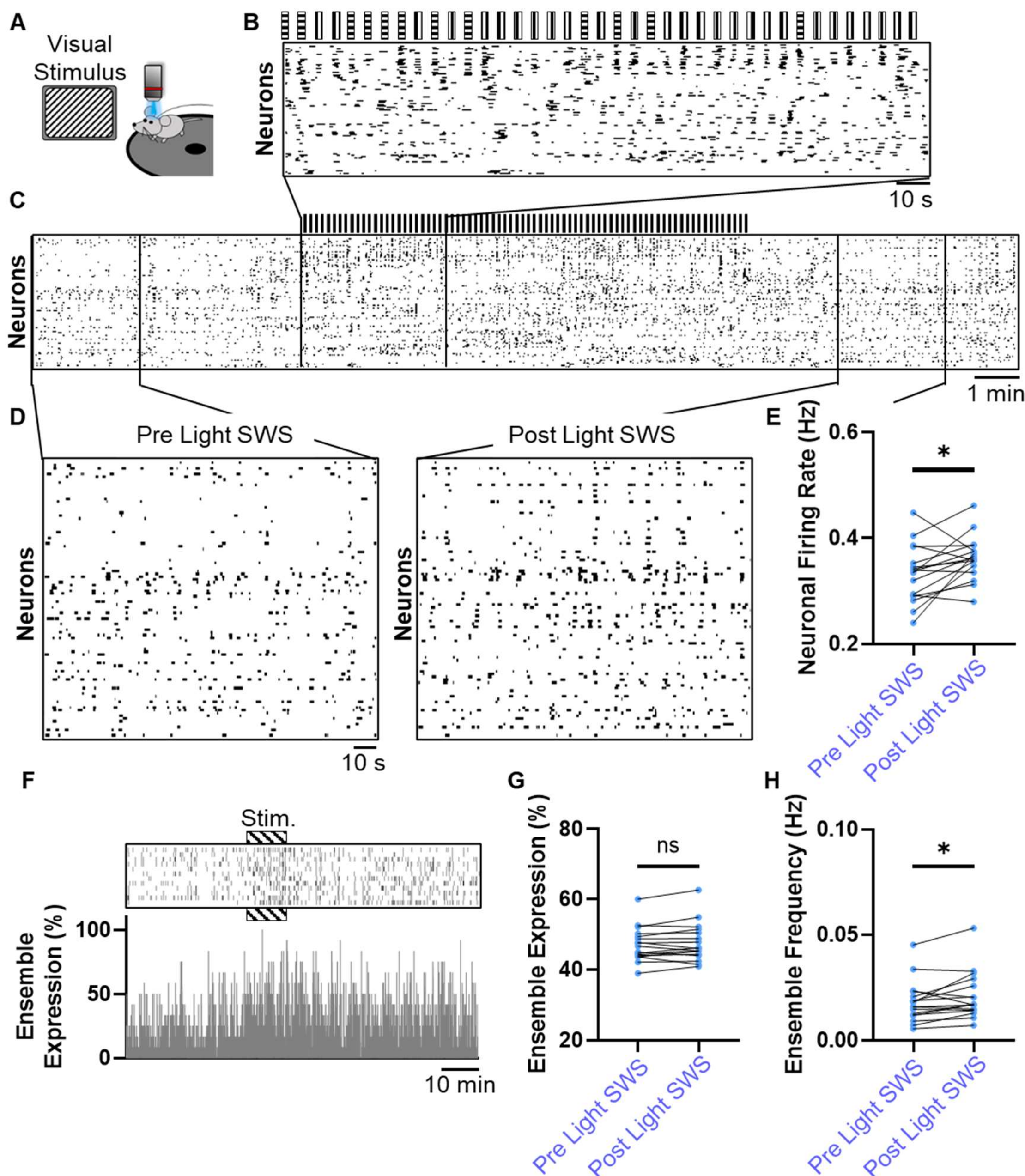

Fig. 4. Neuronal ensembles reactivate following novel visual stimulation.
A, Scheme to present novel visual stimulation. **B**, Sorted raster based on ensembles during visual presentation. **C**, Neuronal raster with rest periods before and after visual presentation. **D**, Neuronal raster during sleep in the pre rest phase (left) and neuronal raster during sleep in the post rest phase (right). **E**, Average neuronal firing rate during light SWS. **F**, Raster ensemble cells (top) and expression with visual stim. Note: higher activity in post rest phase. **G**, Ensemble expression. **H**, Ensemble frequency Paired t-test. ns ≡ p>0.05, * ≡ p<0.05

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1 **Discussion**

2 In order to understand the function of neuronal ensembles across wake and sleep, we
3 measured spontaneous and visually evoked neuronal network activity in V1 in different wake and
4 sleep states. Upon the introduction of a visual presentation to evoke neuronal ensemble activity
5 in V1, we found that ensembles extracted from the stimulus period had increased reactivation in
6 subsequent light SWS, and this followed a general trend of increased neuronal activity in that
7 sleep stage. When comparing the cortical functional architecture between wake and sleep states
8 we found changes that distinctly separated wake from REM sleep from SWS stages. In addition,
9 these functional architectures across wake and sleep states were conserved following visual
10 evoked activity. Analyzing the synchronous firing of neurons to form neuronal ensembles using
11 NMF, we found that neuronal ensembles present in SWS were similarly active to those found in
12 wake states, yet the frequency of ensembles and their expression were reduced in REM sleep,
13 further suggesting a dichotomy between network processing in these specific sleep stages.

14 The effect of sleep could be observed at the network level in the parameters of functional
15 connectivity and average clustering coefficient. An increase in the nonzero functional connectivity
16 of the pairwise connections during sleep states, compared to wake suggests network states of
17 heightened similarity in firing patterns. Sleep states are widely understood to be synchronous
18 states compared to desynchronized waking states [8,25], as is characterized by sleep states being
19 dominated by low frequency oscillations in the local field potential [7,26]. Interestingly, the increase
20 in functional connectivity of nonzero pairwise connections also came with a decrease in the
21 average clustering coefficient. This suggests offline processing as being selective for specific
22 arrangements of neurons over the desynchronized activity in wake. Previous work has shown that
23 sleep increased the firing of hippocampal neurons specially found within ensembles over
24 nonensemble neurons [24], which is in line with our findings.

25 The encoding of percepts on cortical neuronal networks is believed to occur by neuronal
26 ensembles during the repeatable cofiring of neurons. Using NMF, we discovered that neuronal
27 ensembles were active across all vigilance states and that specific sleep stages showed distinct
28 ensemble activity. While SWS was similar to wake in ensemble frequency and expression, we
29 found that ensembles present during REM sleep had reduced expression and frequency of
30 occurrence comparatively. This reduction in neuronal ensemble quality during REM sleep is in
31 line with previous accounts of REM being involved in synaptic downscaling [14]. Recent work has
32 uncovered REM to be involved in emotionally-charged or arousing memory processing [18].
33 Perhaps incorporating arousing stimuli in our paradigm would alter the way ensembles are treated
34 in REM sleep.

35 Passive viewing of visual stimuli had the effect of increasing the spontaneous reactivation
36 of visually evoked neuronal ensembles. Neuronal ensembles classified during the stimulus block
37 became spontaneously active across all behavioral states, but, interestingly, only those in light
38 slow-wave sleep showed an increase in the frequency of neuronal ensemble occurrences. The
39 increased frequency of stimulus-classified neuronal ensembles may have been spurned by an
40 overall increase in neuronal firing rate, as this was also specifically increased during light slow-
41 wave sleep. It may be that light slow-wave sleep places the brain in a quiescent state without
42 major neuromodulatory control such as REM sleep and deep SWS driven largely by acetylcholine,
43 serotonin and norepinephrine [25].

44 Taken together, our results provide a hypothesis that explains the similarity between
45 neuronal ensembles in spontaneous and visually evoked activity. Rather than the original
46 hypothesis that cortical activity during sensory stimulation generated ensembles, our data suggest
47 that visual stimulation itself, by repeated applications, reconfigures cortical circuits and reactivates
48 endogenous ensembles. These ensembles are then reactivated spontaneously during different

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1 wake and sleep states. Overall, our data indicate that cortical circuits are eminently plastic and
2 can react to repeated sensory stimuli by reconfiguring its correlational architecture during slow-
3 wave sleep.
4

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Materials and Methods

Proper animal use and care

All the procedures for handling and sacrificing animals were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals. We used both female and male transgenic animals (VGluT1-GCaMP6s) that were 2-6 months of age, kept on a continuous 12h light/dark cycle and freely available to food and water.

Stereotaxic surgery for cranial window, ECoG and EMG

Mice were anesthetized with 1.5-2% isoflurane and placed in a stereotaxic atop a heating pad maintained at 37° C, and faux tears were applied to prevent corneal dehydration. Carprofen (5 mg/kg), Dexamethasone (0.6 mg/kg), and Enrofloxacin (5 mg/kg) was administered intraperitoneally. Hair was removed from the scalp and ethanol and chlorhexidine was applied. Lidocaine was administered locally to the scalp before an incision was made down the midline of the scalp to expose the skull. A screw was placed over the right frontal plate. Next, a hole was drilled over the primary visual cortex [27] (V1; in mm from bregma: $-3.4_{a\text{-}p}$, $2.1_{m\text{-}l}$) and a tungsten electrode was placed to record electrocorticogram (ECoG). Wires were places in the nuchal muscle to record electromyography (EMG), and a titanium headplate was cemented onto the exposed skull using dental cement. A 3 mm craniotomy was made over the contralateral primary visual cortex (in mm from bregma: $-3.4_{a\text{-}p}$, $-2.1_{m\text{-}l}$), and the dura was removed. Finally, a 3 mm glass coverslip was placed on the exposed cortex and fixed using super glue.

In vivo two-photon calcium fluorescence imaging

In vivo imaging was performed in layers 2/3 (100 – 300 µm below the cortical surface) of the exposed mouse cortex with a custom two-photon system comprised of Ti:sapphire lasers (MaiTai DeepSee at 920 nm) for imaging a 31 Hz resonance galvanometer two-photon microscope to capture 512x512 digital images (500 μm^3). Videos were obtained for 1-2 hours.

Electrophysiological recordings

Electrocorticogram (ECoG) was recorded using an A-M Systems Model 3000 at a bandpass filter of 1 Hz – 1 kHz. Electromyography (EMG) was recorded using a Warner Instruments DP-301 with a bandpass filter of 1-100 Hz. Both signals were sampled at 5 kHz and recorded with a PC running MScan.

Sleep automatic rating

Sleep rating was done automatically. The ECoG frequency content and EMG activity was monitored to determine brain state based on number of standard deviations (SD) over the mean: below 1 SD EMG and over ½ or 1 SD delta activity (1-4 Hz) denote light and deep SWS respectively, below ½ SD EMG with over ½ SD theta (6-10 Hz) divided by delta activity denotes REM sleep. and all other as locomotion or quiet wake [28]. Only epochs that lasted at least 15 seconds were accepted.

Visual stimulation

Drifting Gabor patches were presented to the visual field of the mouse on a monitor connected to a PC running the Psychtoolbox [29] (psychtoolbox.org). Gabor patches were presented in pseudorandom orientation 2 s with 3 s interstimulus of black screen and repeated 120 times over a 10-minute span.

Calcium image processing and analysis

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1 All images were frame averaged by 3 to create videos at 10.3 Hz and registration was
2 performed using TurboReg in ImageJ. Next, regions of interest (ROIs) were created automatically
3 from thresholding correlation images and ROI maps underwent manually correction using custom
4 MATLAB scripts. Fluorescent traces and event detection from ROIs were quantified as in [30].
5 Functional connectivity between a neuron pair was analyzed by taking the cosine similarity of
6 vectors of rasterized activity. For individual states of vigilance, only frames that included that
7 specific state was used for cosine similarity. Clustering coefficients were quantified by taking all
8 nonzero similarity matrices as adjacency matrices as input into the additional MATLAB function
9 clustCoeff() [31]. Neuronal ensembles were detected using Non-negative Matrix Factorization
10 (NMF) [23,24] [REF], where a raster of neuronal activity over the entire experiment for spontaneous
11 activity or a raster during the stimulation block was input into the MATLAB function nnmf() to
12 detect K ensembles determined from the 95th percentile of a latent determined from performing
13 PCA on a shuffled raster.
14

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7 **Author Contributions**

8 J.L. and R.Y. contributed to project conception, project design, and manuscript writing. J.L.
9 performed the experiments and analyzed the results. R.Y. directed the project and secured
10 resources and funding.

11 **Competing Interests statement**

12 The authors declare no competing interests.

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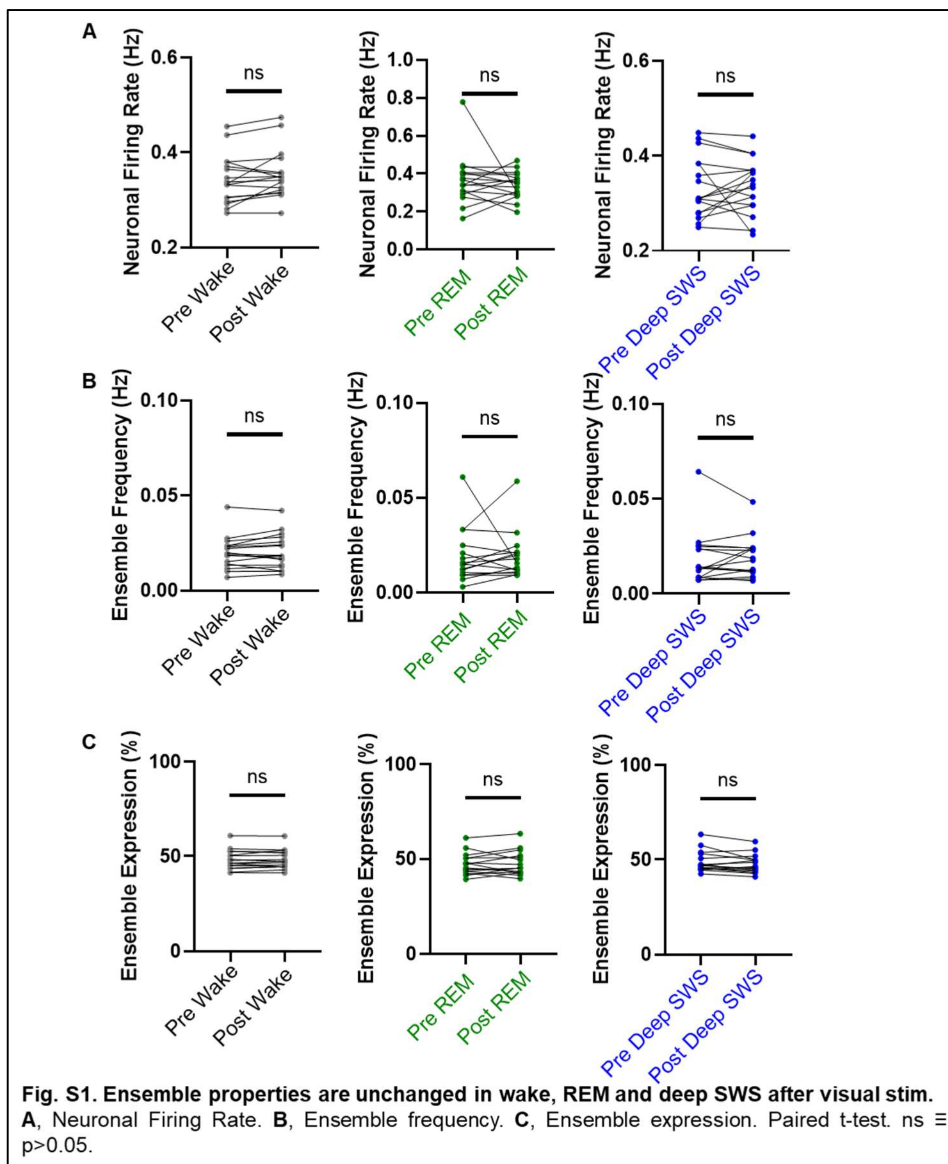

Fig. S1. Ensemble properties are unchanged in wake, REM and deep SWS after visual stim. **A**, Neuronal Firing Rate. **B**, Ensemble frequency. **C**, Ensemble expression. Paired t-test. ns ≡ p>0.05.

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1 **Figure Legends**

2 **Figure 1. Imaging neuronal activity during sleep in a head-restrained mouse.**
3 **A**, Head-restrained imaging and electrophysiology. **B**, Infrared imaging of mouse across wake
4 and sleep states with ECoG traces, frequency spectrograms, EMG traces. **C**, GCaMP6s
5 expression in layer 2/3 of the primary visual cortex. **D**, Neuronal Ca^{2+} traces from C across wake
6 and sleep states.

7 **Fig. 2. Neuronal functional architecture across wake and sleep.**
8 **A**, Matrices denoting functional connectivity between pairs of neurons measured by cosine
9 similarity of rasterized traces across wake and sleep states. **B**, Functional connectivity not
10 including pairs that were equal to zero. **C**, Graphs from binarized functional connectivity matrices
11 in A. Dotted lines are connections with functional connectivity above 0.35 and solid lines are above
12 0.5. **D**, Average clustering coefficient. Paired t-test after ANOVA. * ≡ p<0.05 , ** ≡ p<0.01, *** ≡
13 p<0.001

14 **Fig. 3. Neuronal ensembles are differentially active across brain states.**
15 **A**, GCaMP6s positive neurons. **B**, NMF extracted ensembles overlayed on ROIs. **C**, Traces from
16 the first four ensembles from like colored cells in B. **D**, Sorted raster of neuronal activity. **E**, Raster
17 of cells within ensemble 4 in C across wake and sleep (top), and its expression of proportion of
18 cells active (bottom) with 35% ensemble threshold. **F**, Ensemble frequency. **G**, Ensemble
19 expression. Paired t-test after ANOVA. ns ≡ p>0.05 , * ≡ p<0.05, ** ≡ p<0.01

20 **Fig. 4. Neuronal ensembles reactivate following novel visual stimulation.**
21 **A**, Scheme to present novel visual stimulation. **B**, Sorted raster based on ensembles during visual
22 presentation. **C**, Neuronal raster with rest periods before and after visual presentation. **D**,
23 Neuronal raster during sleep in the pre rest phase (left) and neuronal raster during sleep in the
24 post rest phase (right). **E**, Average neuronal firing rate during light SWS. **F**, Raster ensemble cells
25 (top) and expression with visual stim. Note: higher activity in post rest phase. **G**, Ensemble
26 expression. **H**, Ensemble frequency Paired t-test. ns ≡ p>0.05, * ≡ p<0.05

27 **Fig. S1. Ensemble properties are unchanged in wake, REM and deep SWS after visual stim.**
28 **A**, Neuronal Firing Rate. **B**, Ensemble frequency. **C**, Ensemble expression. Paired t-test. ns ≡
29 p>0.05.

30