Title: Behavior and arousal state controls neurovascular-coupling

Authors: Kevin L. Turner1,2, Ravi Teja Kedarasetti2,3, Kyle Gheres2,4, Patrick J. Drew\*1,2,4,5

Affiliations:

Department of Biomedical Engineering, The Pennsylvania State University, University Park, PA

Center for Neural Engineering, The Pennsylvania State University, University Park, PA

Department of Engineering Science and Mechanics, The Pennsylvania State University, University Park, PA

Graduate Program in Molecular, Cellular, and Integrative Biosciences, The Pennsylvania State University, University Park, PA

Department of Neurosurgery Engineering, The Pennsylvania State University, University Park, PA

\*Corresponding author, [pjd17@psu.edu](mailto:pjd17@psu.edu)

**Abstract**

Hemodynamic signals in the brain are used to infer neural activity, and bilateral correlations in hemodynamic signals have been observed in the absence of any overt stimulus or task. However, recent studies have suggested that the nature and strength hemodynamic signals depend on arousal state. Here, we monitored neural activity and hemodynamic signals in un-anesthetized, headfixed mice to understand how sleep and wake states impact cerebral hemodynamics. In parallel with electrophysiological recordings, we used intrinsic optical signal imaging to measure bilateral changes in cerebral blood volume (CBV). We concurrently monitored body motion, whisker movement, muscle EMG, and cortical LFP to classify the arousal state of the mouse into awake, NREM sleep, or REM sleep. We found that mice regularly fell asleep for a few minutes at time during imaging. During both NREM and REM sleep, mice showed large increases in CBV relative to the awake state. During NREM sleep, the amplitude of bilateral low-frequency oscillations in CBV increased markedly. Bilateral correlations in neural activity and CBV were highest during NREM sleep, and lowest in the awake state. Our results show that hemodynamic signals in the cortex are strongly modulated by arousal state and emphasize the importance of behavioral monitoring during studies of spontaneous activity.

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**Methods**

Processed data is available at **[box link]** analysis code is available at [**github link]**. Unprocessed/original data is available upon request.

**Animal procedures**

All procedures involving the use of animals were conducted in accordance and with approval from the Institutional Animal Care and Use Committee (IACUC) of Pennsylvania State University. All data was acquired from 20 C57BL/6J mice (Jackson Labs, strain 000664) comprised of 11 males and 9 females between the ages of 3 and 8 months of age. Of these 20 animals, 14 were used in IOS experiments and 6 were used in two-photon experiments. Any animals that were excluded from a specific analysis will be noted as such. Mice were given food and water ad libitum and housed on a 12-hr light/dark cycle, remaining individually housed after surgery and throughout the duration of experiments. All experiments were performed during the animal’s light cycle period. Sample sizes are consistent with previous studies1–3. The experimenter was not blind to the conditions of the experiments, data, or analysis.

**Surgery**

*Electrode, EMG, and window implantation procedure for intrinsic optical signal (IOS) imaging experiments.* Mice were anesthetized under Isoflurane (5% induction, 2% maintenance) vaporized in oxygen during all surgical procedures. The incision site on the scalp was sterilized with betadine and 70% ethanol, followed by the resection of the skin and connective tissue. A custom-machined titanium headbar for head-fixation (<https://github.com/DrewLab/Mouse-Head-Fixation>) was adhered atop the occipital bone of the skull with cyanoacrylate glue (32402, Vibra-Tite) posterior to the lambda cranial suture. A self-tapping 3/32” #000 screw (J.I. Morris) was implanted into the center of each frontal bone. A stainless-steel wire ﻿(A-M Systems, #792800) was wrapped around one of the frontal screws to serve as a ground for neural recordings. Two ~4 mm x ~2 mm polished and reinforced thinned-skull windows were bilaterally implanted caudal to the bregma cranial suture above the left and right somatosensory cortices as previously outlined4,5. The skull is thinned and then polished with 4F and 3F grit. A PFA-coated tungsten stereotrode ﻿(AM systems, #795500) is inserted ~700 μm in the z-axis into the whisker representation of somatosensory cortex (~2 mm caudal, ~3-3.5 mm lateral from bregma) at 45﻿° from the horizontal along the rostrocaudal axis. This is mirrored for left and right hemispheres. A third tungsten stereotrode is implanted ~1500 μm in the z-axis into the CA1 region of the left hippocampus (~2.5 mm caudal, 4-4.5 mm lateral from bregma) at 45° from the vertical along the mediolateral axis. Each electrode is positioned using a ﻿micromanipulator (Sutter Instruments, MP285) through a small craniotomy made at the edge of the thinned area for the barrel electrodes, and slightly caudal the thinned-area for the left hemisphere hippocampal electrode. Each craniotomy was sealed with cyanoacrylate glue, and a #0 coverslip (Electrode Microscopy Sciences, #72198) was placed atop the thinned portion of the window. The skin above the neck is resected and a pair of PFA-coated 7-strand stainless-steel wires (AM systems, 793200) were inserted into each nuchal muscle for EMG recording. The skin is then re-attached back to the edge of the occipital bone (3M, VetBond). Dental cement (Lang Dental, Ortho-Jet) was used to seal the edges of the window and provide structural rigidity to the electrodes, screws, and headbar.

*Electrode, EMG, and window implantation procedure for two-photon laser scanning microscopy (2PLSM) experiments.* As described previously, mice were anesthetized with isoflurane and the surgical site cleaned. A titanium headbolt is implanted in a similar fashion, along with two frontal screws and ground wire. A third self-tapping 3/32” #000 screw is implanted into the left parietal bone. Instead of bilateral polished and reinforced thinned-skull windows, a single ~4 mm x ~ 5mm window above the right hemisphere somatosensory cortex is implanted following thinning and polishing. There are no electrodes under the window, as they can scatter the laser and cause heating of the local tissue. Tungsten stereotrodes are implanted into the left hemisphere barrel cortex and left hemisphere hippocampus in a fashion similar to above. Stainless-steel EMG wires are implanted, and the entire area sealed with dental cement. Following surgery, animals were given 2-3 days to recover before habituation.

**Histology**

Following the conclusion of experiments, animals were heavily anesthetized under 5% isoflurane for several minutes. A transcardial perfusion with heparinized saline was performed for several minutes, followed by 4% ﻿paraformaldehyde. Fiduciary marks were made at the corner of each cranial window. The extracted brains were allowed to sit in a solution of 4% PFA/30% sucrose for several days before being coronally section (~60 μM per section) with a freezing microtome. Sections were stained for the presence of cytochrome oxidase (CO). The whisker barrels are visible in layer IV of the sections and allow the verification of the electrode’s presence in the tissue6,7. The hippocampus is also brightly stained and allows the verification of the hippocampal electrode’s relation to CA1.

**Physiological data acquisition**

All data from IOS experiments were acquired with a custom LabVIEW program (2018, National Instruments, Austin TX). Data from 2PLSM experiments were acquired with ﻿Sutter MCS software (Sutter Instruments, Novato, CA) and a different custom LabVIEW program designed to synchronize with the Sutter MCS software. All IOS and 2PLSM experiments were perform in sound-dampening boxes. Both custom LabVIEW programs can be viewed at <https://github.com/DrewLab/LabVIEW-DAQ>

**Habituation**

All animals were gradually acclimated to being head-fixed over the course of three habituation sessions of increasing duration. In the initial session (15-30 min), animals were not exposed to any whisker stimulation and the efficacy of the cortical, hippocampal, and EMG electrodes was determined. Pending clean electrical signals and a tolerance of the head-fixation, animals were habituated for two more sessions of 60 and 120 minutes. During these subsequent sessions, the whiskers were stimulated with directed air puffs. Any animals who showed excessive, visible distress/struggling throughout the habituation were removed from future experiments. Following habituation, IOS animals were run for six imaging sessions lasting of 3-5 hrs, and 2PLSM animals were run for up to six imaging sessions depending on the quality of the thinned-skull window.

**Intrinsic optical signal (IOS) imaging**

Mice (n = 14) were briefly (<1 min) anesthetized with 5% isoflurane and transferred to the head-fixation apparatus with the body being supported by clear plastic tube. Animals were given 30 minutes to wake up prior to data collection to give the vasculature time to return to baseline. Changes in total blood volume was measured by illuminating each cranial window with two collimated and filtered 530 ﻿± 5 nm LEDs (Thor Labs, FB530-10, M530L3). The 530 nm wavelength is an isosbestic point in which oxy- and deoxy- hemoglobin absorb the light equally. We use the changes in the amount of light reflected from the surface of the brain as a measurement of total hemoglobin concentration. The reflected light is imaged with a Dalsa 1M60 Pantera CCD camera (Phase One, Cambridge MA) positioned above the mouse’s head. The magnification of the lens allows simultaneous collection of data from both the left and right cranial windows. The light entering the camera (green) was filtered using a mounted filter (Edmund Optics, Barrington NJ, #46540) to remove the red light used in whisker tracking. Images for tracking changes in total hemoglobin (256 × 256 pixels, 15 µm per pixel, 12-bit resolution) were acquired at 30 Hz1,2. All IOS total hemoglobin data were then low-pass filtered (1 Hz, Butterworth, order = 4, Matlab function: butter, filtfilt).

**Electrophysiology**

Neural activity was recorded simultaneously in both IOS and 2PLSM as the differential potentials between the two leads of either the PFA-coated tungsten microwires ﻿(A-M Systems, #795500)1,8for cortical and hippocampal stereotrodes. EMG activity was identically recorded with PFA-coated 7-strand stainless-steel microwires (AM systems, 793200). Stereotrode tungsten microwires were threaded through polyimide tubing ﻿(A-M Systems, #822200) with an interelectrode spacing of ~100 ﻿µm. The tungsten microwires were crimped to gold pin connectors, with impedances typically between 70 and 120 ﻿kΩ at 1 kHz. EMG stainless-steel microwires were fabricated in a similar fashion, but with an interelectrode spacing of several mm and much lower impedance, typically ~1 to 10 ﻿kΩ at 1 kHz **[double check]**. Each signal was amplified and hardware bandpass filtered between 0.1 Hz and 10 kHz ﻿(World Precision Instruments, DAM80) and then digitized at 20 kHz ﻿(National Instruments, Austin TX, PCIe-6341 for IOS experiments, PCIe-6321 and PCIe-6353 for 2PLSM experiments). The power in each neural band was calculated by digitally bandpass filtering the raw signal ﻿(Matlab function: butter, filtfilt; filter order = 4). Each result was squared, low-pass filtered below 10 Hz, and resampled from 20 kHz down to 30 Hz.

**Two-photon laser scanning microscopy (2PLSM)**

Mice (n = 6) were briefly (<1 min) anesthetized with 5% isoflurane and retro-orbitally injected with 100 µL of 5% (weight/volume) fluorescein isothiocyanate–dextran (Sigma-Aldrich, FD150S-1G) dissolved in sterile saline. Mice were then head-fixed to a similar head-fixation set-up as during IOS experiments and given 30 min to wake up prior to data collection. Imaging was done on a Sutter Movable Objective Microscope with a Nikon CFI75 LWD 16X W Objective

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**Author Contributions**

**Competing financial information**