We developed a protocol for simultaneous magnetic resonance spectroscopy (MRS) and polysomnography (PSG) recordings while subjects are in sleep. The approach is useful to estimate plasticity-stability balances by measuring neurochemical changes in the brain during sleep. We detail the steps needed to minimize artifacts in PSG recordings and the setup and coregistration of MRS data to sleep stages. We also describe useful information for various types of electroencephalogram (EEG) experiments in magnetic resonance imaging (MRI) environments.

**Highlights**
- Avoid instructions that may cause subjects to feel pressured to sleep well
- Introduce an adaptation sleep session before a main sleep session
- Segment PSG data and align them with MRS data for MRI artifact removal
- Assign sleep stage to each MRS segment to obtain E/I balance for the stage
Protocol

Coregistration of magnetic resonance spectroscopy and polysomnography for sleep analysis in human subjects

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SUMMARY

We developed a protocol for simultaneous magnetic resonance spectroscopy (MRS) and polysomnography (PSG) recordings while subjects are in sleep. The approach is useful to estimate plasticity-stability balances by measuring neurochemical changes in the brain during sleep. We detail the steps needed to minimize artifacts in PSG recordings and the setup and coregistration of MRS data to sleep stages. We also describe useful information for various types of electroencephalogram (EEG) experiments in magnetic resonance imaging (MRI) environments.

For complete details on the use and execution of this protocol, please refer to Tamaki et al. (2020b).

BEFORE YOU BEGIN

Brief explanation of the aim of this paper

The balance between excitatory and inhibitory neurotransmitters (or E/I balance) in the brain is assumed to provide important information about the brain plasticity (Hensch, 2005). Transient changes in the E/I balance, which are indicated by the ratio between excitatory and inhibitory neurotransmitters in early visual cortex areas using magnetic resonance spectroscopy (MRS), are closely associated with visual plasticity and stability in humans (Shibata et al., 2017; Bang et al., 2018). In our study (Tamaki et al., 2020b), visual plasticity was indicated by performance improvement, whereas visual stability was demonstrated by resilience to retrograde interference.

Importantly, our recent study showed that the transient changes in E/I balances occur in the visual cortex during sleep in correlation with visual plasticity and stability (Tamaki et al., 2020b). In our paper (Tamaki et al., 2020b), our hypothesis was that E/I balance during nonrapid eye movement (NREM) sleep increases in correlation with visual plasticity, whereas the E/I balance during rapid eye movement (REM) sleep decreases in correlation with visual stability. We conducted simultaneous MRS and polysomnography (PSG) to observe E/I balance changes during NREM sleep and REM sleep relative to wakefulness in the visual cortex after training in visual perceptual learning.

We found that coregistration between MRS data and sleep stages based on PSG was challenging, as the MRS and PSG measurements were obtained independently. This article describes the procedure for simultaneous MRS and PSG data acquisition and how to coregister E/I balance changes in sleep stages such as NREM sleep and REM sleep.
Rationale for the subject screening process and adaptation sleep session

Human sleep is known to be affected by various factors, including age, physical or psychiatric disease, irregularity of sleep-wake cycles and learning and memory experience before sleep. These factors may obscure the true effect of the main independent variable when uncontrolled. Thus, a careful screening procedure is extremely important (see Screening process below) to reduce the contamination effects and to reveal the true main effects of a variable.

Another important procedure to reveal true main effects is the introduction of an adaptation session before the main sleep session. It has been documented that naive, healthy and well-screened subjects do not sleep well in the very first sleep session at a research laboratory, known as the first-night effect (FNE) (Tamaki et al., 2016; Tamaki and Sasaki, 2019; Agnew et al., 1966). The FNE is observed even during daytime nap experiments (Tamaki et al., 2016). Thus, an extra sleep session, called an adaptation session, before the main sleep experiment is important. An adaptation session allows subjects to become familiar with the experimental environment, alleviating the FNE. In the case of simultaneous MRS and PSG experiments (Tamaki et al., 2020b), subjects were asked to sleep inside the MRI scanner with all electrodes attached in the adaptation session in the same way as they would in the main experiment (see Adaptation session below).

Polysomnography and sleep stages

PSG consists of an electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG) and electrocardiogram (ECG) recordings. PSG is required for standardized sleep scoring, which objectively determines sleep stages, such as wakefulness (W); NREM sleep stage 1 (N1), 2 (N2), 3 (N3); and REM sleep stage (R). Sleep scoring was performed for every 30-s epoch of PSG data. In other words, every 30 s, one sleep stage was assigned. See these studies (Rechtschaffen and Kales, 1968; Iber et al., 2007) for detailed rules of sleep scoring. The definition of sleep onset differed depending on researchers. In our lab, sleep onset was defined as the first N2 stage after the lights were turned off (Tamaki et al., 2020b).

Performing PSG inside a highly magnetic environment, such as an MRI scanner, is challenging because MRI scanner artifacts and ballistocardiogram artifacts contaminate PSG data obtained simultaneously with MRI data (Yotsumoto et al., 2009). Moreover, electrodes may produce heat due to the strong magnetic field, which could potentially damage the EEG equipment (including electrodes) and be dangerous to subjects. Thus, it is important to use electrodes that are shown to be safe to use inside a magnetic environment. In our study (Tamaki et al., 2020b), we used a multi-channel MRI-compatible EEG cap (32- or 25-channel BrainCap MR with Multitrodes, Brain Products GmbH, Gilching, Germany), since they were confirmed to be safe. In particular, we have made a customized PSG cap based on the MRI-compatible EEG cap. We customized the cap since default MRI-compatible EEG caps are not good enough for PSG, as the default caps are not designed to have electrodes for EOG and EMG. Moreover, we found that artifacts coming from the MRI scanner (see below) on polysomnogram are greatly reduced if we use a single amplifier for all EEG, EOG, EMG, and ECG recordings than if we use two amplifiers, one for an EEG cap and another for EOG and EMG. The electrodes for EOG, EMG, and ECG recordings were drop-down cables that were long enough to reach the eyes, chin, and shoulder blade, respectively. Figure 1 shows a custom-made MRI-compatible PSG cap.

These data were recorded by a software (BrainVision Recorder, Brain Products GmbH, Gilching, Germany) together with an MRI-compatible amplifier (BrainAmp MR, Brain Products GmbH, Gilching, Germany) and an MRI-compatible battery (PowerPack, Brain Products GmbH, Gilching, Germany).

Before conducting simultaneous MRS and PSG experiments in human subjects, we performed preliminary phantom experiments and confirmed that there was no temperature increase around the electrodes during MRS scans. Temperature testing experiments using phantoms are strongly recommended to be performed before human subjects are included in experiments for the safety, not only
for simultaneous MRS and PSG recordings but also for any experiments, including simultaneous EEG and functional MRI measurements.

The study was approved by the institutional review board at Brown University. All participants gave written informed consent.

Screening process
First, the eligibility criteria were shown in the flyer. Second, potential participants contacted our research staff, and a more detailed prescreening process took place. After eligibility was confirmed, individuals completed two questionnaires that inquired about their sleep characteristics (see below). Participants completed these two questionnaires after providing consent, as each questionnaire asks detailed and private information about the subjects.

**Timing: Approximately 1 week prior to the adaptation session**

1. Preconsent eligibility screening
   a. Age; 18–30 years old human subjects (both female and male)
   b. No prior experience of being subjects for visual perceptual learning experiments
   c. Normal or corrected-to-normal visual acuity
   d. No physical, psychiatric or sleep disease, no medication, by self-report
   e. No irregular sleep schedule, checked by the Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al., 2003) and Sleep-wake habits questionnaire (Tamaki et al., 2019, 2020a), which was created in our lab

   **Note:** Those whose sleep/wake time differed by more than 2 h between weekdays and weekends are determined as having irregular sleep-wake habits and regarded as ineligible.

   **Note:** Because the start time of the sleep session was consistent across subjects (see below), we recruited subjects whose circadian timing appear to be similar to each other and who sleep before 2AM regularly.
f. No frequent action video game playing determined by video game questionnaire (Tamaki et al., 2019, 2020a; Berard et al., 2015; Green and Bavelier, 2003)

g. Able to undergo EEG and MRI experiments (for instance, not too sensitive to surgical tapes or no metal in the body)

h. Able to wear an MRI-compatible EEG cap snugly (see Troubleshooting 1)

*Note:* Criteria were also shown in the flyer briefly and confirmed thoroughly at the prescreening session.

*Note:* We did not screen for the ability to nap on command, as this may indicate that a person is chronically sleep deprived.

2. Postconsent questionnaires
   a. Morningness-Eveningness questionnaire (MEQ) (Horne and Ostberg, 1976)
   b. Pittsburg Sleep Quality Index (PSQI) (Buysse et al., 1989)

   *Note:* The PSQI can be used as a preconsent questionnaire to exclude subjects with unhealthy or disordered sleep patterns. However, we did use the PSQI as a postconsent questionnaire for the following reasons. First, since the PSQI asks about private information of participants, their consent before administration would be required. Second, with their consent, we were allowed to collect their responses in the PSQI as data, and to analyze them. Third, there were a sufficient set of questionnaires at the preconsent stage to exclude subjects with unhealthy and disordered sleep patterns.

**Adaptation session**

An adaptation session should take place approximately 1 week after the screening process. This is because the adaptation session needs to start after confirmation of regular sleep-wake cycles (see below) for about approximately a week. In addition, at least a 1-week interval between the adaptation session and the main experimental session is preferable. This is because daytime naps may temporarily disrupt a normal sleep-wake cycle, so the main sleep session may be disrupted by the negative carry-over effect of the adaptation session if the interval between the adaptation and main sleep sessions is too short (e.g., only 1 day apart). A 1-week interval would be enough to diminish the impact of the adaptation session on a regular sleep-wake cycle.

*© Timing: 1 week*

3. One week before the adaptation session, instruct participants to maintain regular sleep-wake habits
   a. A sleep log; ask subjects to keep their sleep and wake schedule consistent and record their sleep/wake times.
   b. Also, use a wrist actigraph device (GT9X-BT, ActiGraph, Pensacola, FL) to record subjects’ sleep/wake times.

*© Timing: 1 day*

4. On the day before the adaptation session, instruct subjects to refrain from alcohol consumption, unusually excessive physical exercise, and naps.

*© Timing: 120 min*

5. Ask the subjects to sleep inside the MRI bore together with MRS scans and PSG. The procedure is described below. See “Part 1. Main Experiment” for the procedure for simultaneous MRS and PSG acquisition.
**Note:** If a sleep log or an actigraph data indicated that the sleep/wake cycles are irregular (see the criteria in the preconsent eligibility screening above) for the scheduled subject, postpone and reschedule the experiment so that the sleep/wake cycles would be more stable and regular.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited data      | Tamaki et al. (2020b) | https://www.nature.com/articles/s41593-020-0666-y |
| Data generated in the previous study | Tamaki et al. (2020b) | n/a |
| Experimental models: Organisms/strains | Tamaki et al. (2020b) | n/a |
| Human subjects      | Tamaki et al. (2020b) | n/a |
| Software and algorithms | Provencher (1993) and Provencher (2001) | n/a |
| LCmodel             | Brain Products GmbH, Gilching, Germany | n/a |
| Brain Vision Recorder| Brain Products GmbH, Gilching, Germany | n/a |
| Brain Vision Analyzer 2 | Brain Products GmbH, Gilching, Germany | n/a |
| EEGLAB with the FMRIB plug-in | The University of Oxford | n/a |
| Psychomotor vigilance test (PVT) | Dinges and Powell (1985) | n/a |
| Other               | Siemens, Prisma with a 64-channel head coil | n/a |
| MRI 3T              | Siemens automatic shimming routine with manual mode | n/a |
| Shimming            | Siemens, Prisma with a 64-channel head coil | n/a |
| Multielectrodes MRI-compatible PSG cap based on an MRI-compatible EEG cap | Siemens automatic shimming routine with manual mode | n/a |
| An MRI-compatible amplifier | Siemens, Prisma with a 64-channel head coil | n/a |
| An MRI-compatible battery | Siemens automatic shimming routine with manual mode | n/a |
| A wrist actigraphy device | Siemens, Prisma with a 64-channel head coil | n/a |
| Munich Chronotype Questionnaire (MCTQ) | Roenneberg et al. (2003) | n/a |
| Sleep-wake habits questionnaire | Tamaki et al. (2019, 2020a) | n/a |
| Video game questionnaire | Tamaki et al. (2019, 2020a), Berard et al. (2015), and Green and Bavelier (2003) | n/a |
| Morningness-Eveningness questionnaire (MEQ) | Horne and Ostberg (1976) | n/a |
| Pittsburg Sleep Quality Index (PSQI) | Buysse et al. (1989) | n/a |
| Stanford sleepiness scale (SSS) | Hoddes et al. (1972, 1973) | n/a |
| Pillow              | n/a    | n/a |
| Towels              | n/a    | n/a |
| Cotton              | n/a    | n/a |
| Cushions            | n/a    | n/a |
| Gauze               | n/a    | n/a |
| Bouffant cap        | n/a    | n/a |
| Ear plugs           | n/a    | n/a |
| Alcohol bottle (67%)| n/a    | n/a |
| EEG prep pad        | professional disposables int, B59800 | n/a |
| Cotton swabs        | n/a    | n/a |
| EEG gel             | Abralyt HiCl (V19), Easycap | n/a |
| Chest band          | n/a    | n/a |

### MATERIALS AND EQUIPMENT

- MRI 3T (Siemens, Prisma with a 64-channel head coil)
- LCmodel (Provencher, 1993, 2001)
Multielectrodes MRI-compatible PSG cap based on an MRI-compatible EEG cap (32- or 25-channel BrainCap MR with Multitrodes, Brain Products GmbH, Gilching, Germany)

An MRI-compatible amplifier (BrainAmp MR, Brain Products GmbH, Gilching, Germany)

Brain Vision Recorder (Brain Products GmbH, Gilching, Germany)

Brain Vision Analyzer 2 (Brain Products GmbH, Gilching, Germany)

An MRI-compatible battery (PowerPack, Brain Products GmbH, Gilching, Germany)

EEGLAB (The University of Oxford) with the FMRIB plug-in

A wrist actigraphy device

Questionnaires
  - MCTQ (Roenneberg et al., 2003)
  - Sleep-wake habits questionnaire (Tamaki et al., 2019, 2020a)
  - Video game questionnaire (Tamaki et al., 2019, 2020a; Berard et al., 2015; Green and Bavelier, 2003)
  - MEQ (Horne and Ostberg, 1976)
  - PSQI (Buysse et al., 1989)
  - Stanford sleepiness scale (SSS) (Hoddes et al., 1972, 1973)

Psychomotor vigilance test (PVT) (Dinges and Powell, 1985).

Pillow, towels, cotton, gauze, bouffant cap, ear plugs, alcohol bottle (67%), EEG prep pad, cotton swabs EEG gel, and chest band (to stabilize EEG cap) (Figure 2)

**STEP-BY-STEP METHOD DETAILS**

**Part 1: Main experiment. PSG preparation**

© Timing: 120 min

Approximately 1 week after the adaptation session, start the main experiment. Here, we describe the procedures used for the main sleep session in Experiment 1 in the previous study (Tamaki et al., 2020b), in order of the timeline. Subjects were trained for a visual perceptual learning task,
and then simultaneous MRS and PSG experiments were initiated. See our previous study (Tamaki et al., 2020b) for details of the procedures for the visual task.

1. Before the sleep session, there were several behavioral sessions (Figure 3) where a standard texture discrimination task (TDT) was used.

2. Prior to test sessions, sleepiness was measured both by the Stanford sleepiness scale (SSS) (Hoddes et al., 1972, 1973) and psychomotor vigilance test (PVT) (Dinges and Powell, 1985). Sleepiness tests were used to test whether performance and sleepiness were correlated. Thus, PVT and SSS were administered 4 times in total.
   a. The SSS was completed on a computer, coded in MATLAB.
   b. The PVT was performed on a computer and was implemented with open-source Psychology Experiment Building Language (PEBL) software (Dinges and Powell, 1985).

3. After Test #2, the electrodes for PSG were attached to the subjects, using an MRI-compatible PSG cap (Figure 1). When a 32-channel PSG cap was used, 23 electrodes were used for EEG, 2 electrodes were used for horizontal EOG, 2 electrodes were used for vertical left EOG, 2 electrodes were used for vertical right EOG, 2 electrodes were used for EMG (the mentum), and 1 electrode was used for ECG to the lower shoulder blade. The electrode configurations were customized for our study. See Troubleshooting 1.
   a. EEG and ECG signals were referenced to the Fz electrode. Electrode impedances were kept at or below approximately 5 kΩ for EEG and ECG
   b. The ground electrode was placed at AF4. Electrode impedances were kept approximately at 10 kΩ for EOG and EMG
   c. All data were recorded using an MRI-compatible amplifier (BrainAmp MR, Brain Products GmbH, Gilching, Germany), an MRI-compatible battery (Powerpack, Brain Products GmbH, Gilching, Germany) and a recording software (BrainVision Recorder, Brain Products GmbH, Gilching, Germany) at a sampling rate of 5000 Hz.

Note: The electrodes for the reference and ground were placed in the MRI-compatible PSG cap. Avoid the Cz position referencing or grounding because it is easy for it to become detached from the head.

Note: In our experience, the good impedance level of electrodes remains for 2 h. Thus, we recommend to make sure that all the electrodes are within the acceptable levels of impedance before the MRS scan starts. Once the concurrent MRS and PSG scan starts, it is impossible to check or fix impedance levels of electrodes, unless the concurrent MRS and PSG scan is stopped. This is because the PSG recording software needs to be switched to the impedance measurement mode. If PSG is stopped, we would also have to stop the MRS scan, otherwise the MRS scan would occur without concurrent PSG recording.

Part 2. Instruction regarding when to sleep

© Timing: 5 min
This part started after electrodes were attached but before subjects entered the MRI gantry.

**Note:** Instructions regarding sleeping are important. In our design, turning the lights off served as a signal for the start of the sleep session. Thus, we needed to convey this information to subjects. However, at the same time, we do not want subjects to feel pressured to sleep, as such obligatory feelings may actually bother sleep onset.

4. Instruct the subjects when to sleep.
   a. Tell the subjects that they should feel free to fall asleep after lights are off following completion of PSG preparation.
   b. Tell the subjects to remain awake as much as possible before lights-off even if they felt sleepy because the period of wakefulness is required to be measured by MRS for subsequent normalization. In addition, in this way, the vigilance level at the start of the sleep session could be consistent across subjects. See Troubleshooting 2.

△ **CRITICAL:** We did not give strong or definitive instructions in a way that may have caused the subjects to feel pressured. Do not use demanding instructions, including “you need to fall asleep”, “please sleep deeply” or “we need high-quality sleep data”.

**Part 3: A sleep session with repetitive MRS scans**

- **Timing:** 120 min

  **Note:** A sleep (nap) session was conducted in the early afternoon. Subjects were scanned using a 3T Siemens Prisma scanner (Siemens) with a 64-channel head coil.

Part 3 describes the procedure after subjects entered the MRI room, with the PSG cap placed on their heads. See Troubleshooting 3.

5. Place the subject on the patient table of the MRI scanner.
   a. Have the subject lay down with their heads fixed with thin pillows/gauze.

  **Note:** The starting time of the sleep session was fixed in the early afternoon (around 1–2 pm) across subjects. We recruited subjects who were not extreme morning or evening type and wake up at a consistent time regularly, based on information reflected in questionnaires including the MCTQ.

△ **CRITICAL:** The electrodes placed around the occipital region may hurt the participants as the scanning time goes by, due to the pressure from the occipital electrodes to the inion. It is important for subjects to sleep without discomfort and head motion during the MRI measurements. Thus, good protection around the back of the head is key for successful long scans during sleep sessions. Place cotton around the occipital electrodes so that the head weight would not be concentrated on these particular electrodes.

   b. Stabilize subjects’ heads with cushions and gauze, and ensure that there would be no space left between subjects’ heads and the head coil to reduce head motion.
   c. Use a thin back cushion and knee cushion upon the subjects’ request. Use several blankets to keep the subjects warm and to initiate sleep during the scan.

△ **CRITICAL:** Instructions to keep subjects’ head still throughout the scan are important. Unlike postprocessing for functional magnetic resonance imaging, motion correction was not possible for MRS data analysis. This lack of motion correction posed the possibility that the data would be unusable when a large amount of head motion occurred. Thus, careful instructions for participants to keep their heads still is extremely important. Some of the
instructions we provide informed participants that the data may not be usable if they moved their heads even slightly to the left or right.

**Note:** Head motion is a serious problem which leads to unusable data. It may be a good idea to add a mock-scanner training to reduce head motion by giving feedback to subjects when head motion is detected (Epstein et al., 2007) prior to the sleep experiments with simultaneous MRS and PSG. However, the mock-scanner training may increase the alertness of the subjects and make it more difficult for them to fall asleep. Thus, we would need to investigate whether the mock-scanner training impacts the quality of sleep. Another possibility for motion correction for PSG recording taken in the MRI environment may be to use a carbon-wire loop based artifact correction (van der Meer et al., 2016).

△ **CRITICAL:** The electrode cords must not form loops, as this could have led to heating and damage to the electrodes.

6. After moving a subject to the inside of the MRI bore, connect the PSG cap to the MRI-compatible amplifier via the flat cable inside the MRI bore (Figure 4).
   a. Pull the bundle of electrodes from the PSG cap out from the hollow of the head coil.
   b. Lay out the bundle of electrodes and the flat cable as straight as possible to reduce the artifacts in the PSG data.
   c. Place the MRI-compatible amplifier on a stack of paper in the MRI bore (just outside the head coil) so that the height of the flat cable from the amplifier would be at the same height as the bundle of electrodes.
   d. Place the MRI-safe battery for the amplifier above the amplifier.
   e. Place a heavy sandbag on the MRI-safe battery to stabilize the tower of amplifier and battery.
   f. Place another stack of paper on to the plastic case that was on a folded bed sheet to lift up the line of the electrode bundle, the connector and the flat cable and to stabilize the plastic case.
   g. Stabilize the bundle of electrodes further by pieces of surgical tape attached to the stack of paper.

7. Provide instructions again, while the experimenter is looking the subjects in the eyes through a mirror attached to the coil, reaffirming that it is important that participants keep their heads still and reiterating the meaning of lights being turned off.
a. Examples of instructions: “How are you? This is the last moment you can move, so please try to find a position where you find comfortable without moving your head. I’m going to check the PSG recording; I’ll be right back.”

8. Test the impedance value of each electrode again, as placing and fixing the subjects’ heads in the head coil might move the electrodes.
   a. If the impedance of electrodes is all OK, give the instructions regarding the lights being turned off and keeping their heads still. For example, “Everything looks fine, so we will start the scan shortly. During the scan please try not to move as much as possible. When we start the main scan, we will turn the lights off, and this is the signal for you to take a nap. Do you have any questions? When you have a question or when you want to talk to us, please do not hesitate to squeeze the ball. Have a great nap!”
   b. If an unacceptable amount of impedance is noted, the subject should be pulled off from the bore, and the problematic electrodes should be fixed. Most of the time, the problem is that an electrode is slightly shifted or moved up. Adding more EEG gel and fixing the electrode by a piece of surgical tape suffices.

9. MRI procedures before MRS
   a. Scout: quick anatomical scan (one slice each for sagittal, horizontal and transverse orientations; slice thickness 7 mm, TR = 8.6 ms, TE = 4.0 ms, flip angle 20°, FoV = 280 mm).
   b. Collect high-resolution anatomical images by an MPRAGE sequence (256 slices, voxel size = 1 × 1 × 1 mm, 0 mm slice gap, TR = 1900 ms, TE = 3.02 ms, flip angle = 9°, FoV = 256 mm) and use them for localization of the voxel of interest (VOI) for the subsequent MRS sequences.
   c. VOI placement: Looking at the MPRAGE images carefully, manually place the VOI on the most posterior part of the occipital lobe, covering the calcarine sulci that correspond to early visual areas bilaterally (Shibata et al., 2017) (Figure 5). Carefully place the VOI to include the least amount of white matter, as lipids in the white matter may alter the shape of the spectra.
   d. Shimming: use a vendor-provided automated tool (defined by the full width at half maximum of the water peak). See Troubleshooting 3.

10. MRS
   a. Conduct a quick MEGA-PRESS sequence test (TR = 1.25 s, TE = 68 ms, 32 spectral averages, for edit-on and edit-off) to check whether the spectrum is acceptable, using the LCmodel (Provencher, 1993, 2001).
b. Acquire an unsuppressed water spectrum (TR = 1.25 s, TE = 68 ms, 16 spectral averages) as a standard water concentration reference for single-voxel proton MRS (Klose, 1990; Oeltzschner et al., 2016; Gasparovic et al., 2006).

c. Up until this point, the procedure takes approximately 20–30 min. Since we had a 2 h-MRI slot, we would have remained 90–100 min for a sleep session.

d. When ready to start the sleep session, tell the subjects “Have a good nap!”, which is meant to be the start of the sleep session.

e. Then, turn off the lights and started MRS scans. For the sleep session, run the 5-s dummy scans for the steady state of longitudinal magnetization and 10-min water-suppressed MEGA-PRESS sequence (TR = 1.25 s, TE = 68 ms, 240 spectral averages for each edit-on and edit-off, and VOI = 2.2 × 2.2 × 2.2 cm³) repeatedly until the sleep session is over (approximately 9–10 repetitions).

f. Stop the MRS scan when 9 or 10 times of MRS are conducted just before the 2 h MRI slot is over.

Note: The MEGA-PRESS sequence (Mescher et al., 1998; Edden and Barker, 2007; Hu et al., 2013) was employed for J-difference editing of the 3.0 ppm GABA resonance. We used this sequence because MEGA-PRESS allowed us to measure concentrations of both GABA and Glx simultaneously from the VOI. Thus, the concentrations of GABA and Glx were acquired from the same scan during the same sleep stages, according to the procedure used in previous studies (Muthukumaraswamy et al., 2009; Stagg et al., 2009, 2011, 2014; Robertson et al., 2016; Henry et al., 2011). In contrast, in a previous study (Shibata et al., 2017), a PRESS sequence was used for measurement of glutamate concentration measurements, and a MEGA-PRESS sequence was used for GABA measurements.

Note: In the study (Tamaki et al., 2020b), we used the Glx signal, which is a combined signal from glutamate with glutamine, as an excitatory neurotransmitter. This is because clear dissociation between the glutamate and glutamine may be difficult with a 3T machine. It is estimated that the contribution of glutamine to Glx is less than 15% (Floyer-Lea et al., 2006), so that the Glx mostly represents glutamate.

Note: In the MEGA-PRESS sequence, we used a shorter TR and a larger VOI size than a previous study (Shibata et al., 2017) to increase the signal-to-noise ratios.

Optional: In our study, because we wanted to test the role of REM sleep in visual perceptual learning, we had an NREM sleep-only condition without REM sleep, in which subjects were woken up before REM sleep started when the PSG display suggested that NREM sleep was ending and stopped the sleep session. The amount of artifacts that contaminated onto the online PSG data with MRS was smaller than the functional MRI scanner artifacts. Thus, while the online PSG display was noisier than the normal PSG data taken at a normal earthly magnetic field, some of PSGs were still able to be readable, especially between TRs (1.25 s) of the MEGA-PRESS sequence. Based on the online PSG, we terminated the sleep session when sleep stage N1 or N2 reappeared following stage N3 after NREM sleep lasted more than 40 min and when PSG showed desynchronized EEG activity and decreased EMG activity, a sign that indicates the end of NREM sleep and the start of REM sleep.

Part 4: Postprocessing. First PSG segmentation by 10-min MRS scans

© Timing: 120 min

11. Segmenting PSG by the start of MRS recording
a. It is very important to locate the starting point of each MRS recording on the PSG data chronologically. The scanner artifacts caused by MRS is easily detected in the PSG data. This
allows us to align the start of each MRS measurement on the PSG recording. Since the most of simultaneous MRS and PSG sessions takes approximately for 90 min, there will be 9 times of starting point of MRS on the PSG data. The duration of the sleep session was 90 min in our study (Tamaki et al., 2020b), because the duration of one sleep cycle which includes NREM sleep and REM sleep is estimated as 90 min in human (Rechtschaffen and Kales, 1968). In our study (Tamaki et al., 2020b), the average (± SEM) time spent in NREM sleep was 43.7 ± 3.3 min, and that in REM sleep was 5.8 ± 1.3 min during the 90-min sleep session.

b. Omit PSG data that correspond between MRS scans for later coregistration.
c. Because of these intervals between MRS scans, the artifact removal process described below works better after PSG was segmented.

12. PSG recordings taken inside the MRI bore are contaminated by two types of artifacts: scanner and ballistocardiogram artifacts. Both types of artifacts were removed in the postprocessing phase in our study (Tamaki et al., 2020b).

a. Remove scanner artifacts using Brain Vision Analyzer 2 (Brain Products GmbH, Gilching, Germany) by subtracting an averaged artifact waveform, followed by adaptive noise cancellation to reduce any residual artifact (Allen et al., 2000). The steps taken in Brain Vision Analyzer 2 for scanner artifact removal with MRS are slightly different from those with functional magnetic resonance imaging (fMRI). This is because trigger information that is synchronized with volume acquisition is available in fMRI, but not with MRS. Take the following three steps:
   i. Use a “detection method” in Brain Vision Analyzer 2 to detect the scanner artifacts in the PSG recording. Because scanner artifacts in PSG recording appear with a much larger amplitude than artifacts induced by spontaneous oscillatory activity, the detection method utilizes the peaks of scanner artifacts.
   ii. After selecting all the channels for artifact removal, select “continuous” for the scan type in Brain Vision Analyzer 2, since the MRS scans are carried out continuously.
   iii. After the baseline correction, reduce the sampling rate to 250 Hz for the next step for a ballistocardiogram artifact removal. While EEG data are usually recorded with high sampling frequency (e.g., 5000 Hz), it would take extremely long time for a ballistocardiogram artifact removal with such a large data size. The reduced sampling size (250 Hz) is sufficient for the ballistocardiogram artifact removal. Apply a low-pass filter at 100 Hz.

b. Remove ballistocardiogram artifacts by detecting heart-beat events and subtracting an artifact template from the PSG data processed by EEGLAB (The University of Oxford) with the FMRIB plug-in. Brain Vision Analyzer 2 could also be used.

13. Subsequently, rereference EEG data to TP9 and TP10 using EEGLAB for sleep stage scoring.

14. Sleep stage scoring for each PSG segment according to these studies (Rechtschaffen and Kales, 1968; Iber et al., 2007).

a. Each PSG segment should be approximately 10-min long. One sleep stage is assigned for a 30-s epoch. Assign one of sleep stages (wakefulness, N1, N2, N3, REM sleep, and artifacts, which is unsuccessful denoising that precluded sleep scoring).

Note: The timing for the lights off should be identified in the PSG data.

Note: We also conducted frequency analysis on the EEG data in a previous study (Tamaki et al., 2020b). Please see the previous study for details about the frequency analysis.

Note: The FMRIB plug-in processes data based on triggers synchronized with volume acquisition. However, because there was no trigger information available with MRS, the FMRIB plug-in was not used for the removal of scanner artifacts in our analysis.

Part 5: Postprocessing. How to assign sleep stages to MRS data, including decision-making guidance for further segmentation in a 10-min segment

© Timing: 15 min
After PSG segmentation, assign one of the sleep stages to the MRS data. Here, we explain the procedure when there are 9 MRS recordings during the sleep session.

15. Identify the 10-min segment of PSG that correspond to one MRS scan. There will be 9 segments that are of 10 min each.
   a. As mentioned above, on PSG data, identify the start of each MRS scan, which lasts approximately 10 min. If there are 9 times of MRS scans, there should be 8 time-intervals (up to 10 s) between each MRS scan on the PSG data, as each MRS is manually started. Do not use these PSG data corresponding to the intervals of MRS. One MRS scan and one PSG segment are the same length (approximately 10 min).

16. Combine sleep stages N1, N2 and N3 together and label them as “NREM sleep” in each PSG segment, after sleep stage scoring (as mentioned above).
   a. In total, we classify sleep stages into 4 categories: NREM sleep, REM sleep, wakefulness and artifacts (which is not wakefulness, NREM sleep or REM sleep).

17. Follow the guidelines below for the decision whether to consider a whole 10-min segment as one segment or to further split the segment into multiple segments.
   a. Do not further split the segment in the following two cases.
      i. One 10-min MRS scan corresponds to a single sleep stage (wakefulness, NREM sleep, or REM sleep)
      ii. More than 80% of the 10-min MRS scan is dominated by one of the sleep stages.
      iii. Coregister one sleep stage label to a 10-min MRS segment.
   b. Perform further segmentation of 10-min MRS data when one 10-min MRS scan corresponds to multiple sleep stage labels.
      i. When none of the wakefulness, NREM sleep, or REM sleep states in a 10-min PSG segment exceeds 80% of the segment, split one 10-min MRS scan into five 2-min MRS segments using the raw data (twix files).
      ii. Split the 10-min PSG segment that is originally coupled with the 10-min MRS segment into five 2-min PSG segments. Thus, each 2-min MRS segment is coupled with its corresponding 2-min PSG segment.
      iii. For each 2-min MRS segment, register the mode (the most frequent) sleep stage. Note that there should be 4 (each 30-s) sleep stages in the 2-min PSG segment.
      iv. If the same number of different sleep stages is observed in a 2-min PSG segment, follow the identification hierarchy: wakefulness > NREM sleep > REM sleep. For instance, if there were 2 epochs of wakefulness and 2 epochs of REM sleep, label the segment wakefulness.
      v. This hierarchy is implemented for the following two purposes. The first purpose is to remove any MRS segments containing a wakefulness stage from MRS segments identified during sleep stages. This reduces possible contamination of the MRS data for a sleep state with that for a wakefulness state. The second purpose is to obtain MRS data for REM sleep with a reduced possibility of contamination by an NREM sleep state.
      vi. Remove an MRS segment from further analyses if either the number of PSG segments contains artifacts or the number of epochs shows arousal.
      vii. This way you classify each MRS segment (2-min or 10-min) as either wakefulness, NREM sleep or REM sleep.

Note: Splitting data into five 2-min segmentations may occur in the first part of sleep due to the shift from wakefulness to sleep onset then in the later phase of sleep due to the shift from NREM sleep to REM sleep.

Part 6: Postprocessing. Calculation of the mean E/I balance for each of NREM sleep and REM sleep

© Timing: 15 min
Start this part after completion of coregistration between MRS and PSG segmentation. Here, we explain how to obtain the E/I balance for NREM sleep and REM sleep when there are 9 MRS scans, and the two out of nine 10-min MRS-PSG segments are split into five 2-min segments during the sleep session. Thus, the example contains seven 10-min and ten 2-min MRS-PSG segments, for a total of 17 MRS-PSG segments. Assume that there are 5 MRS-PSG segments labeled as wakefulness, 8 as NREM sleep and 4 as REM sleep.

18. For each MRS-PSG segment (2 min or 10 min), using the LCmodel, obtain the concentrations of NAA, Glx, and GABA. Use NAA as a reference metabolite.
   a. Divide the amounts of Glx and GABA by the amount of NAA. The results refer to as the concentrations of Glx and GABA, respectively. Although NAA would be canceled out when measuring the E/I balance (see b next), this step is necessary to measure the concentrations of Glx and GABA.
   b. Calculate the E/I balance by dividing the concentration of Glx by GABA for each of 17 MRS-PSG segments.

19. Calculate the baseline E/I balance by averaging the E/I balance for MRS-PSG segments labeled as wakefulness (in this example, using 5 MRS-PSG segments).
20. In each of the remaining 12 MRS-PSG segments, calculate the E/I balance by normalizing to the baseline.
   a. \( \frac{E/I_{\text{segment}} - E/I_{\text{baseline}}}{E/I_{\text{baseline}}} \)

21. Obtain the mean E/I balance for NREM sleep by averaging the normalized E/I balance across 8 MRS-PSG segments labeled NREM sleep.
22. Obtain the mean E/I balance for REM sleep by averaging the normalized E/I balance across 4 MRS-PSG segments labeled REM sleep.

**Note:** While creatine has sometimes been used as a reference metabolite in other MRS studies, we used NAA as the reference metabolite. NAA was chosen because the creatine singlet cancels out in the final spectra in the MEGA-PRESS sequence. At any rate, in the calculation of E/I balances, the contribution of the reference metabolite is canceled out.

**Note:** An alternative way is to average the MRS data from all the segments for each of sleep stages first and then using the LCmodel to obtain the concentrations of Glx and GABA.

**Note:** In the calculation of the E/I balance, the weighted average based on the duration is another option.

**EXPECTED OUTCOMES**

In our previous study, we found that after visual training, the E/I balance increased during NREM sleep and decreased during REM sleep. In contrast, when there was no learning prior to the sleep session, while the E/I balance increased during NREM sleep, the E/I balance during REM sleep did not decrease. Thus, the impact of learning prior to the sleep session was larger for REM sleep than NREM sleep. Please see Figure 2 in the previous study (Tamaki et al., 2020b). See Troubleshooting 5.

**LIMITATIONS**

The spatiotemporal resolution of MRS is limited. First, we obtained MRS signals on the order of minutes (e.g., 2 or 10 min). Much faster metabolic changes are difficult to capture. Second, we used the 2.2 cm cubic size for the VOI. Much smaller volumes may be problematic for obtaining robust MRS signals. Research that requires MRS measurements on a smaller resolution may need to use a scanner with larger power than 3T. Third, the present simultaneous MRS and PSG recordings allowed us to collect MRS data from only one VOI. Thus, it was difficult to study interactions between several brain regions during sleep at the same time. However, there are techniques that allow us to collect MRS data from multiple regions (for instance, magnetic resonance spectroscopic imaging (Duyn et al., 1993)). Future technical developments are necessary to incorporate these techniques for simultaneous MRS and PSG experiments.
Another limitation may be about the duration of experiments. It was time and labor consuming to perform a simultaneous MRS and PSG experiments, as electrodes preparation takes time. This also increases the entire experimental time, which could make subjects tired.

**TROUBLESHOOTING**

**Problem 1**
Participants need to be able to wear an MRI-compatible PSG cap properly. It is difficult to predict exactly how long it takes to obtain acceptable electrode impedance values, because individuals’ properties of hair (including hair thickness) and skin may interfere with and increase the impedance values (step 3).

**Potential solution**
It is a good idea to schedule experiments and allow sufficient time, as the time needed for PSG preparation may vary. Hair conditioner and hair creams used in participants’ hair would make it difficult to lower the impedance values. Thus, we state that it would save the preparation time if their hair was dried without hair conditioner and hair creams before the experiment. In addition, some of the people who have very sensitive skins may not be suitable for participants, because alcohol used during PSG preparation may be too stimulating.

**Problem 2**
Sleeping inside an MRI bore may be a novel, unusual or awkward experience for a subject (Adaptation session).

**Potential solution**
First, introducing an adaptation session is effective for subjects to become accustomed to the setting. Second, we provided the subjects with enough information about MRI and EEG processes in details in advance to alleviate any anxiety the subjects may have had. At the same time, we did not put any pressure on subjects regarding their obligatory sleep (e.g., subjects needed to exhibit a good sleep) because such pressure might increase the subjects’ alertness.

**Problem 3**
An electrode at the Inion may become painful for a subject (step 5).

**Potential solution**
When this happened, we pulled out the subject from the bore and readjusted the pillow or cotton around the electrode so that the head weight would not concentrate on the electrode. For instance, placing more cotton around the electrode fixed by a piece of surgical tape often worked.

**Problem 4**
How do we know that the quality of the MRS data is acceptable? (step 10-a)

**Potential solution**
Immediately after the shimming value turned out to be acceptable (below 20 Hz) (Zeinali-Rafsanjani et al., 2018), we conducted a quick MRS measurement before the main sleep session. We also quickly analyzed the MRS data using the LCModel to double check that the quality of the MRS signal was acceptable. This was done by checking the Camer-Rao lower bounds (or %SDs). %SD shows a measure of fitting error, thus, the lower the better. %SD smaller than 20 is recognized as an acceptable fit (Kreis, 2004). If the MRS quality was not acceptable, we repositioned the VOI (e.g., slightly shifted the position of VOI) and performed shimming again before starting the main sleep session. If the VOI contained a large amount of white matter, cerebrospinal fluid, skull, or cavities of the brain, it would worsen the signal quality of MRS. After the scan, we checked the quality of the MRS data in the following two manners. First, we measured the mean full-width-at-half-maximum linewidth for NAA (NAA linewidth), which determines the resolution available to discern spectral features, thus,
lower the better (Shibata et al., 2017; Robertson et al., 2016). The NAA linewidth was around or below 10 Hz in previous studies (Shibata et al., 2017; Robertson et al., 2016). In our study, it was below 10 Hz (Tamaki et al., 2020b). Second, we measured the frequency drift which indicates head motion, thus, lower the better (Shibata et al., 2017; Robertson et al., 2016). The frequency drift was around 1 Hz in previous studies (Shibata et al., 2017; Robertson et al., 2016) and in our study (Tamaki et al., 2020b).

Problem 5
It is often the case that sleep inertia occurs immediately after a sleep session (step 10-f).

Potential solution
When there is a behavior session scheduled after the sleep session, it is a good idea to have a break for approximately 30 min and pay attention to subjects’ sleepiness. Otherwise, sleepiness caused by sleep inertia may interfere with the performance of behavior measures. We had the subjects wash their hair to remove the EEG gel during the break to disperse sleep inertia.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuka Sasaki (yuka_sasaki@brown.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The data generated in the previous study are available at the following site. https://www.nature.com/articles/s41593-020-0666-y

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AUTHOR CONTRIBUTIONS
M.T., T.W., and Y.S. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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STAR Protocols
Protocol

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