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The Editors
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October 24th, 2018

Dear Editors.

Thank you for your letter dated July 31st, 2018 about your decision on our article, 58613_R0_062518 “Optogenetic manipulation of neural circuits during monitoring sleep/wakefulness states in mice”. We found the reviewers’ comments to be constructive and helpful to improve our manuscript.

I am pleased to resubmit our revised manuscript, which, we believe, is totally improved over the previous version. We have addressed all of reviewers’ concerns.

We attach a point-by-point response to the reviewers' specific concerns, and our changes to the previously submitted version of the text are mentioned. Thank you for your consideration of our revised manuscript and patience for revision process. We hope that our revised version of manuscript could be suitable for publication in JOVE.

Sincerely yours,

Takeshi Sakurai, MD, PhD

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TITLE:

Optogenetic Manipulation of Neural Circuits During Monitoring Sleep/wakefulness States in Mice

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KEYWORDS:

Neuroscience, Optogenetics, Sleep recording, EEG/EMG, NREM sleep, REM sleep, Sleep/wakefulness states

SUMMARY:

Here, we describe methods of optogenetic manipulation of particular types of neurons during monitoring of sleep/wakefulness states in mice, presenting our recent work on the bed nucleus of the stria terminalis as an example.

ABSTRACT:

In recent years, optogenetics has been widely used in many fields of neuroscientific research. In many cases, an opsin, such as channel rhodopsin 2 (ChR2), is expressed by a virus vector in a particular type of neuronal cell in various Cre-driver mice. Activation of these opsins is triggered by application of light pulses which are delivered by laser or LED through optic cables, and the effect of activation is observed with very high time resolution. Experimenters are able to acutely stimulate neurons while monitoring behavior or another physiological outcome in mice. Optogenetics can enable useful strategies to evaluate function of neuronal circuits in the regulation of sleep/wakefulness states in mice. Here we describe a technique for examining the effect of optogenetic manipulation of neurons with a specific chemical identity during electroencephalogram (EEG) and electromyogram (EMG) monitoring to evaluate the sleep stage of mice. As an example, we describe manipulation of GABAergic neurons in the bed nucleus of the stria terminalis (BNST). Acute optogenetic excitation of these neurons triggers a rapid

transition to wakefulness when applied during NREM sleep. Optogenetic manipulation along with EEG/EMG recording can be applied to decipher the neuronal circuits that regulate sleep/wakefulness states.

INTRODUCTION:

Sleep is essential for optimal cognitive function. Recent findings also suggest that disturbances in sleep are associated with a wide range of diseases[1–3]. Although the functions of sleep are as yet largely unresolved, substantial progress has been made recently in understanding the neural circuits and mechanisms that control sleep/wakefulness states[4]. In mammals, there are three states of vigilance: wakefulness, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep. Wakefulness is characterized by fast EEG oscillations (5–12 Hz) of low amplitude with purposeful and sustained motor activity. NREM sleep is defined by slow oscillations (1–4 Hz) of high amplitude (delta waves), with lack of consciousness and purposeful motor activity. REM sleep is characterized by relatively fast oscillations (6–12 Hz) of low amplitude and almost complete bilateral muscle atonia[5].

Borbely proposed a theory of sleep-wakefulness regulation known as the two process model[6, 7]. A homeostatic process, also referred to as process S, represents sleep pressure that accumulates during wakefulness and dissipates during sleep. Another process, referred to as process C, is a circadian process, which explains why vigilance levels fluctuate in the 24-hour cycle. In addition to these two processes, allostatic factors are also important for regulation of sleep/wakefulness[8,9]. Allostatic factors include nutritional state and emotion. Fear and anxiety are usually accompanied by an increase in arousal along with autonomic and neuroendocrine responses[10–12]. The limbic system is believed to play a role in regulation of fear and anxiety, and mechanisms underlying autonomic and neuroendocrine responses have been studied extensively, but the pathway by which the limbic system influences sleep/wakefulness states has not yet been revealed. A large number of recent studies using opto- and pharmacogenetics have suggested that neurons and neuronal circuits that regulate sleep/wakefulness states are distributed throughout the brain, including the cortices, basal forebrain, thalamus, hypothalamus, and brain stem. In particular, recent advances in optogenetics have allowed us to stimulate or inhibit specific neural circuits *in vivo* with high spatial and temporal resolution. This technique will allow progress in our understanding of the neural substrates of sleep and wakefulness, and how sleep/wakefulness states are regulated by circadian processes, sleep pressure, and allostatic factors, including emotion. This paper aims to introduce how to use optogenetic manipulation combined with sleep/wake recording, which could have the potential to update our understanding of the connectomes and mechanisms in the brain that play a role in the regulation of NREM sleep, REM sleep, and wakefulness. Understanding of this mechanism by which the limbic system regulates sleep/wakefulness states is of paramount importance to health, because insomnia is usually associated with anxiety or fear of being unable to sleep (somniphobia).

The BNST is thought to play an essential role in anxiety and fear. *GAD 67*-expressing GABAergic neurons are a major population of the BNST[12,13]**.** We examined the effect of optogenetic manipulation of these neurons ($GABA^{BNST}$) on sleep/wakefulness states**.** One of the greatest advances in neuroscience in recent years has been methods that enable manipulation of neurons

with particular chemical identities *in vivo*, with high spatial and temporal resolution. Optogenetics is highly useful for demonstrating causal links between neural activity and specific behavioral responses[14]. We describe optogenetics as a method to examine the functional connectivity of defined neural circuits in the regulation of sleep/wakefulness states. By utilizing this technique, great progress has been achieved in understanding the neuronal circuits that regulate sleep/wakefulness states[15–19]. In many cases, opsins are specifically introduced into neurons with particular chemical identities in selective brain regions by a combination of Cre-driver mice and Cre-inducible AAV-mediated gene transfer. Further, focal expression of photo-sensitive opsins such as channelrhodopsin 2 (ChR2)[20] or archaerhodopsin (ArchT)[21] combined with a Cre-loxP or Flp-FRT system allows us to manipulate a selective neuronal population and specific neural pathway[22].

We describe here experiments on GABAergic neurons in the BNST as an example. To express opsins in a designated neuronal population, appropriate Cre driver mice and Cre-dependent virus vectors are most frequently used. Transgenic or knock-in lines in which opsins are expressed in particular neuronal populations are also useful. In the following experiments, we used *GAD67-Cre* knock-in mice[23] in which only GABAergic neurons express Cre recombinase with a C57BL/6J genetic background, and an AAV vector which contains ChR2 (hChR2 H134R) fused with EYFP or EYFP as a control with a "FLEx (Flip-excision) switch"[24]**.** The procedure specifically describes optogenetic excitation of GABAergic neurons in the BNST during monitoring of sleep/wakefulness states[25].

PROTOCOL:

All experiments here were approved by the Animal Experiment and Use Committee of the University of Tsukuba, complying with NIH guidelines.

1. Animal Surgery, Virus Injection, Electrode for EEG/EMG, and Optical Fiber Implantation

CAUTION: Appropriate protection and handling techniques should be selected based on the biosafety level of the virus to be used. AAV should be used in an isolated P1A graded room for injection, and the tube carrying AAV must be sterilized with an autoclave after all the volume is used up.

NOTE: See **Figure 1.**

1.1. Disinfect the surgical equipment with the autoclave.

1.2. Anesthetize mice with isoflurane using an anesthetic vaporizer. Observe until the mouse has reached the desired depth of anesthesia, determined by loss of response to pinching the tail with forceps. Apply ophthalmic ointment to the eyes to prevent them drying.

1.3. Disinfect the surgical field with iodine solution or 70% EtOH and dry sufficiently. Allow the virus to thaw on ice as the surgery is being performed. Cover the surgical area with absorbent lab

bench paper.

1.4. Fix the mouse's head in the stereotactic apparatus with ear bars and a nose pinch. After confirming the head is held stably, make a midsagittal incision in the scalp to ensure the positions of the bregma and lambda are located at the same level on a horizontal line.

1.5. To avoid a positioning gap, appropriately adjust the levels of the nose pinch and ear bars up and down. The bregma and lambda refer to the intersection between the sutura saggitalis and sutura coronalis or sutura lambdoidal, respectively (**Figure 2**).

1.6. Use Serafin clamps to hold the skin to maintain access to the cranium. After exposure of the skull, disinfect the surface of the skull with iodine or 5% H_2O_2, to enable the cranial sutures including the bregma and lambda to be visualized more clearly.

1.7. Prepare AAV vector injection:

1.7.1. Wash the inside of a 10 mL syringe (see **Table of Materials**) sequentially with 70% EtOH, 100% EtOH, and sterilized water, 5 times each. Secure the syringe in the clamp of a microinjection pump arm and make sure all solution in the syringe is discharged.

1.7.2. Carefully aspirate 2 µL of mineral oil without air bubbles, then aspirate the designated volume of the virus solution. After aspiration, manipulate the plunger button and confirm that virus solution emerges at the tip of the needle.

NOTE: The injection volume of the virus solution was determined in pilot experiments using the same mouse strain and same virus product. The relation between the volume of virus solution and extent of the infection area should be estimated in advance.

1.8. Inject AAV vector:

1.8.1. Adjust the tip of the microinjection needle on the bregma and note the coordinates as the original point. Move the tip to the designated injection site (for the BNST: anteroposterior + 0.2 mm, mediolateral ± 1.0 mm, dorsoventral - 4.2 mm) and place the tip of the needle onto the position. Put a mark on the skull and drill holes of approximately 2 mm in diameter using a dental drill with a 0.7 mm carbide cutter. Be careful not to damage the dura or brain tissue.

1.8.2. After removing blood from around the holes with a cotton swab, slowly move the needle into the position of the BNST. Slowly inject the designated amount of virus solution (0.07 µl/min) with a mechanical microinjector. After completing the injection, leave the needle for 5 min to allow the solution to sufficiently infiltrate the BNST tissue. Carefully take out the needle.

1.8.3. For bilateral injection, repeat steps 1.8.1-1.8.2 on the other side. Throughout the procedure, keep the skull moist with applications of sterile saline.

NOTE: We used custom EEG/EMG implants (W: 5 mm, D: 7 mm, H: 1 mm) with four EEG electrodes (4 mm), two EMG electrodes (2 mm; cut the 4 mm electrode to 2 mm with nippers) and 6 electrodes (4.5 mm) (**Figure 2A**).

1.9. Solder two stainless steel wires (see **Table of Materials**) from which 1 mm of the insulation is stripped off both ends to the EMG electrodes. Adjust the center of the electrodes to the bregma and mark the position of each EEG electrode (anteroposterior ± 1.5 mm, mediolateral ± 1.0 mm), and determine the position of the implant (**Figure 2B**).

1.10. Implant optical fibers:

1.10.1. Attach an optic fiber ferrule to the manipulator and rotate the manipulator arm so that it has an angle of ±30° against a horizontal line (this process is only needed to avoid interference between electrodes and optic fibers, such as in the case of BNST stimulation). Put the fiber tip on the bregma and record the coordinates.

1.10.2. Move the tip to the targeted insertion line and mark the position on the skull. Also put additional marks near the insertion site for the anchor screws. Drill the skull on each site with a dental drill to insert the optic fiber and fix the screw. Fix the screw on the skull. Be careful not to break the dura or damage any tissue by screw.

1.10.3. Insert the optic fiber gently until reaching above the BNST with a manipulator. The ferrule should rest on the remaining cranium (**Figure 1B**).

1.10.4. Apply photocurable dental cement (see **Table of Materials**) to cover the fiber and the screw. The reaction time to solidify the glue should be specified by the manufacturer's manual (Our material needs exposure to light for at least 10 s with specific wave length photo-generator. It is unnecessary to dry the glue after this).

1.10.5. In this step, make sure that no materials (screw or glue) occupy the mounting space for the electrodes. In addition, avoid making any interruption in the cement for the ferrule connecting to the optic fiber and cable. Repeat steps 1.10.1-1.10.4 on the opposite side for bilateral stimulation.

1.11. Drill holes for EEG/EMG electrodes. Insert the tips of the electrodes into the holes. Hold the implant and apply cyanoacrylate adhesive to the space between the skull and the electrodes. Insert again with attention not to interfere with any materials.

1.12. Cover the circumference of the electrodes and optic fibers with cyanoacrylate adhesive followed by application of cyanoacrylate accelerant on the adhesive. This step avoids causing any interruption at the ferrule-to-optic cable and electrode-to-lead wire connecting zone (**Figure 1C**).

NOTE: Cyanoacrylate adhesive and its accelerant are harmful to the mouse eye. Pay attention not to cause spillage of these chemical substances. Also, be careful not to strongly touch the

electrodes and the fibers in order to avoid unexpected deviation immediately after adhesive solidification.

1.13. Expose the mouse neck muscles and insert the wires for the EMG electrode under the muscle. Adjust the length of EMG electrode so that it locates just under the nuchal muscles. Light connection between tip of electrode and muscle fascia is enough to catch the EMG signal.

1.14. Apply cyanoacrylate adhesive to fill the implants and solidify the adhesive with acceleration liquid. Then, put the mouse on a heat pad for recovery until the postural reflex appears. Adjust heat pad temperature to animal resting body temperature (36.0 °C in ZT 0-12 in case of C57BL6 mice; do not exceed 38.0 °C).

NOTE: An antibiotic is not required for sterile surgery.

1.15. Keep the mice in a home cage for a recovery period of at least 7 days.

2. EEG/EMG Monitoring with Photo-Excitation of Targeted Neurons in Specific Sleep States

CAUTION: This protocol includes use of class 3B laser equipment or LED devices. Experimenters should be aware of safety information. Protective eye goggles are required.

2.1. Before connecting the laser cable to the optic fiber, adjust laser intensity with a scaler. Tether the tip of the laser cable to an unused optic fiber with a ferrule and confirm that there is no space at the junction between the fiber and the cable.

2.2. Turn on the main switch of the laser and wait 20 min for it to warm up.

2.3. Emit the laser to the intensity checker and adjust laser intensity to 10 mW/mm^2. Change the laser mode to transistor logic and confirm that light pulses are emitted from the fiber controlled by the pattern regulator which is set at 10 ms for duration, 40 ms for rest, 20 times for cycle, and 20 times repeat (that is, 20 Hz of 10 ms light pulses for 20 s).

2.4. After the recovery period, move the mice to the experimental chamber for recording EEG/EMG. House mice at a constant 23 °C with a 12 h light/dark cycle with food and water available *ad libitum*.

2.5. Connect the implanted electrode and cable adaptor which is tethered to a slip ring to avoid entanglement. It is recommended to cover the junction with light-impermeable material such as aluminum foil to prevent laser leakage. If a bilateral experiment is required, use a slip ring with a bifurcate attachment for the cables.

2.6. In this protocol, we assess latency to wakefulness from NREM sleep or REM sleep, so the recording time should be limited in optimized zeitgeber time (ZT0 is defined as the time when the light is on). This protocol was conducted between ZT4 - ZT10. Let the mice stay freely in the

experimental chamber for at least 1 h as acclimatization.

2.7. During the experimental period, monitor EEG and EMG signals in the same recording screen and evaluate the mouse's state as wakefulness, NREM sleep or REM sleep. Use the gain control for each wave to make it easier to distinguish each state.

2.8. For measurement of NREM sleep to wakefulness latency, observe stable NREM sleep for 40 s or stable REM sleep for 30 s, then turn on the switch of the pattern generator for photostimulation (this protocol generates 20 Hz of 10 ms light pulses for 20 s). Confirm laser emission to the implanted optic fibers.

2.9. Record EEG/EMG signals until the sleeping state changes to wakefulness. If two or more experimental trials are needed, limit optogenetic manipulation to once a day because photostimulation is an artificial intervention which might affect sleep/wakefulness architecture.

2.10. After the experiment, deeply anesthetize and perfuse with sterile saline and paraformaldehyde (PFA) for sampling the whole brain for immunohistochemical analysis[26].

3. Analysis of Latency Time from NREM Sleep to Wakefulness.

3.1. After recording EEG/EMG signals, transfer the signal data to a computer for scoring sleep/wakefulness states. This protocol describes the method for EEG analysis with recording software (see **Table of Materials**).

3.2. Start the sleep sign application and click the **File** tab and select **Open** to choose the recorded data (.kcd file). Click the **Sleep** tab to select **Epoch time** for adjusting the time window for each epoch (we use 1 epoch/4 sec).

3.3. Manually score sleep/wakefulness states based on EEG/EMG signals, according to the following criteria: wakefulness, high EMG and low EEG voltage with high frequency; NREM, low EMG tone and high EEG voltage with high δ (0.5–4 Hz) frequency; and REM, EMG indicates muscle atonia and low EEG voltage with high θ (6–9 Hz) frequency. A state that does not consecutively continue for 16 s (*i.e.* 4 epochs) is not defined as a state change because it is not a stable state.

3.4. Click and hold the left mouse button on the first epoch and drag the cursor until the specific trend of more than 16 s (4 epochs) is ended. Then, release the left mouse button and choose the appropriate state (wakefulness or NREM sleep or REM sleep) in the pop-up window. Repeat this procedure to score all EEG/EMG signals recorded in the file.

3.5. Find the exact time of stimulation in the epoch where the EEG shows NREM or REM sleep, and the epoch showing state transition following the stimulation point. Count the number of epochs between the periods just after stimulation and just before state transition.

3.6. Then multiply counted number of epochs by 4 s (A). In the epoch of stimulation, take a screen

shot and measure the width between the stimulation point and the end of the epoch. Then, divide the measured length by the entire epoch length and multiply by 4 s (B). Similarly, calculate the duration of NREM sleep in the epoch of state change, which means the time between the start of the epoch and the state change point (C).

3.7. Sum A, B and C to obtain the latency from NREM sleep to wakefulness. The same procedure is used for analysis of state transition from REM sleep to wakefulness.

REPRESENTATIVE RESULTS:

The present study showed the effect of optogenetic excitation of $BNST^{GABA}$ neurons on sleep state transition. ChR2-EYFP was focally expressed in GABA neurons in the BNST. An *in situ* hybridization histochemical study showed that ChR2-EYFP was colocalized in neurons expressing GAD 67 mRNA signals, indicating that these are GABAergic neurons. Immunohistochemical slice samples confirmed the position of the optic fiber, whose tip was just above the BNST[25].

Figure 3A shows representative EEG/EMG traces before and after photostimulation during NREM sleep. High voltage and slow frequency EEG with no EMG signals represent NREM sleep. Photostimulation (10 ms pulses at 20 Hz for 20 sec) was applied following stable NREM sleep. Stimulation triggers acute transition to wakefulness (low voltage and high frequency EEG with active EMG signals) about 2 s after stimulation in ChR2-expressing mice. Control mice (EYFP) did not show transition after stimulation (latency of waking from NREM: EYFP, 295.39 ± 106.61 sec, $n = 6$; ChR2: 2.71 ± 0.59 sec, $n = 6$; $t_{10} = 2.35$, $p < 0.05$; **Figure 3B**, upper). These data suggest that excitation of $BNST^{GABA}$ neurons during NREM sleep triggers rapid induction of wakefulness. On the other hand, photostimulation during REM sleep had no effect (EYFP: 36.45 ± 13.08 sec, $n = 6$; ChR2: 37.29 ± 15.19 sec, $n = 6$; $t_{10} = 0.04$, $p = 0.484$; **Figure 3B**, bottom) so a transition effect only emerged in NREM sleep.

FIGURE AND TABLE LEGENDS:

Figure 1: Procedure to inject AAV, implant optic fibers and EEG/EMG implants. (A) Experimental procedure of virus injection. EYFP-fused ChR2 or EYFP (for control) gene incorporated in AAV vector whose transcription is gated by Cre recombinase was bilaterally injected into the BNST. **(B)** Optic fibers were inserted toward the BNST at a 30° angle to the horizontal to prevent collision with the electrode. Two screws were inserted around it. **(C)** EEG/EMG recording device was implanted after secured placement of the optic fibers. **(D)** At the end of the operation, the entire surgical area should be covered with cyanoacrylate adhesive and strongly fixed with accelerant. Make sure not to apply any agent to the region connecting the electrode and ferrules.

Figure 2: Custom EEG/EMG electrode and electrode pins insertion sites. (A) Top: Out of 6 electrode pins, the external two pins are cut down to 2 mm. Bottom: EEG/EMG electrodes. **(B)** These electrodes and EMG conduction wires are then soldered. The connecting zone should be isolated with any insulation like cyanoacrylate adhesive. Insertion sites of electrodes are relative to the bregma (anteroposterior ± 1.5mm, mediolateral ± 1.0 mm). EMG wires are inserted under the neck muscle with removal of insulation protecting the wire at the insertion site (1 mm).

Figure 3: Effect of GABA^{BNST} stimulation on state transition in NREM sleep and REM sleep. (A) Representative EEG and EMG wave and EEG power spectrum. Photostimulation (10 ms pulses at 20 Hz for 20 sec) was applied to ChR2-expressing GABA^{BNST} neurons following 40 s NREM sleep. Wakefulness was rapidly induced after a few seconds. The EEG showed low voltage and high frequency with EMG bursting. The EEG power spectrogram also showed transition from low to high frequency. **(B)** Optogenetic excitation of GABA^{BNST} neurons showed rapid transition from NREM sleep to wakefulness (upper), but this effect was not seen in the case of applying the same manipulation in REM sleep (bottom). *$p < 0.05$, Welch's *t*-test.

DISCUSSION:

We here presented a method to evaluate the effect of optogenetic stimulation of neurons with particular chemical identities on state transitions of sleep/wakefulness and gave an example of manipulation of BNST^{GABA} neurons. Our data showed that optogenetic excitation of BNST^{GABA} neurons results in immediate transition from NREM sleep to wakefulness.

Various experimental designs are available because of the development of numerous types of optogenetic tools. It is possible to activate or inhibit neuronal activity of particular neurons using different kinds of opsins, such as ChR2, SSFO, halorhodopsin, ArchT, and iChloC[27]. ChR2 can activate neurons a few milliseconds after photo-stimulation and this can be used to evoke action potentials in a phase-lock manner by a pulse generator to examine the acute impact in specific sleep stages. A stably activating opsin such as stable step function opsin (SSFO), which induces depolarization of neurons for 15 to 30 min after stimulation, might also be useful for some kinds of experiments designed to observe a semi-chronic effect[28]. Depolarized cells with SSFO might become more sensitive to various physiological neuronal input and be deactivated by applying long wavelength light. Furthermore, we can activate axons by implantation of optic fibers at the site of an axonal projection. Fiber stimulation could provide information on the function of a particular axonal projection pathway.

EEG/EMG recording during optogenetic manipulation is a less invasive method to determine the direct consequences of selective excitation/inhibition of neural circuits on sleep/wakefulness states in mice. With this method, many neuronal populations and neural circuits have been shown to be involved in the regulation of sleep/wakefulness states. Towards further development of this technique, it is possible to implant multiple fibers to manipulate multiple pathways simultaneously, or this could be also used in combination with fiber photometory or miniscopes to monitor neuronal activities.

In conclusion, it is anticipated that optogenetics will accelerate progress in unlocking the mystery of sleep regulation by the brain and the development of innovative therapies for refractory insomnia and other sleep disorders.

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Figure 1

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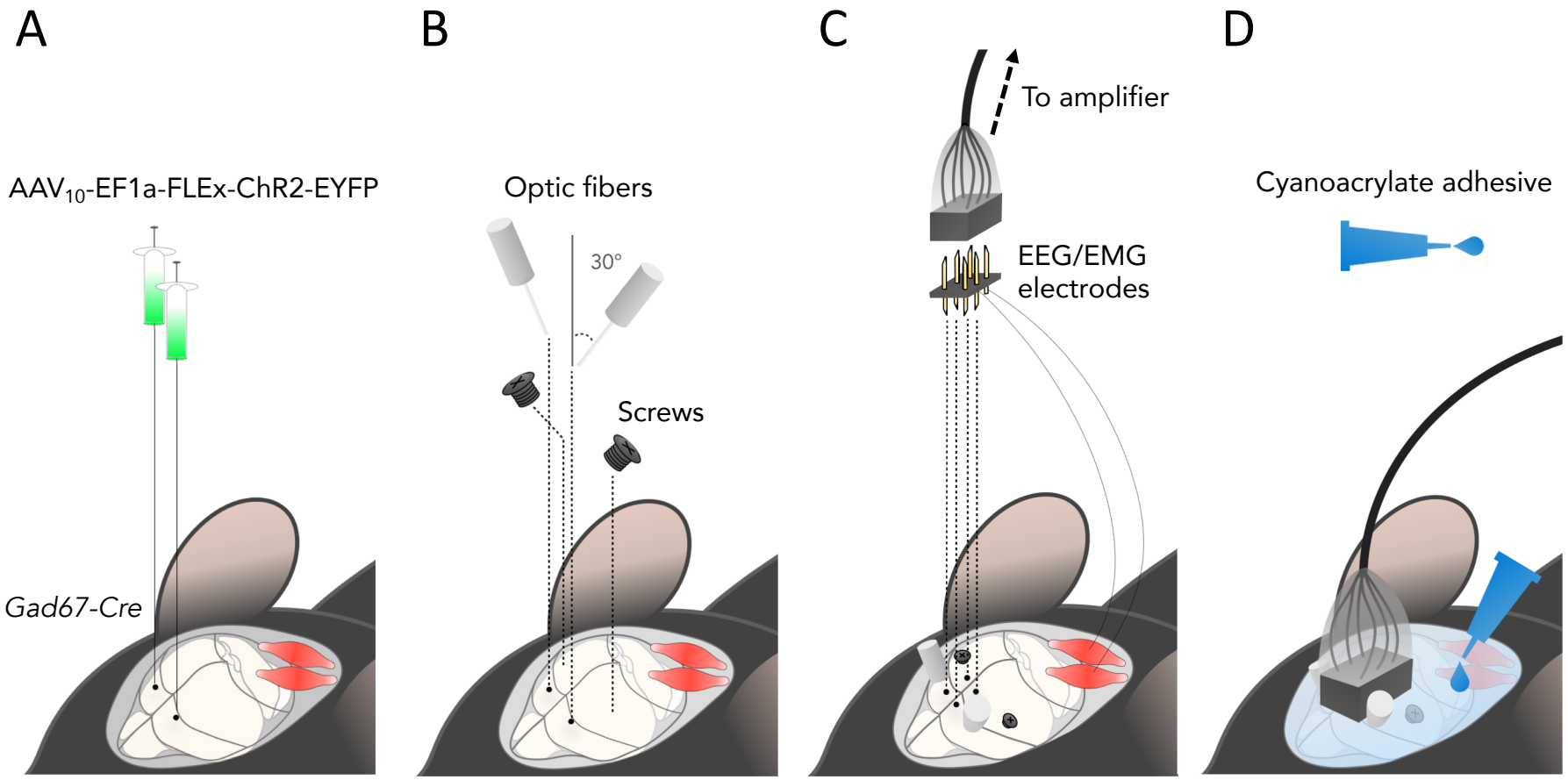

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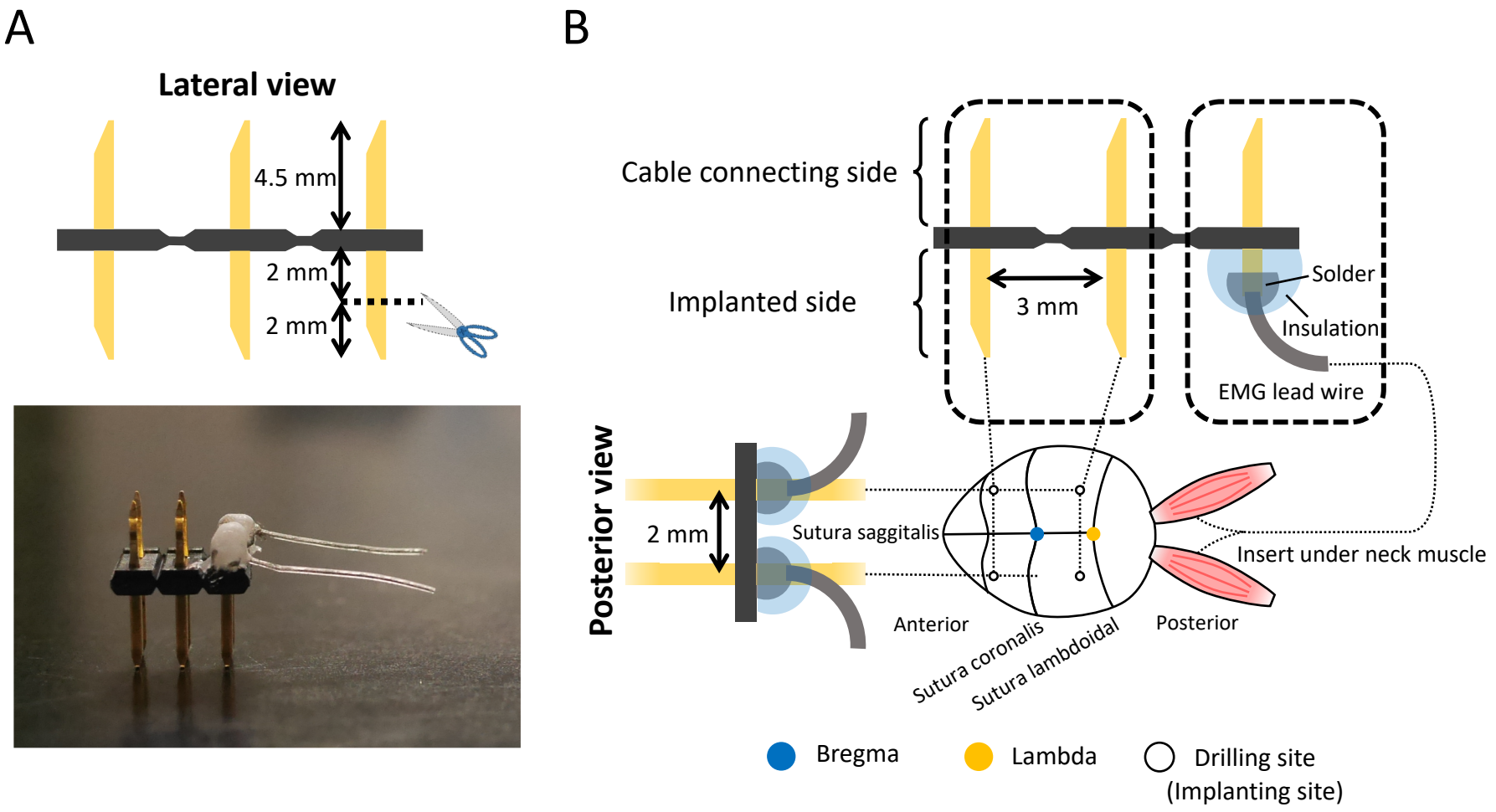

Figure 3

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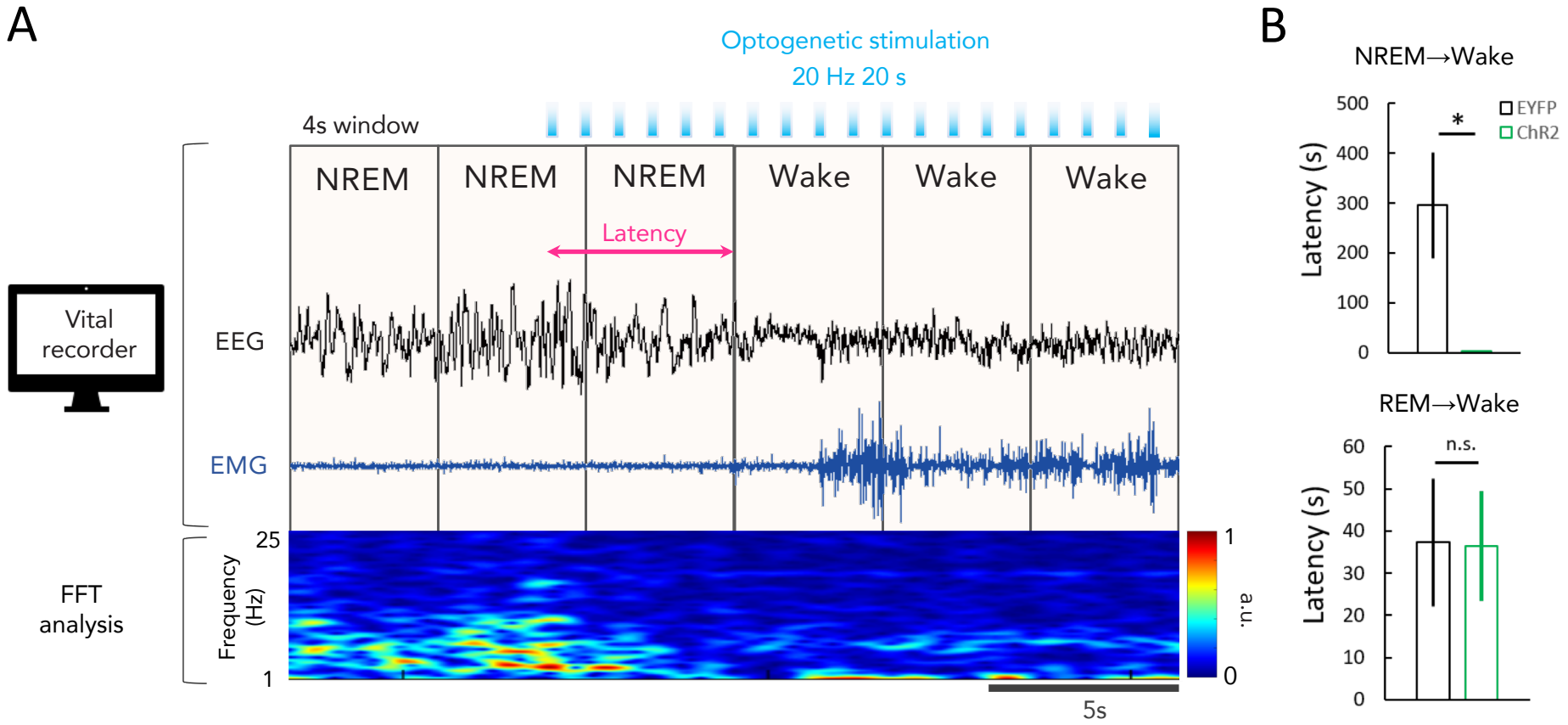

Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1x1 Fiber-optic Rotary Joints	Doric	FRJ 1x1 FC-FC	for optogenetics
6-pin header	KEL corporation	DSP02-006-431G	
6-pin socket	Hirose	21602X3GSE	
A/D converter	Nippon koden	N/A	Analog to digital converter
AAV $_{10}$-EF1α-DIO-ChR2-EYFP			3.70×10^{13}(genomic copies/ml)
AAV $_{10}$-EF1α-DIO-EYFP			5.82×10^{13}(genomic copies/ml)
Ampicillin	Fuji film	014-23302	
Amplifier	Nippon koden	N/A	for EEG/EMG recording
Anesthetic vaporizer	Muromachi	MK-AT-210D	
Automatic injecter	KD scientific	780311	
Carbide cutter	Minitor	B1055	ϕ0.7 mm. Reffered as dental drill, used with high speed rotary micromotor
Cyanoacrylate adhesion (Aron alpha A) and acceleration	Konishi	#30533	
Dental curing light	3M	Elipar S10	
Epoxy adhesive	Konishi	#04888	insulation around the solder of 6-pin and shielded cable
Fiber optic patch cord (branching)	Doric	BFP(#)_50/125/900-0.22	
Gad67-Cre mice	provided by Dr. Kenji Sakimura		Cre recombinase gene is knocked-in in the *Gad67* allele
Hamilton syringe	Hamilton	65461-01	
High speed rotary micromotor kit	FOREDOM	K.1070	Used with carbide cutter
Interconnecting sleeve	Thorlab	ADAF1	ϕ2.5 mm Ceramic
Isoflurane	Pfizer	871119	
Laser	Rapp OptoElectronic	N/A	473nm wave length
Laser intesity checker	COHERENT	1098293	
Laser stimulator	Bio research center	STO2	reffered as pulse generator in text
Optic fiber with ferrule	Thorlab	FP200URT-CANNULA-SP-JP	
pAAV2-rh10	provided by PennVector Core		
pAAV-EF1α-DIO-EYFP-WPRE-HGHpA	Addgene	plasimid # 20296	
pAAV-EF1α-DIO-hChR2(H134R)-EYFP-WPRE-HGHpA	provided by Dr. Karl Deisseroth		
Patch cord	Doric	D202-9089-0.4	0.4m length, laser conductor between laser and rotary joint
pHelper	Stratagene		
Photocurable dental cement	3M	56846	
Serafin clamp	Stoelting	52120-43P	
Shielded cable	mogami	W2780	Soldering to 6-pin socket for EEG/EMG recording
Sleep recording chamber	N/A	N/A	Custum-made (21cm× 29cm × 19cm) with water tank holder
Sleep sign software	KISSEI COMTEC	N/A	for EEG/EMG analysis
Slip ring	neuroscience,inc	N/A	for EEG/EMG analysis
Stainless screw	Yamazaki	N/A	ϕ1.0 x 2.0
Stainless wire	Cooner wire	AS633	0.0130 inch diameter
Stereotaxic frame with digital console	Koph	N/A	Model 940
Syringe needle	Hamilton	7803-05	
Vital recorder software	KISSEI COMTEC	N/A	for EEG/EMG recording

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Author(s): Shota Kodani, Shingo Soya, Takeshi Sakurai

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Reviewer #1:

Manuscript Summary:

Kodani et al., describe how optogentics can be used as a research tool to activate discrete neuronal populations with millisecond time scale. In their paper they show the activation of GABAergic neurons in the BNST using optogenetics in GAD67-cre mice and describe that this leads to rapid transitions from NREM sleep to wakefulness, but does not induce transitions from REM sleep to wakefulness. Kodani and co-workers deliver a very detailed description of the procedures necessary for the surgical implantation of the optical fibers and EEG/EMG recording electrodes. Although others authors have described this process before (e.g. Zhang et al., 2010 PMID: 20203662), it may be very valuable to publish this article in conjunction with the video.

Major Concerns:

The text absolutely needs editing to correct spelling and grammar throughout.

Answer: We have a native English speaker to edit of the entire manuscript.

Minor Concerns:

Please re-check part 3.4 (lines 241 and following) for clarity on how to get the latency to wake.

Answer: We modified the sentences in part 3.4 to clarify the method to get the latency time to wake as follows. “3.5. Find the exact time of stimulation in the epoch where the EEG shows NREM or REM sleep, and the epoch showing state transition following the stimulation point. Count the number of epochs between the periods just after stimulation and just before state transition. 3.6. Then multiply counted number of epochs by 4 sec (A). In the epoch of stimulation, take a screen shot and measure the width between the stimulation point and the end of the epoch. Then, divide the measured length by the entire epoch length and multiply by 4 sec (B). Similarly, calculate the duration of NREM sleep in the epoch of state change (C). 3.7. Sum A, B and C to obtain the latency from NREM sleep to wakefulness. The same procedure is used for analysis of state transition from REM sleep to wakefulness.” (p7, ll. 297-308).

line 280 should be "20 sec", not "20 min"

Answer: We corrected (p8, ll. 351).

For some materials used you add the brand (e.g. for SleepSign) - it may be valuable for the reader to know where other materials can be purchased

Answer: According to the reviewer's suggestion, we modified the table 'Materials'.

Reviewer #2:

Manuscript Summary:

Kodani et al., follow up on their publication from 2017 in which optogenetic excitation of GABAergic neurons of the bed nucleus of the stria terminalis (BNST) triggers a transition from non-rapid eye movement sleep to wakefulness in mice. This is an interesting methodology in that these neurons have never been stimulated this way before during specific sleep stages to produce the effect of immediate transition to wakefulness. However, the experimental protocol is not very innovative in technical novelty in that there are at least 70 other Jove papers that deal with optogenetic activation of a specific cell type. Furthermore, there are papers such as Clegern et al., 2012, where sleep monitoring and optogenetic stimulation have already been combined. Also, the paper is lacking important background information needed to justify the technique as well as significant details in methodology and clarity about the technique are absent.

Major Concerns:

1) Despite the previous publication by the authors, it is not obvious from the introduction why stimulating the BNST would have an effect on sleep. The only justification is that "the BNST is thought to play an essential role in anxiety and fear." What does this have to do with sleep?

Answer: Considering the sleep disorder, it is necessary to understand the neural mechanism that how limbic system affects the sleep/wake regulation. Our results suggest for the first time that an activation of the BNST could partly explain the mechanism by which fear and anxiety influence sleep/wakefulness states.

2) The authors describe multiple times in the introduction the advantages of optogenetics and how these allow to control specific neuronal populations. It is unnecessary to repeat this so many times, the countless publications using this technique make this amply clear. However, it is not very useful in a methods paper to describe optogenetics in such general terms. Instead, it would be helpful to write about how the specific use of the technique is useful. Furthermore, there are many potential issues that arise with optogenetics when stimulating in vivo (see "Optogenetics the Age of Light" 2014 by Hausser).

Answer: Following another reviewer's suggestion, we added discussion about the many potential of optogenetics in "discussion" section.

3) There is little detail about the electrodes implanted. These are described as custom made. However, often the most difficult element of these in vivo methods is to make the devices that are attached to the head. It would be great if the authors would submit images of the headstages and how they connect to the electrodes that are being implanted, see Bender 2018, JOVE.

Answer: We added images of electrode in the figure 2A.

4) In the third paragraph of the protocol section (from line 106) there is absolutely no need to describe the cre-lox system in detail. The authors are not molecular biology specialists, cre-lox with virus has been used in thousands of publications and no new techniques are presented here in terms of expression of the virus. This is one of many examples in the manuscript that show a lack of focus and organisation in the paper.

Answer: We deleted the related sentences following your suggestion.

5) In line 127 in the protocol section it is said to "Disinfect the surgical equipment with 70% EtOH". It is definitely not correct aseptic technique to just use ethanol. This is very unlikely to kill all the microbes. Instruments must be autoclaved or a cold sterilant must be used.

Answer: We modified the sentence as follows 'Disinfect the surgical equipment with the autoclave.' (p3, ll. 126)

6) It is not impressive that in line 129 the actual anaesthetic is not mentioned.

Answer: We mentioned the name of anaesthetic (p3, ll. 128).

7) The discussion is too general as it is focused on optogenetic techniques not described in the manuscript. It would be best to describe what this technique is able to do? How this technique specifically could be used in future experiments? How could stimulating BNST GABAergic neurons further be used to uncover the mechanisms of sleep?

Answer: We already wrote the advantage of this method in the discussion section (p9, ll. 366-377).

Minor Concerns:

1) Citations are needed for the following sentences in the indicated lines:

43-44 Recent findings also suggest that disturbances in sleep…

Answer: We added reference in the sentence (p2, ll. 47).

44-46 Although the functions of sleep have been…

Answer: We added reference in the sentence (p2, ll. 49).

49-52 NREM sleep is defined by slow oscillations…

Answer: We added reference in the sentence (p2, ll. 55).

2) In line 50, "lack of consciousness". Is there really consciousness in any sleep state? Can dreams really be described as that?

Answer: Based on the previous studies and definition of sleep state, there is no consciousness in the sleep state. REM sleep is the candidate for dreams, but we don't think it's necessary to describe that in here.

3) The concluding paragraph of the introduction is very unclear. What are other examples of sleep-wake optogenetic stimulation? It is best again, to focus on this particular method. Simplify this last paragraph.

Answer: We modified the sentences as follows "In conclusion, it is anticipated that optogenetics will accelerate progress in unlocking the mystery of sleep regulation by the brain and the development of innovative therapies for refractory insomnia and other sleep disorders." (p9, ll. 387-389)

4) What is the JAX reference number of the GAD67-cre line used?

Answer: ID of this line is 5051631 (MGI). However, it is not available in JAX.

5) Line 123. What is proper protection and handling techniques for viruses of this biosafety?

Answer: We added the sentences explaining this (p3, ll. 121-124).

6) Line 183. Much greater description of the EMG technique is needed. What do electrodes look like? Images would be helpful and high detail of implantation should be required.

Answer: We added another figure to show the detail of electrodes (figure 2).

7) Line 206, what does the "zeitgeber time" mean?

Answer: Zeitgeber time is a standard of time based on the period of a zeitgeber (light which could reset the circadian rhythm of the mice. Under standard light-dark cycles, the time of lights on usually defines zeitgeber time zero (ZT 0) for diurnal organisms and the time of lights off defines zeitgeber time twelve (ZT 12) for nocturnal animals. We added the information in the manuscript as follows " In this protocol, we assess latency to wakefulness from NREM sleep or REM sleep, so the recording time should be limited in optimized zeitgeber time (ZT0 is defined as the time when the light is on)." (p6, ll. 253-255).

8) Lines 214-215. Was the recording shut off immediately after animals woke?

Answer: It depends on the research design whether post stimulation effects are required to evaluate or not. Only the latency is required, the recording should be ceased. However, in our original article of the representative results, we compared two manners of stimulation, one and ten times of photo stimulation, to investigate whether many times of $BNST^{GABA}$ neurons stimulation had an effect on the wakefulness/sleep architecture. Thus, we recorded and analyzed additional hour of EEG/EMG data. It is beyond the theme of this methodological article, so we don't think it's necessary to describe.

9) Paragraph in line 241. Why does one multiply by 4 sect? To take a screenshot of data seems rather imprecise? Would it not be better to get access to the data with analysis software and get the precise time?

Answer: We could not integrate the laser system and recording software because the software is commercial one. We modified the related sentences as follows "3.5. Find the exact time of stimulation in the epoch where the EEG shows NREM or REM sleep, and the epoch showing state transition following the stimulation point. Count the number of epochs between the periods just after stimulation and just before state transition. 3.6. Then multiply counted number of epochs by 4 sec (A). In the epoch of stimulation, take a screen shot and measure the width between the stimulation point and the end of the

epoch. Then, divide the measured length by the entire epoch length and multiply by 4 sec (B). Similarly, calculate the duration of NREM sleep in the epoch of state change (C). 3.7. Sum A, B and C to obtain the latency from NREM sleep to wakefulness. The same procedure is used for analysis of state transition from REM sleep to wakefulness." (p7-8, ll. 297-308).

10) Paragraph in line 252. It would be useful to have the methodology for the in situ hybridization to confirm co-expression.

Answer: We don't think it is useful to describe that in this manuscript, which is focusing on the method for optogenetic manipulation during monitoring sleep states. Methods to identify the AAV-infected cells depend on the experiments (cell markers, type of virus).

Reviewer #3:

Manuscript Summary:

The title of the paper is very misleading. As indicated at the end of the introduction (line 94), the authors "describe here methods for analyzing the consequence of optogenetic manipulation on sleep/wakefulness". The important text there is 'methods for analyzing the consequence'. The only portion of the paper that is of any value at all is where the authors are describing analytical tools that will assess the effect of any manipulation that is hypothesized to disrupt sleep. They are not describing methods for optogenetic manipulation of the brain at any meaningful level.

Major Concerns:

Due to the lack of detail, readers will not be able to implement most of the techniques discussed here without performing a significant amount of research on their own. The table of materials is incomplete. It does not contain catalog or item numbers for the instrumentation listed. This and the fact that other essential materials, including the are not included in the table make the table all but useless. Other items that should be listed in the table (including their sources and catalog numbers) are: Cre-driver mice; Cre-inducible AAV-constructs; metal pins, screws and other parts of the mouse/machine interface; class 3B laser equipment; LED devices; electrode for EEG/EMG; optical fibers; anesthetic vaporizer; stereotactic apparatus; Serafin clamps; microinjection pump; dental drill and bit;

optic fiber ferrule; anchor screw; photocurable dental cement; intensity checker (I assume they mean a LASER power meter when they use this atypical term); fiber optic bifurcator. Other experimental procedures/techniques that are absent: stereotaxic coordinates for EEG, EMG, type of anesthesia used;

Answer: We modified and edit the text and table to include details of the experiments.

Minor Concerns:

Line 35: abbreviations EEG, EMG are not identified

Answer: We added the abbreviations (p1, ll. 38).

Line 37: 'osteogenic' should be 'optogenetic'

Answer: We corrected the typo (p1, ll. 40).

Line 54: The lengthy discussion of the two process model of sleep is unwarranted, since the methods presented in this article are not targeting either of the two processes underlying sleep, but rather one wake-promoting nucleus that impinges on the nuclei that regulate the two processes underlying sleep homeostasis.

Answer: We think it's necessary to include introduction about how sleep/wakefulness states are thought to be regulated.

Line 69: "virus vectors for neuronal tracings," are in fact vectors for targeting; whether or not the application is tracing of pathways depends on the proteins engineered into the viral construct.

Answer: We deleted the related sentences to avoid the duplication of information.

Line 75: somniphobia is misspelled.

Answer: We corrected (p2, ll. 80).

Line 323: "Merck & Sharp Corp" is a misnomer for Merck & Co.

Answer: We corrected the misspelling (p10, ll. 397).

Line 153: What is the exact genetic background of mice used? The stereotaxic coordinates for the BNST are likely strain dependent and. The authors need to acknowledge this explicitly.

Answer: We added the information (p3, ll. 115).

Line 154: The drill bit should not crack the skull but rather penetrate it.

Answer: We modified the sentence in line (p4, ll. 162-163).

Line 162: Are the EEG electrodes simply wires with no screw/pin on the end?

Answer: We didn't use any screw/pin on the end.

Line 164: What gauge wire?

Answer: We added the information (0.0130 inch diameter) in 'Materials' file.

Line 172: What are the specifications for the anchor screw (dimensions, material)?

Answer: We added the information (ϕ 1.0 x 2.0) in 'Materials' file.

Line 173: How can one not break the dura when inserting an optic probe at a depth of 4.2 mm relative to bregma?

Answer: It might be misunderstanding that we have to break the dura for optic probe insertion, but we have to be careful not to break the dura when we fix the screw on the skull (p4, ll. 162-163).

Line 179: What size drill bit is used?

Answer: We added the information in 'Materials' file.

Line 186: Prophylactic antibiotic use is not recommended (at least in the U.S.). Antibiotics should only be used in the case of post-surgical infection.

Answer: We deleted the sentences of antibiotics and added the sentences as follows. "An antibiotic is not required for sterile surgery." (p6, ll. 235-236).

Line 237: It is unclear whether states are categorized in 16-second or 4-second epochs due to poor use of language.

Answer: We explained how to define these states as follows "A state that does not consecutively continue for 16 sec (i.e. 4 epochs) is not defined as a state change because it is not a stable state." (p6, ll. 228-229).

Reviewer #4:

Manuscript Summary:

This protocol is interesting and should be of wide interest to researchers who study sleep, physiology, and neural circuits. In this protocol, the authors describe how to optogenetically target neural circuits underlying sleep/wake regulation. As an example, the authors optogenetically target GABAergic neurons of the BNST via viral-mediated transfer of ChR2-YFP to this neuronal population.

Major Concerns:

This protocol requires major revisions and needs to be more clearly written and described for the readership who wants to learn how to perform this technique. More diagrams and figures would aid in visualizing various steps of the protocol. First, a detailed list of equipment and catalog numbers is required. The authors have only listed two pieces of equipment. Second, this protocol needs to be written from more of a beginner's frame of mind. The viewers may have not performed rodent brain surgery before. For example, define bregma and lamda and what they are in the context of this surgery. Third, control experiments need to be discussed. In addition to ChR-YFP (experimental), YFP virus (control) should also be done in parallel. cfos/YFP FISH/IHC should be performed after optogenetic activation of a neuronal population to confirm neuronal activation...especially for novice experimenters. Fourth, this manuscript requires extensive copy-editing. There are many typos. For example, the word "osteogenic" was incorrectly used in line 37, when the authors should have stated "optogenetic". These major concerns addressed, this protocol would useful to the sleep research community in describing functional interrogation of neuronal populations.

Answer: Thank you for valuable suggestions. Firstly, we added detailed material information in table 'Material'. Secondly, we made schemes more detailed in order to make it understandable for broad range of researchers. Thirdly, we added the sentence describing the use of EYFP as a control (p3, ll.115-116). Fourthly, we corrected typos and grammatical errors.

Minor Concerns:

-It is important to discuss either in the protocol introduction or in one of the steps for AAV control of non-ChR2 viral vector, such as YFP/GFP alone. Need to describe how you confirm GABAergic neurons of BNST are optogenetically activated (e.g. Fos/YFP immunostaining).

Answer: We mentioned the use of EYFP as a control (p3, ll.115-116). We used the fos expression to check the activation of GABAergic neurons of BNST. We don't think it's necessary to discuss about this in here.

-Protocol intro: A diagram of the genetic loxP and loxP2722 cassettes described in the introduction of the protocol would be helpful for the general readership.

Answer: Following another reviewer's suggestion, we deleted the sentences to describe the details about Cre-loxP system. Because its already well known and we concluded that we should delete this in the manuscript.

-1.4 Define bregma and lamda for someone who is not use to rodent brain surgery.

Answer: Thank you for pointing out improving suggestion. We made new figure and legend (Fig 2, p8, ll. 341-347) for the description of the custom electrode and the location of implantation. Also, we added the following sentences to describe the definition of the bregma and the lambda. "After confirming the head is held stably, make a midsagittal incision in the scalp to ensure the positions of the bregma and lambda are located at the same level on a horizontal line. 1.4. To avoid a positioning gap, appropriately adjust the levels of the nose pinch and ear bars up and down. The bregma and lambda refer to the intersection between the sutura saggitalis and sutura coronalis or sutura lambdoidal, respectively (Fig 2)." (p.4, ll. 135-141).

-1.5 Injection of how much AAV vector? Provide recommended range of volume and concentration. Provide recommendations on serotype.

Answer: It is important to determine the volume of virus. There is no formula for calculation to decide the volume because the titers of virus varies. However, pilot experiments are recommended to estimate the relationship between injected volume and infection area. So, we added the following sentence. "NOTE: The injection volume of the virus solution is determined in pilot experiments using the same mouse strain and same virus product. The relation between the volume of virus solution and extent of the infection area should be estimated in advance." (p4, ll. 154-157).

-1.5 What does flashing the inside of 10mL Hamilton syringe exactly mean?

Answer: We modified the sentence as follows "As preparation of the injection of AAV vectors, we wash inside of 10ml syringe (see Table 'Materials') sequentially with 70 % EtOH, 100 % EtOH and sterilized water 5 times for each." (p4, ll. 147-148).

-1.6 From the perspective of someone who has never performed this surgery, what does it look like when you have done it correctly versus incorrectly? (one sentence is fine). You

describe removing blood around the drill hole with the cotton swab, but how much is to be expected if you have done it correctly at the described drill site.

Answer: A little amount of bleeding, i.e. about 1 - 2 droplets of blood is usually observed due to disruption of fine capillaries. On the other hand, relatively large amount of bleeding is occurred near the suture of skull because of the existence of the sinus venosus. Considering these, we added sentence as follows, “Be careful not to damage the dura or brain tissue.” (p4, ll. 163)

-1.6 How is the Hamilton syringe injected to achieve 0.07ul/min? Physically by hand or with a nano-pipetter?

Answer: In this protocol, mechanical micro injector was used for accurate velocity and amount of virus injection. We modified the sentence as follows. “Slowly inject the designated amount of virus solution (0.07 μl/min) with a mechanical microinjector.” (p4, ll. 166-167).

-1.7 step is hard to follow by text.

Answer: We created another figure (figure 2) to explain this. This figure legend was previously mentioned in the answer to your concern 1.4. In addition, we also modified the figure 1 for readers to understand easily about the surgical steps to implant the device.

-1.7 Are there any special consideration that need to be described when doing bilateral stimulation instead of unilateral stimulation? (e.g. tangling of wires/fibers). How are optical fibers secured? Dental cement? How long does it take for the dental cement to dry.

Answer: We added the information of glue we used for fixing fiber in ‘Materials’ file and modified the sentences as follows “The reaction time to solidify the glue should be followed by manufacturer’s manual (Our material needs exposure to light for at least 10 sec with specific wave length photo-generator. It is unnecessary to dry the glue after this).” (p5, ll. 196-198)

-1.8 What type of glue? Describe manufacturer and catalog number in materials list

Answer: We added glue information at the table of materials list and revised the text. We also describe precaution to apply fixing glue as follows “Hold the implant and apply

cyanoacrylate adhesive to the space between the skull and the electrodes. Insert again with attention not to interfere with any materials. 1.19. Cover the circumference of the electrodes and optic fibers with cyanoacrylate adhesive followed by application of cyanoacrylate accelerant on the adhesive. This step avoids causing any interruption at the ferrule-to-optic cable and electrode-to-lead wire connecting zone (Fig 1C). 1.20. NOTE: Cyanoacrylate adhesive and its accelerant are harmful to the mouse eye. Pay attention not to cause spillage of these chemical substances. Also, be careful not to strongly touch the electrodes and the fibers in order to avoid unexpected deviation immediately after adhesive solidification." (p5, ll. 205-217)

-1.9. How deep do you insert the wires? When do you know you've gone too far?

Answer: We modified the sentences as follows. "Adjust the length of EMG electrode so that it locates just under the nuchal muscles. Light connection between tip of electrode and muscle fascia is enough to catch the EMG signal." (p6, ll. 220-221)

-1.10. What is the temperature range of the heatpad? Can you list some heatpad recommendations?

Answer: We listed the heatpad in table "materials" and modified the sentences as follows, "Heat pad temperature should be adjusted to animal resting body temperature (36.0 ℃ in ZT 0-12 in case of C57BL6 mice and not to exceed 38.0 ℃)." (p6, ll. 225-226)

-1.10 What antibiotics should be applied? Locally? In food? How often and how much? What manufacturer and catalog number?

Answer: We deleted the sentence and added the sentence to suggest that antibiotics is not required in this surgery. "An antibiotic is not required for sterile surgery." (p6, ll. 228)

-2.1 How do you know when you have used too much intensity? How was 20Hz determined for BNST stimulation? Is that the inherent firing rate of GABAergic neurons in BNST? Describe to audience how they should determine stimulation frequency. Should it grossly parallel the firing rate of the neuronal population being targeted.

Answer: We used 20Hz based on the previous observation (Kim et al., 2013). They used multi-unit recording method to show that baseline firing of dorsal BNST neurons is

around 15 Hz. Ideally, it's better to use electrophysiology to see the inherent firing rates and transfer this information to optical stimulation protocol. It is sometimes challenging to determine proper stimulation protocol. Preliminary examination with several different stimulation frequencies to see the consequences would be help.

-2.3. 40 seconds for NREM and 20 seconds for REM - Why not both for 40 seconds, or both for 20 seconds? Discuss rationale.

Answer: It is well known that REM sleep period is much shorter than NREM sleep. We previously published the mean duration of NREM and REM in WT suggesting that NREM duration bout is more than 200 sec, however REM duration is less than 100 sec in light period (Hondo et al., 2011, plos one). With consideration of these results, we used different definition to apply laser during NREM and REM sleeps.

-2.3. I'm not sure what this means in line 215-216: "Experiment with light pulses should be limited one time for each day because photostimulation could affect sleep/wakefulness architecture." Explain this more clearly.

Answer: We modified the sentences as follows. "If two or more experimental trials are needed, optogenetic manipulation should be limited to once a day because photostimulation is an artificial intervention which might affect sleep/wakefulness architecture." (p7, ll. 267-270).

-2.4. This is not a complete sentence in line 219-220: "Analysis of the latency time from NREM sleep to wakefulness."

Answer: We deleted a duplicated sentence (p7, ll. 274).

-3.2 In line 231 it state that an epoch is 16 second, but in line 230 you state an epoch is 4 second. Please clarify.

Answer: We explained how to define these states as follows "A state that does not consecutively continue for 16 sec (i.e. 4 epochs) is not defined as a state change because it is not a stable state." (p7, ll. 288-290).

-Figure 1. It is not quite clear to me what all the items in C are? Please label.

Answer: We modified the figure 1 and added label to the objects. Also, we revised the sentences in figure 1 legend as follows. “(D) At the end of the operation, the entire surgical area should be covered with cyanoacrylate adhesive and strongly fixed with accelerant. Make sure not to apply any agent to the region connecting the electrode and ferrules. (p8, ll. 336-339)