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# Simultaneous Monitoring of Wireless Electrophysiology and Memory Behavioral Test as a Tool to Study Hippocampal Neurogenesis

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**TITLE:**

# Simultaneous Monitoring of Wireless Electrophysiology and Memory Behavioral Test as a Tool to Study Hippocampal Neurogenesis

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**KEYWORDS:**

electrophysiology, neurogenesis, standardization, troubleshooting, wireless technology, behavioral observation, novelty-seeking behavior, behavioral research, memory, long-term memory, short-term memory, memory and learning tests

**SUMMARY:**

The protocol presented here provides information on the simultaneous electroencephalography (EEG) and behavioral assessment in real-time. We have discussed all steps involved in this protocol as an attractive solution for researchers in many fields of neuroscience, particularly in learning and memory areas.

**ABSTRACT:**

Brainwaves amplitude obtained from electroencephalography (EEG) has been well-recognized as

a basis for cognitive capacity, memory, and learning on animals and humans. Adult neurogenesis mechanism is also linked to memory and learning improvement. Traditionally, researchers used to assess learning and memory parameters in rodent models by behavioral tasks. Therefore, the simultaneous monitoring of behavioral changes and EEG is particularly interesting in correlating data between brain activity and task-related behaviors. However, most of the equipment required to perform both studies are either complex, expensive, or uses a wired setup network that hinders the natural animals' movement. In this study, EEG was recorded with a wireless electrophysiology device along with the execution of a novel object recognition task (NORT). The animal's behavior was monitored simultaneously by a video tracking system. Both recordings were analyzed offline by their timestamps which were synched to link EEG signals with the animal's actions. Subjects consist of adult Wistar rats after medium-term environmental enrichment treatment. Six skull screw electrodes were fixed in pairs on both hemispheres over frontal, central, and parietal regions and were referenced to an electrode located posterior of the nasal bone. NORT protocol consists of exposing the animal to two objects for 10 min. After 2 h and 24 h, one of the objects was replaced with a novel one. Exploration time for each object was monitored by a behavioral tracking software (BTS) and EEG data recording. The analysis of the EEG synced with behavioral data consists of estimations of alpha and beta relative band power and comparisons between novel object recognition versus familiar object exploration, between three experimental stages. In this manuscript, we have discussed electrodes manufacturing process, epidural electrodes implantation surgery, environmental enrichment protocol, NORT protocol, BTS setup, EEG – BTS coupling for simultaneous monitoring in real-time, and EEG data analysis based on automatic events detection.

**INTRODUCTION:**

Behavioral test is crucial in neuroscience research for a large amount of information generated in an in vivo context. In this regard, researchers have been widely using different behavioral tests to analyze sensory-motor function, social interactions, anxiety-like and depressive-like behavior, substance dependence and various forms of cognitive functions[1]. Manual recording of behavioral tests might be difficult, exhausting, and inaccurate even for most expert observers. Even though some efforts have been made to develop free and open-source software for behavior registration (e.g., sexrat male[2] app for sexual behavior), several alternatives allow the automatic and real-time behavioral recording of different animal species from fish[3] to rodents[4–6]. Video tracking is a valuable method for quick and accurate behavior recording used in a wide variety of applications[7]. A more potential feature in the behavioral recording area is to explore neural activity during the behavioral manifestation. Simultaneous recording of neuronal activity (from single cells to the major brain areas) and behavioral tasks could show us how the brain generates specific behavioral patterns[8]. Behaviors are a sequence of minor components that could reveal correlates between the neural activity and movements or actions. If neuronal activity and behavioral patterns could be simultaneously recorded through multiple timescales, they could explain how each brain state correlates to each particular behavior (for a more in-depth examination of behavioral recording, see Datta et al., 2019 review[8]). Therefore, synchronized recording of behavioral and neuronal activity at the desired scale (from neurons to large areas of the brain) is considered as an extremely useful tool. There are several systems intended to integrate behavioral recordings with other measurements as neural activity[4,5].

Although electroencephalography is considered one of the most widely used techniques in the field of clinical and research neuroscience, the relatively high mobility, as well as the size of the EEG recording device, makes this technique unique and challenging for detection in case of in vivo models[9]. Some solutions to this problem have been developed e.g., the use of cables and swivel-devices that allow animals to move freely in the arena. Nevertheless, cable-based systems often impose problems to conduct studies, e.g., during the transfer of an animal from one cage to another, hindrance or entanglement of the animal with the cables is observed. Telemetric devices have been developed for wireless electrophysiological recordings to increase the flexibility of the recording situation[10,11]. However, such systems have shown considerable limitations due to their low number of recording channels and low sampling rates[11]. In this study, we used a commercially available wireless system that sends EEG signals from the animal through a Wi-Fi connection with a freely-moving rodents system[12]. The apparatus weighs 6 grams and stands up to 16 channels recorded at 1 kSps. This system allows EEG or spike recording in the animal environment, with a reduced disturbance, serving as an economical solution compared to the traditional electrophysiological systems in the market. In addition, we have synchronized this data using a video tracking software to provide correlation between EEG and behavioral patterns. This synchronization is done offline by alignment and interpolation of data and events based on timestamps generated by both systems and is processed on MATLAB.

Adult neurogenesis is defined as the proliferation, survival, and differentiation in neurons of newly generated cells in the dentate gyrus of animals[13,14]. This process is known to be associated with memory and learning improvement which increases adult neurogenesis in rodents through enriched environment (EE) conditions[15]. EE consists of housing rodents in small groups inside a large cage provided with toys and tubes, where animals have novel and complex but no biological relevance[15]. Although EE stimulates hippocampal neurogenesis, it also varies in many factors such as age, animal strain, specific stimulation conditions, or neurogenesis detection procedure. In middle-aged mice exposed to EE housing for seven days, the birth of new granular cells (GC) in the hippocampal dentate gyrus (DG) has been reported[16]. Studies attempting to ablate adult neurogenesis in adult rats selectively have suggested that new granular cells of about 1 - 2 weeks of age are required in the learned response[17]. Around 2 or 3 weeks after the GC are born in adults DG, several characteristic features such as dendritic spines, which are essential for excitatory synaptic transmission[18], begin to appear. Zhao et al. performed a quantitative analysis to show that the peak of spine growth occurs during the first 3 - 4 weeks[19]. Several electrophysiological in vivo studies suggest that only three weeks of EE housing conditions produces alterations in the DG's synaptic transmission and increase cell excitability[20]. Also, it has been reported that exposure to an enriched environment at 1–4 weeks after BrdU injections significantly increased the density of BrdU/NeuN cells in the DG granular layer in mice[21]. These authors suggest that a critical period exist between one and three weeks after EE exposure since a substantial increase in the number of new neurons was observed[21]. Studies of adult hippocampal neurogenesis (AHN) in humans has been controversial since there was no direct evidence. However, a recent report described the developmental stages of AHN in the human adult brain, identifying thousands of immature neurons in the DG, and thereby demonstrating the importance of AHN during aging in humans[22]. Based on the evidence mentioned previously, the study of AHN in animal models is

more important than ever (for a more in-depth examination of AHN, see Leal-Galicia et al., 2019 review[15]).

As previously mentioned, the hippocampus has been linked to a fundamental function in learning and memory capacities. The formation of memories goes through three distinct processes: encoding (memory acquisition), consolidation (memory storage), and retrieval (memory recognition)[23]. Recognition memory in humans is tested using the visual paired comparisons task[24]. The fundamentals of human and animal models of memory and amnesia are the behavioral tests that assess the ability to recognize a previously presented stimuli[25,26], as the visual paired comparisons task does in humans. Therefore, one of the most used behavioral tests for assessing the ability of a rodent to recognize a previously presented stimulus, that is to say, the learning and memory capacity is the spontaneous novel object recognition task (NORT)[23,27]. NORT protocol consists of two identical novel objects in a familiar arena for 10 min in the acquisition trial. After a specific time between 0[28] and 48 hours[29] (variable time according to each protocol), the animal is returned to the same arena containing one of the same familiar objects, and one novel object. The animal spontaneously explores the novel object if the familiar object was memorized[26]. The preference ratio is commonly used in assessing exploration performance. It is determined by dividing the total object exploration time from the exploration time of the novel or familiar object. The NORT has some advantages over other recognition memory tests. Most importantly, it requires no external motivation, reward, or punishment. It does not generate stressful conditions. Finally, no training is needed to evoke the behavior of exploratory objects (for a more in-depth examination of NORT, see ref.[23]).

Therefore, simultaneous recording of multiple data modalities and their integration in the study of learning and memory, as an effect of adult hippocampal neurogenesis is highly attractive and provides a compelling solution for researchers in the field. The present work will expose all processes involved in the simultaneous behavioral video-tracking assessment (novel object recognition task) and wireless electroencephalography recording. Here we have reviewed the electrode manufacturing process, epidural (skull screw) electrodes implantation surgery, environmental enrichment protocol (for hippocampal neurogenesis induction), following NORT protocol, BTS setup, EEG – BTS coupling for simultaneous monitoring in real-time, and EEG and behavior data analysis executed on MATLAB computing environment.

**PROTOCOL:**

All procedures follow the Guide for the Care and Use of Laboratory Animals (NIH Publications N°. 8023, revised in 1978) implemented by National Health Institutions and local Mexican laws to reduce the number of animals used for animal welfare and prohibition of animal suffering. The Ethics Committee of the Universidad Iberoamericana approved the experimental protocols for the use of animals in this study.

## 1. General setup

1.1. Install the Behavioral Tracking Software on a computer according to the manufacturing instructions.

1.2. Mount the camera directly above the device, so it faces downward. The camera should be connected to the computer.

1.3. Install the driver's software required by the camera (following the manufacturing instructions).

1.4. If the camera includes zoom lens, adjust them to fit perfectly in the camera's display.

1.5. Turn off the camera autofocus (AF) mode following the manufacturing software.

1.6. Ensure that the camera is working correctly in real-time and test the video capture mode until it is ready to use.

## 2. Environmental enrichment protocol (see Figure 1)

NOTE: Three-month old male Wistar rats were used for this experiment and were maintained under natural dark-light conditions.

2.1. Place sawdust bedding in a transparent acrylic square arena (50 x 50 x 50 cm).

2.2. Put three different kinds of toys on the arena for rodents to interact with (e.g., activity wheels, double deck, stairs, etc.).

2.3. Add four 2-inches and four curved gray opaque PVC tubes.

2.4. Provide food and water dispensers with *ad libitum* access to animals.

2.5. Place three rodents per cage inside the vivarium room under regular conditions.

2.6. Leave the animals in this arena for the time required according to the corresponding protocol. In this experiment, animals should stay inside the arena for 20 days.

NOTE: After the electrode implantation surgery, animals do not go back to environmental enrichment treatment. Instead, they were put in single cages until the novel object recognition test is completed.

## 3. Electrodes manufacturing process

3.1. Cut a piece of copper wire at approximately 2 cm and use a sandpaper to rub approximately 0.5 cm from each end.

3.2. Roll one end of the copper wire to the head of a small-sized screw (electrodes) and make sure it is firmly fixed since this is a crucial step. Correct contact between both materials must be

guaranteed to avoid artifacts in the EEG signals.

3.3. Insert the other end on the connector's terminal tip and make sure it is properly fixed by reinforcing using fine forceps. This tip should connect with an amplifier cable.

3.4. Measure the suitable conductivity from the tip to screw using a multimeter. This process guarantees that the electrode connection is properly installed.

**4. Epidural (skull screw) electrodes implantation surgery**

NOTE: After 20 days of environmental enrichment treatment, the animals will undergo surgery following the procedure described below:

4.1. Inject a cocktail of Ketamine/Xylazine (90/10 mg/kg, i.p.) to the animal.

NOTE**:** To avoid airway obstruction, wait until the rat stops moving, then take it out of the housing cage and place the animal on a flat surface.

4.2. Once the rat is entirely anesthetized, shave the head area of the rat.

NOTE: Make sure that the animal is completely anesthetized before continuing with the surgery. Carefully pinch one of the legs or the tail. If the animal reacts to the stimulus, wait a few more minutes and pinch it again. If the animal does not react to the pinch, go to the next step.

4.3. Place the animal on the stereotaxic apparatus by protecting both ears first with the ear bars (be careful not to hurt the animal's internal ear). Finally, place the front teeth over the bite bar and secure the nose bar.

NOTE: Provide the animal with a heating pad for all the surgery since the anesthesia used in this procedure usually causes hypothermia and breathing problems.

4.4. Clean the top of the head area using a cotton pad/swab soaked with benzalkonium chloride antiseptic solution.

4.5. Administer lidocaine subcutaneously (20 mg/mL) under the skin of the head area (0.5 mL).

4.6. Instill a drop of ophthalmic solution or saline to each animal's eyes every 5-10 min to help them not dry out.

4.7. Using a scalpel, make an incision of approximately 2 cm from anterior to posterior direction to properly expose the skull's top region.

4.8. Retract the skin using bulldog clamps and scrape the tissue overlying the skull.

4.9. Using a cotton swab, clean the exposed skull with a small amount of peroxide solution.

4.10. Identify and record the bregma coordinates obtained.

4.11. Starting from Bregma, using the stereotaxic Paxinos and Watson Atlas[30], locate and mark the position of each of the seven points (coordinates) where the electrodes will be fixed in.

NOTE: In this experiment, F3, F4 screws (+2.0 mm from Bregma, 2.25 mm lateral from midline); C3, C4 screws (−3.0mm from Bregma, 2.75 mm lateral from midline); and P3, P4 screws (−7.0 mm from Bregma, 2.75 mm lateral from midline) were installed. A seventh screw was located posterior of the nasal bone (NZ), as the ground reference (see **Figure 2**).

4.12. Using a variable speed drill tool, make a hole with a tip size 2 (length 44.5 mm) on each one of the marks, be careful not to penetrate the skull fully.

4.13. Insert the electrode into the hole and screw it into the skull carefully.

4.14. Repeat steps 4.11 and 4.12 until all seven screws are fixed in appropriately.

4.15. Fix all 7 screws with a first layer of dental cement. Insert each electrode into a connector. Cover the wires entirely with a second layer of dental cement (it will prevent the animal from pulling screws off), and the bottom of the connector. If necessary, cover with a third layer of dental cement, leaving the EEG connector clean for a proper connection, so that the EEG device can be connected appropriately (see **Figure 3**).

NOTE: After placing each pair of bilateral screws, these could be fixed in with dental cement (optional step).

4.16. Leave the rat in post-operative care overnight. Observe the animal and provide the animal with a heating pad for 1-2 h after surgery since the anesthesia used in this procedure usually causes hypothermia and breathing problems.

4.17. Administer 1 mL of saline solution subcutaneously to prevent dehydration. Inject a nonsteroidal anti-inflammatory (meloxicam 2 mg/kg, s.c.) and an antibiotic (enrofloxacin 5 mg/kg, p.o.) after surgery and for the next 24 h.

4.18. After the surgery, keep the rats in single cages for full recovery during seven days before conducting the behavioral tests.

4.19. Gently manipulate the animal on a periodical basis (at least once a day) to help to reduce the stress in future manipulations. While holding the rat with one hand, finger pressure is gently applied to the back of the animal, sliding the fingers through the fur. Check the head wound, health condition, behavior in general, and body weight for a period of one week after the surgery.

NOTE: If any abnormality or signs of disease/stress are found in the animal, notify the responsible veterinary physician. After this period, perform the Novel object recognition test and EEG recording technique.

## 5. Novel object recognition test (NORT)

NOTE: Seven days after surgery, proceed to behavioral tests. All behavioral procedures, in the presented experiment were performed between the 14 h:00 min and 16 h:00 min, which corresponds to the rat's light cycle.

5.1. Place a vest made of soft fabric (to which the EEG device would be placed during the behavioral test) on the rat. Allow habituation for 2-3 days before conducting the behavioral test.

5.2. Place a black acrylic square arena (50 x 50 x 50 cm) in a dim-light illuminated recording room.

5.3. Fix two identical novel objects to the floor center of the arena using double-sided tape (to prevent its displacement by the animals). Objects must be equidistant from each other and the arena walls.

5.4. Clean each object thoroughly beforehand with 50% ethanol, as well the arena's floor after each trial (to avoid olfactory cues).

NOTE: Always transfer the animals to housing rooms (from the vivarium room to the experimental room) at least half an hour before starting each session. After completing the recording session, leave the animals in the experimental room for an additional hour. This is to avoid the stress that could affect the performance of this test.

5.5. Connect the EEG device, before starting each test. Gently restrain the animal and firmly insert the cable to the connector on the animal's head with the EEG kit attached to the animal's back (see **Figure 4**). Only one position is allowed.

NOTE: Gentle previous manipulation of the animal might help to reduce the stress in animals during the connection procedure. Otherwise, the risk of damage to the device or the animals increases. Fully pre-charge the device battery using a USB-Port.

5.6. Novel object recognition tests phases

5.6.1. Habituation: Handle the animal at 5-min intervals for two consecutive days, and immediately after, place the animal on the arena (without any objects) and allow them to explore for 10 min freely.

NOTE: Before executing any acquisition and memory test sessions, rats were carefully handled and connected to the corresponding EEG device, which was properly fixed before starting the

test.

5.6.2. Acquisition Session: Place the animal on the arena facing one of the walls opposite to the objects. Allow animals to explore freely for 10 min. Go to step 6.13 for test recording using Behavioral Tracking Software.

NOTE: Make sure the EEG device properly holds the vest attached to the back of the rat (to ensure proper tracking of the animal). For additional reinforcement, use masking tape.

5.6.3. Short-term memory test (SMT): Replace one of the objects with any other completely different in shape, color, and texture. Place the animal, 2 h after the acquisition session, in the arena facing one of the walls opposite to the objects. Allow the animal to explore freely for 10 min. Go to step 6.13 for test recording using the Behavioral Tracking Software.

5.6.4. Long-term memory test (LMT): Replace the object used with any other completely different in shape, color, and texture from the short-term memory test. Place the animal 24 h after the acquisition session, in the arena facing one of the walls opposite to the objects. Allow the animal to explore freely for 10 min Go to step 6.13 for test recording using the Behavioral Tracking Software.

## 6. Behavioral tracking software setup

6.1. Open the Behavioral Tracking Software.

6.2. Log in to the account using the institution's user and password.

6.3. Open the tap "**New empty experiment**" and choose a name for the protocol (e.g., "NORT").

6.4. Select "**Video tracking mode.**"

NOTE: In this experiment, the camera is set up to stream the video tracking live. However, there is an additional option to select pre-recorded videos.

6.5. Go to "**Apparatus.**" Define the arena area, by adjusting the orange rectangle to the limits of the projected arena. Determine the object's region, fitting the orange circles at the objects border inside the arena projected from the camera on the screen.

6.6. Setup the scale moving ruler line to a position along the known length of the image (the arena). Enter the length of the object in millimeters in the option "**The length of the ruler line is**" on the Settings panel. In this case, the arena measures 500 x 500 mm.

6.7. Go to "**Tracking and behavior**." Continue to "**Zones**." Click on the "**Add item**" menu and select "**New Zone**." Select the arena area and name the new zone (e.g., "Field").

6.8. Repeat the previous step with the objects' area and name the new zone (e.g., “Objects”).

6.9. Go to “**Animal color**” and select “**The animals are lighter than the apparatus background**” option.

NOTE: White (Wistar) rats were used for this experiment. However, the software has additional options for researchers who use black and spotted rats. Both breeds of animals can be used in the same experiment.

6.10. Go to “**Tracking the animal’s head & tail**” and select “**Yes, I want the animal’s head and tail to be tracked**.”

6.11. Go to “**Testing**” **|** “**Stages**,” and from the menu “**Add item**,” select “**New stage**.” Name the new stage, “**Acquisition**.” Define the duration of the stage (e.g., 600 s).

6.12. Repeat the previous step of “**Short-term memory test**” and “**Long-term memory test**” stages.

NOTE: In this protocol, all stages have the same duration (10 min).

6.13. Go to “**Procedures**.” Define the events to be tracked for each stage (acquisition, short-term memory test, and long-term memory test).

6.14. Start the test (with each animal). Go to “**Tests**” (in the top menu bar) and select “**Add a test (+)**.” Assign a number for the animal to be tested (e.g., “1”).

6.15. Select “**Record**” and name the animals and session (e.g., “M1 Acq”).

6.16. Before placing the animal in the arena, click one time on the “**Play**” button. A message “waiting to start” will be displayed.

6.17. After placing the animal in the arena, click a second time on the “**Play**” button. The test will start and end automatically.

6.18. Repeat steps 6.13-6.16 for the short-term memory test (2 h after the acquisition session) and the long-term memory test (24 h after the acquisition session).

## 7. Wireless electrophysiology device setup

7.1. Connect the modem to a PC host and turn it on. Turn off any other network device on the PC. Preferably, silence any other wireless communication in the registration room like Bluetooth, cell phones, other modems, or even wireless handsets.

7.2. Attach the amplifier to the rat's back, as mentioned in step 5.5.

7.3. Turn on the EEG device by plugging in the battery.

NOTE: 2 s after connecting the device, a red led on the EEG amplifier will blink, indicating that the communication with the modem is active, and then green led will be turned on. If the communication is successful, the LEDs on the modem will start to flash continuously. The amplifier is now ready to send information to the modem.

7.4. Launch the EEG software and set it up according to manufacturer instructions to integrate into the wireless EEG acquisition device

7.5. Press the "**Start Visualization**" button. The EEG software will display the actual signal acquisition.

NOTE: Use "**Windows Task Manager**" to assign the "**Real-time**" priority mode to avoid missing information during experimentation.

## 8. Electroencephalography (EEG) signal recording

8.1. After verifying that the EEG software is acquiring data, launch the Behavioral Tracking software, and set experimental protocol to verify that the animal is in the observation zone and the set-up is working correctly.

8.2. At this point, start the EEG software recording by pressing the "**Start Record**" button. After checking that the acquisition signal is running, start experimentation in the BTS.

8.3. After the experiment ends, return to the EEG software and stop the recording process. The recording will be saved by using a default name consisting of the date of recording using the following format: "yyyy-mmdd-hhmm_SubjectID_Ephys.plx". By default, all recordings are saved in the EEG software (NeurophysData) folder.

8.4. Check that both data files were created. Record the experiment log or change the name to avoid confusion.

## 9. Behavioral task and EEG signal synchronization

9.1. Open MATLAB and execute the command: convert_plx2mat. Such function will open a browser box. The converting functions are provided by the manufacturer and must be added to MATLAB's path.

9.2. Select the *.plx to convert and press "**Enter**" on MATLAB's command line to convert it to default parameters.

9.3. Open the BTS experimentation file and go to "**Protocol**." Click on the option "**Results, reports and data**" select all the events of both objects and click on "Choose the time format for the report," select the third option: "Show events times as real-time in HH:MM:SS.sss - for example 13:20:14.791."

9.4. Now go to "**File**" and click on "**Export**" and "**Export experiment as XML**," check "**Date and time of the test**," finally click on "**Create XML**."

9.5. Go to "**Export test data**" and click on "**Save Data**." A .csv file with events times will be created.

9.6. Repeat steps 9.1 to 9.5 for each file. In our case, the three experiments were: ACQ, STM, and LTM.

9.7. Once the EEG and behavior files are converted, collect them on a single folder. The folder must have six files, the three .mat files, and three .csv, respectively. In our case, files were called: PID_01_ACQ_N.mat, PID_02_STM_N.mat, PID_03_LTM_N.mat, PID_01_ACQ_M.csv, PID_02_STM_M.csv and PID_03_LTM_M.csv. ID refers to an animal's identification number.

9.8. Open "procesa_sujeto.m" function using MATLAB, and adjust the second line to the animal's ID.

9.9. Now move MATLAB to such folder and execute: "procesa_sujeto" to create figures of alpha and beta relative band to power associated with objects recognition on ACQ, STM and LTM stages.

NOTE: "procesa_sujeto" is a function that executes/runs several signal processing analyses. These analyses are summarized as follows in steps 9.10 to 9.15.

9.10. Filter Each EEG signal with a 4th order Butterworth bandpass filter at [5-40] Hz, using phase correction.

9.11. Visually inspect Signals before to the following analysis, and those channels with artifacts derived from defective electrodes placement or misadjustment by animal movements were excluded from further analysis.

9.12. Reference signals to common average to alleviate motion artifacts.

9.13. Segment EEG signals to form epochs of 4 s length synced by timestamps derived from BTS. The target events were the exploration of the object marked by the distance of the animal to objects border. These events are marked on the BTS timestamps and were used as identifiers fix the windows' positions. So, EEG epochs are delimited by 1 s before exploration begins to 3 s after. At this point, no validation about exploration length was used, but it will be considered for future researches.

9.14. Estimate spectral power density on those epochs by using Welch's periodogram method using 1 s window length, an overlap of 90%, Hanning window prior to Fourier transform estimation, with these parameters a resolution of 1 Hz was achieved.

9.15. Assess power spectral on each band by evaluating area under periodogram, and the values presented corresponds to relative energy, it means that the energy of each EEG band was divided by the epoch's total energy. This procedure also reduces erroneous estimations due to artifacts on EEG signals.

**REPRESENTATIVE RESULTS:**

The methods described above were applied to record EEG and rat activity simultaneously after the environmental enrichment treatment. Three-month-old male Wistar rats were under a medium-term environmental enrichment treatment protocol for 20 days, and they were operated to fix six skull screw electrodes paired on frontal, central and parietal regions referenced to a seventh electrode located at NZ. Animals were maintained under natural dark-light conditions, with *ad libitum* access to food and water. This work shows the integration between the EEG system and the behavioral tracking software for a simultaneous live recording. We only used animals treated under EE protocol since we do not pretend to compare the effectiveness of the treatment, but only exemplify the advantages of the equipment. As evidence that the 20 days environmental enrichment housing protocol used stimulates the adult neurogenesis, we present BrdU positive cell count data from animals under EE and animals housed under standard conditions from unpublished data from our lab. Three-month-old male Wistar rats were used. They were injected three times with BrdU with 12 h between each other. Animals were anesthetized (pentobarbital (50 mg/kg, i.p.) and euthanized by transcardial perfusion (see **Figure 5**). To ensure that the vest attached to the EEG device does not limit animal movements, we performed the open field test (OFT) into two groups, one group underwent surgery while wearing the equipment (vest and EEG amplifier), and the other group of animals remained intact without wearing the hardware. We did not find significant differences in the distance traveled by the animals in 10 min of testing (see **Figure 5**). The typical NORT protocol consists of the presentation of two objects, and the replacement of one of them with a new object. The behavioral tracking software monitored exploration time.

The Behavioral Tracking Software recorded a group of animals to evaluate their key performance parameters. Therefore, we used three parameters to evaluate exploration performance. The preference ratio was calculated using the animals' head time spent in the object zone, which reports the total amount of time that the animals' head spent in each object. Also, we calculated a preference ratio for the time spent moving towards the objects, which shows the total amount of time spent on every animal that was moving towards each object zone. Additionally, the spent time per visit to each object was calculated. **Figure 6** shows the three-parameter results mentioned above. In the acquisition trial, there were no distinctions between objects in the three assessed parameters: head time in the object zone for the three trials, time moving towards the objects for the three trials, and time per visit in each object. There were no differences in the STM trial. Meanwhile, in the LTM trial, an exploration preferent ratio significantly higher for the

novel object was seen. Additionally, in the LTM trial, a preference for the novel object in the time spent per visit (panel C) could be seen, as well. **Video 1** shows a representative example of a rat recorded in the experiment while **Video 2** shows a representative example of simultaneous EEG and behavioral recording.

It was possible to match time events tracked with the Behavioral Tracking and the EEG software recording using the computer's clock. **Figure 7** and **Figure 8** show the changes in EEG relative power over alpha and beta bands. These are related to motor control, concentration, and memory, suggesting that exploration is just related to these functions. The results of animal 3 show that alpha power tends to reduce on STM regarding ACQ and LTM, suggesting a desynchronization related to exploration or memory retrieval. The number of object recognition (processed epochs) was low. By this point, it is not possible to determine if a statistical test would validate if such difference is real, or an artifact was able to produce such experimental conditions. Nevertheless, epochs segmentation, labeling, and analysis have become possible by a timeline of simultaneous marking events in animals and EEG outcomes produced for future research projects. Combining these systems prevents a wrongful identification of events by a manual marking process, which has become a significant issue in animal experimentation purposes. The combination of the BTS and electrophysiological (EP) activity could be accurately associated with animal behavior; nevertheless, experimental conditions require the use of advanced signal processing techniques to eliminate motion artifacts and make improvements in the experimental setup effectively.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Examples of enriched environment (EE) conditions cage.** Housing was provided with toys and tubes, in which animals find novel and complex but no biological relevance.

**Figure 2: Positions of epidural electrodes in the rat skull.** The screws were simultaneously used as anchor for the headset and as electrodes. F = frontal; C = frontoparietal; P = parietal; 3 = left; 4 = right; NZ = as the ground reference.

**Figure 3: Representative images of an epidural (skull screw) electrodes implantation surgery.** Image showing implanted intracranial electrodes screws in rats at different stages of the surgery.

**Figure 4: Representative images of a rat along with the experimental setup.** The rat was made to wear the vest attached to the EEG device with an embedded battery, inside the arena used for the NORT protocol. The image shows the headset and the cable connector installed on the head's rat.

**Figure 5: Evidence of movement capability, and adult neurogenesis stimulation by EE protocol.** (**A**) Representative images of the animal activity for 10 min in the Open Field Test (OFT) and the mean distance that animals wearing the equipment/surgery traveled, and animals without the equipment/No surgery. (**B-E**) Representative DG section with BrdU labeled cells (intense dark) for EE and standard housing groups. Panels B and D show a low magnification of the DG, and

panels C and E show the box area at higher magnification. Panels B and C are tissue from the EE housing group, panels D and E are from the standard housing group. The inset illustrates the average numbers of labeled cells in both groups. ML - molecular layer; GCL – granular cell layer; SGZ – subgranular zone; arrows - BrdU+ cells. The graphs show the mean ± SEM. The T-student test was used to compare groups. * p≤0.05. No significant differences were found between groups in the Open Field Test.

**Figure 6: Exploration performance in NORT assessment.** (**A**) Head time in the object zone for the three trials. (**B**) Time moving towards the objects for the three trials. (**C**) Time per visit in each object. The graphs show the mean ± SEM. Two-way repeated-measures ANOVA with Sidak's multiple comparisons test was used in all parameters. * p≤0.05, ** p≤0.01 between the objects in the respective trial.

**Figure 7: Changes over alpha EEG band power associated with exploration**. This figure show changes in relative alpha power, from half second to 2.5 after animal begins the exploration of the objects. The six graphs corresponded to Frontal, Central, and Parietal electrodes (from top to bottom) and left and right sides. Boxplots show the distribution of such time series for each condition combination of an Object: “Familiar” and “Novel,” and stage: “ACQ,” “STM” and “LTM.”

**Figure 8: Changes over beta EEG band power associated with exploration.** This figure show changes on relative beta power, from half second to 2.5 after animal begins the exploration of the objects. The six graphs corresponded to Frontal, Central, and Parietal electrodes (from top to bottom) and left and right sides. Boxplots show the distribution of such time series for each condition combination of an Object: “Familiar” and “Novel,” and stage: “ACQ,” “STM” and “LTM.”

**Video 1: Representative video showing a rat recorded in the experiment.** The rat was inside the arena used for the NORT protocol. The rat was wearing the vest attached to the EEG device with an embedded battery.

**Video 2: Representative video showing simultaneous EEG and behavioral recording.** EEG signal was displayed on the left side while the behavioral test (NORT) was displayed on the right side of the video.

**DISCUSSION:**

Behavioral and electroencephalography research is difficult and challenging by nature. Therefore, the combination of both techniques presents significant critical steps. Thus, both concurrent techniques are not widely used. In real practice, every group around the world performs behavioral tests with special conditions, such as animals, analyzed parameters, or treatments. The above creates significant controversies in the field and the need for developing standard procedures available to everybody. Here, we have prepared this detailed procedure with all the critical steps and methodological considerations that are not usually described or mentioned in most of the published articles. These are discussed below.

Production of the needed materials is a fundamental step in the success of this technique. In this

regard, the electrode needs to be built from scratch using stainless steel screws, copper cables, and silver welder. These materials are difficult to weld together permanently, in such a manner that the conductivity and strength of each electrode must be verified before use. It is possible to use another type of wire for the electrode assembly; however, the copper is flexible enough to manipulate the electrode to insert it into the amplifier connector. In this regard, the use of commercial electrodes is desirable, but their acquisition could be complicated and expensive. The surgery is one of the most critical steps in this protocol. It is highly recommended and even necessary to have an experienced surgeon, particularly for electrodes implantation. Since the surgery frequently requires lengthening anesthesia time and sometimes a welding application during surgery, each laboratory must perform the necessary tests with the appropriate anesthesia (different cocktails can be used) for each strain of rodents, particularly under vivarium conditions, differences between litters, and even individual differences among animals. Proper planning and consideration could prevent losing animals during surgeries. The electrodes implantation is another crucial step. It requires great care to avoid punching the skull and damage meninges or brain tissue. Screws should be placed correctly, that is, completely fixed in the skull otherwise, noise and artifacts will be presented on signals, like those related to a lousy colocation or movement that does not use the EEG recording. Pre- and post-operative treatment and conditions must always be performed and observed to avoid the rodent's suffering. Subcutaneous lidocaine can be used on the head skin before making the incision with the scalpel. A drop of saline to the animal's eyes will help prevent dryness. Also, a saline solution must be administered in the mouth, and after the surgery, 1 mL must be administrated either subcutaneous or intraperitoneally to compensate the animal's fluid balance and prevent dehydration. Immediately after surgery, an anti-inflammatory medication (to reduce pain), as well as antibiotics via subcutaneous or topical antibiotics, must be administrated directly on the periphery of the scalp where the dental cement cap is located (to decrease the probability of infection). Repeat the above procedure 24 h after the surgery. The positioning of the EEG amplifier on the animal's back is the main difficulty for the simultaneous recording. The design and manufacture of a vest are specifically based on the animals' size. The vest must allow the natural movement of the rodent (see **Figure 5**). This latter will guarantee the main advantage of the technique, which is the recording of free movements. Since the animals did not attempt to remove the vest, the head connector, or cables after surgery and during subsequent days, it was presumed that the setup did not generate movement restriction significantly or caused pain or discomfort. For a correct EEG segmentation in epochs based on events marked by the BTS is mandatory to write down a well-defined protocol. The temporary marks could be merged by time series manipulation because both systems use the same clock to set up their timestamps. The above extends the possibilities for animal experimentation incorporating electrophysiological data for analysis.

The technique presented here can be used in any neuroscience research area and with the most common murine species and even other species. The versatility of the Behavioral Tracking Software is one of the most significant advantages since it could be used in a great versatility of mazes as Morris water maze, open-field, novel object recognition, conditioned place preference, hole board, elevated plus maze, Y-maze, radial arm maze, Barnes maze, and others. It can be used up to 16 cameras simultaneously. Additionally, hundreds of different measures (for more

detailed information see the manuals[31,32]) can be reported. Consider that this work describes experimentation for EEG recordings, some other techniques like Local Fields Potentials or single-unit recording are possible. However, users must consider that the general setup and several preparatory steps need to change for other purposes. So, when this technique is used together with EEG Wi-Fi recording, the possibilities are extended, because it adds new perspectives to animal studies like those performed on human beings to evaluate several characteristics of the EEG integration and dynamics, like connectivity, EEG band power, or evoked responses. Unlike human beings, animal experimentation is possible to evaluate drug administration, gene modifications, or expression, among many other experimental paradigms. For EEG analysis, consider that some protocols have a very low number of repetitions of the desired behaviors, which restrains the possibility to average responses and obtain reliable results. Therefore, be careful to design the recording and analysis protocols that it is considered to perform before beginning the experiment. Nevertheless, it must be contemplated that working in animal experimentation is not possible to prevent movement, increasing the complexity of the experimental protocol and considerations for signal analysis and behavioral tasks. Currently, equipment for full tracking systems and EEG recordings are not standardized or modular, which means that their setup is intended to a single protocol and adaptations to explore other behavioral tasks, implying/suggesting higher costs to a large number of laboratories. This situation could be solved by following the options explained in this study. Nevertheless, several improvements could be realized for more reliable experiments. The work can be improved at several steps starting from electrodes fabrication through behavioral and signals processing. Nonetheless, it is demonstrated that animal tracking and EEG acquisition are possible using an affordable high-tech but inexpensive setup.

In summary, the present work is an attempt to help scientists, particularly in the neurosciences field, to be able to use these two techniques that are not commonly used in combination. The simultaneous recording technique of EEG and behavioral testing using Behavioral Tracking Software has many advantages, and it can be particularly useful in many fields of Neuroscience, particularly in learning and memory areas. Considering this equipment has other capabilities as a deep recording of subcortical structures as the hippocampus, but as mentioned, several preparatory steps will change. Wireless equipment solves almost all the limitations of a conventional wire approach, such as animals' mobility problems from one cage to another, hindered or entangled animals with the cables. This setup technique is user-friendly, as described above, and an almost untrained or non-specialized group of experts or individuals can use this software. The price for the EEG equipment is lower than a regular EEG amplifier. Behavioral Tracking Software is also one of the most affordable software for video tracking in the market. This software requires annual licenses. The equipment can be used in more than one experimental setup, different animals, and the type of versatility. We hope that this effort will help the scientific community and provide an easy access to simultaneously study the behavior and electroencephalography.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

Dr. Sylvia Ortega-Martinez works as an employee of Stoelting Co., a company that provided and sponsored the production and open access to this article.

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FIGURE 1

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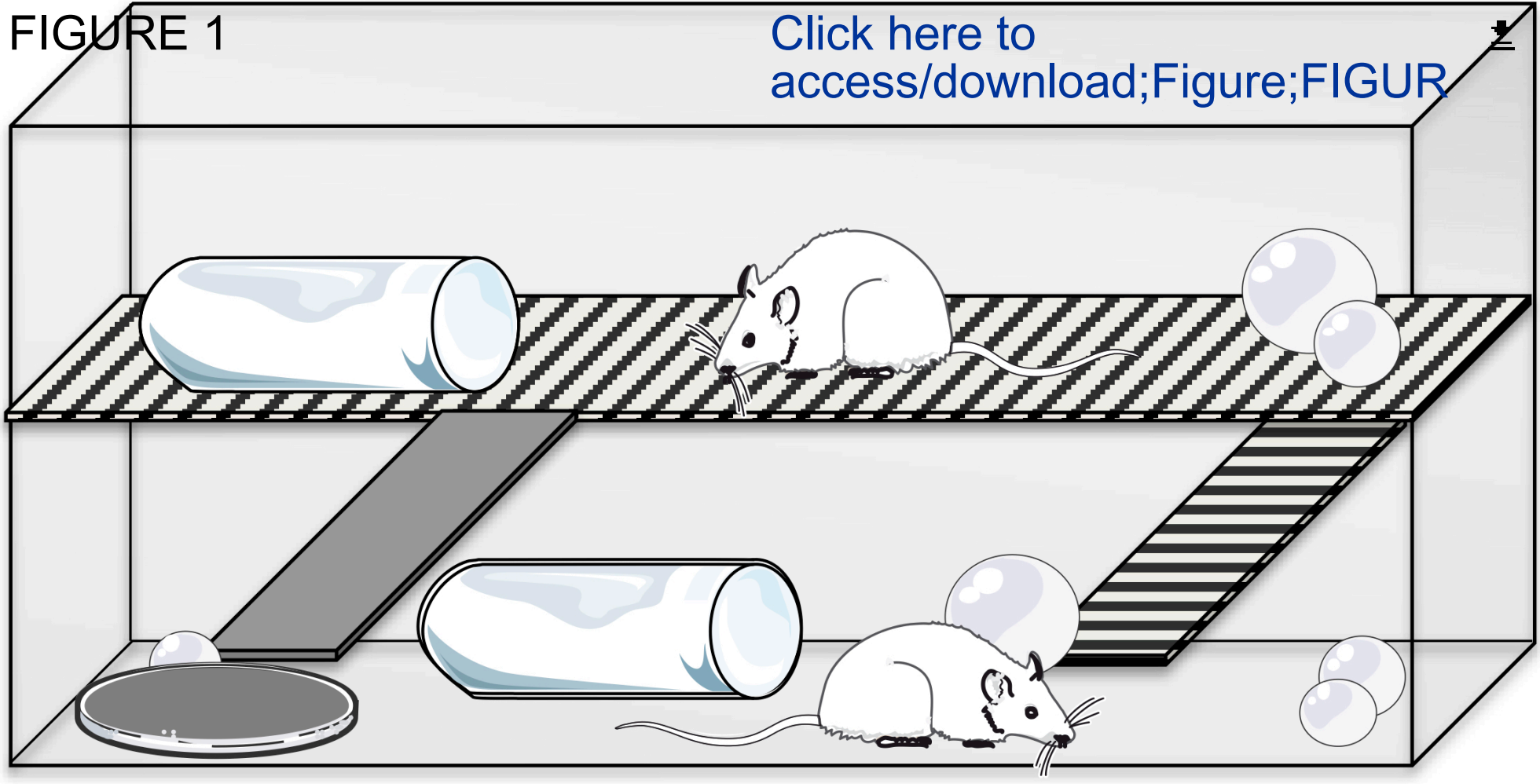


FIGURE 2



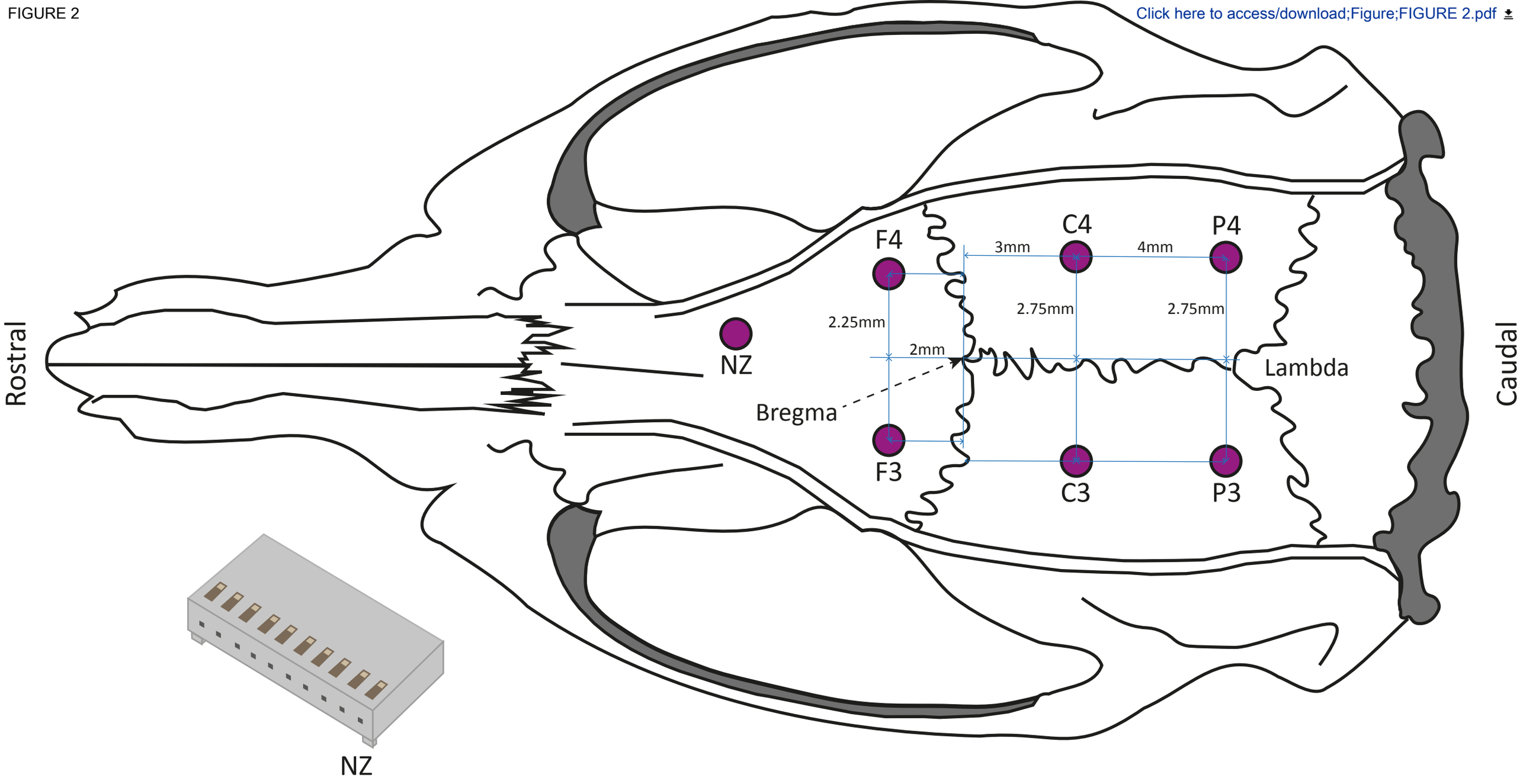
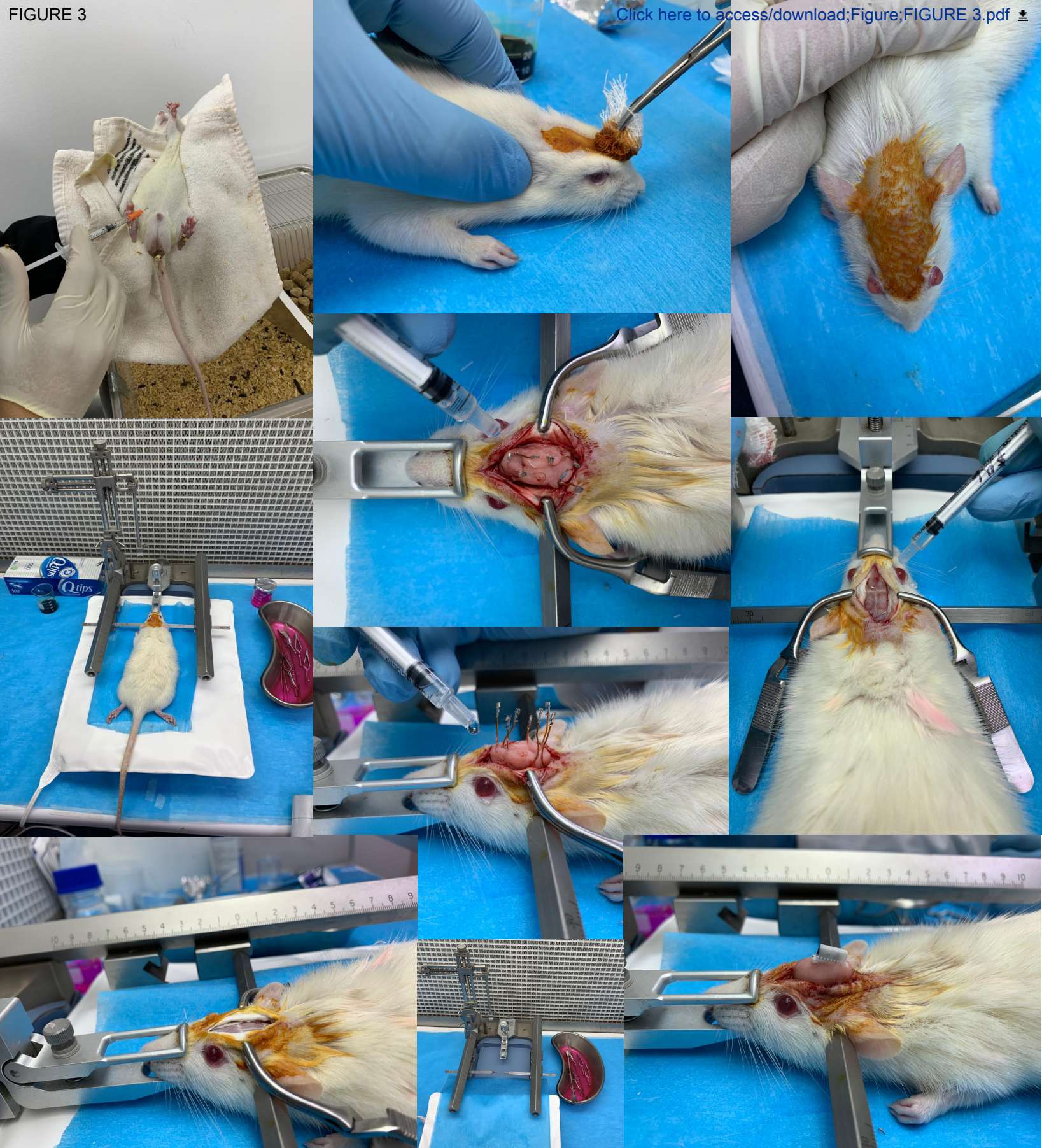
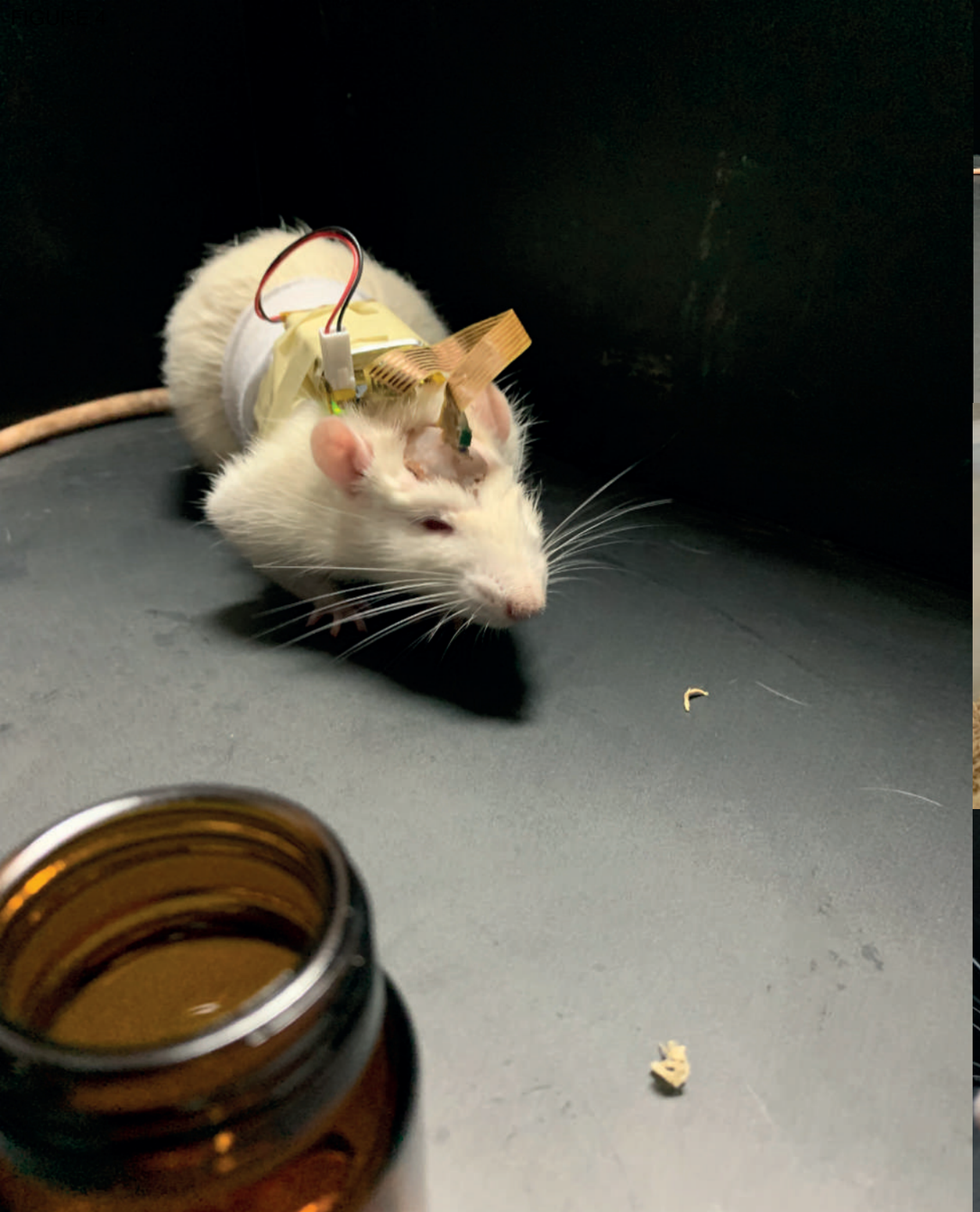


FIGURE 3







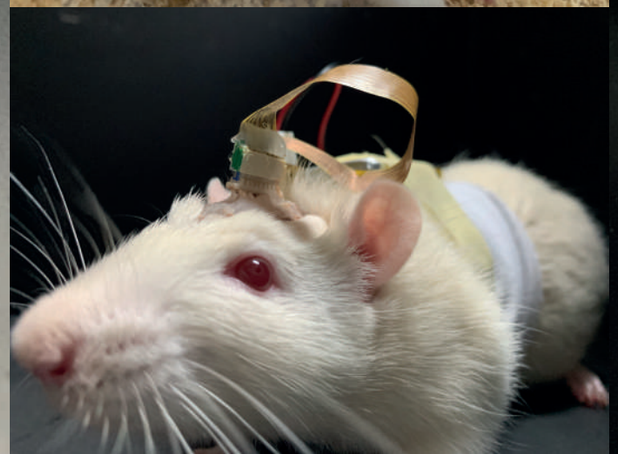


FIGURE 5

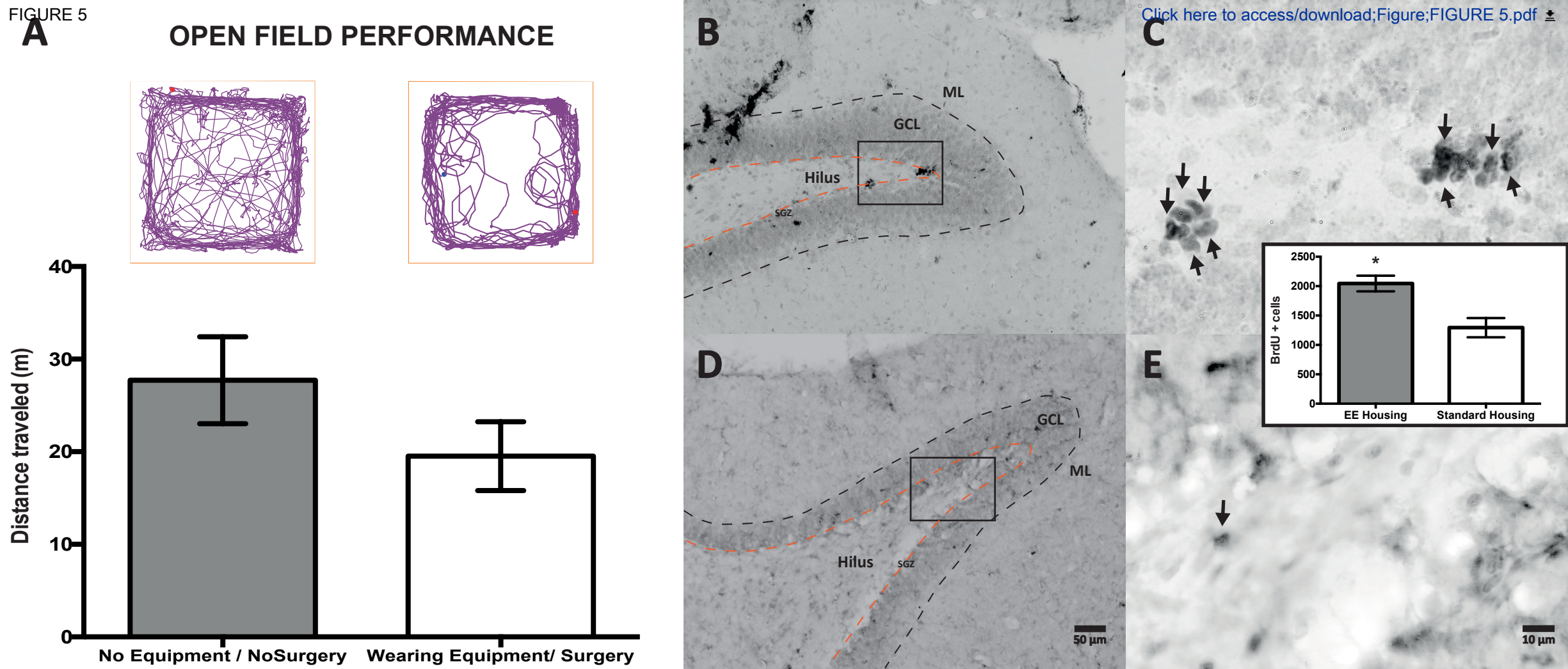


FIGURE 6



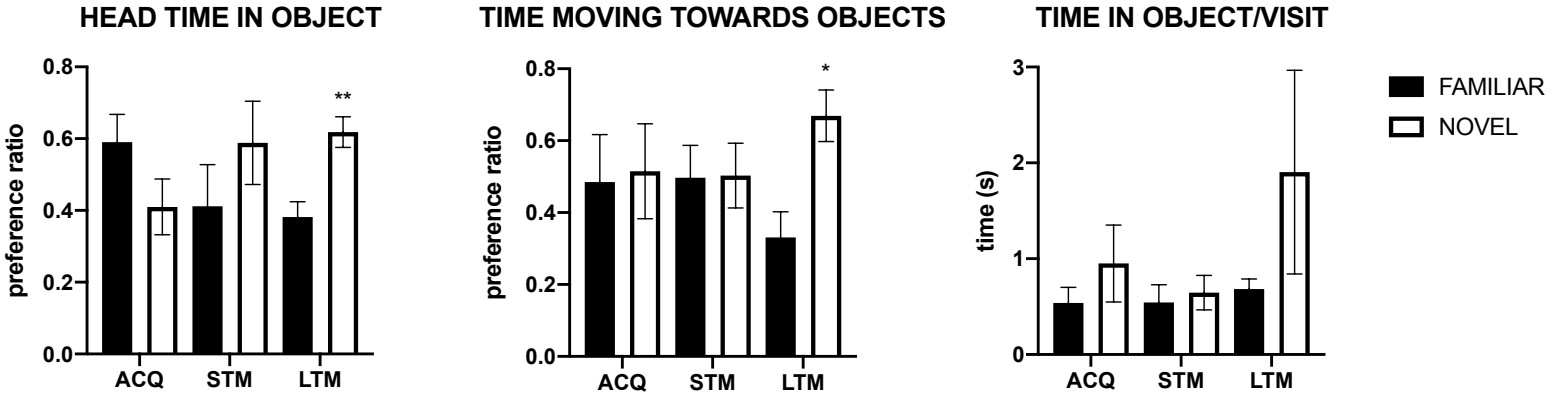


FIGURE 7

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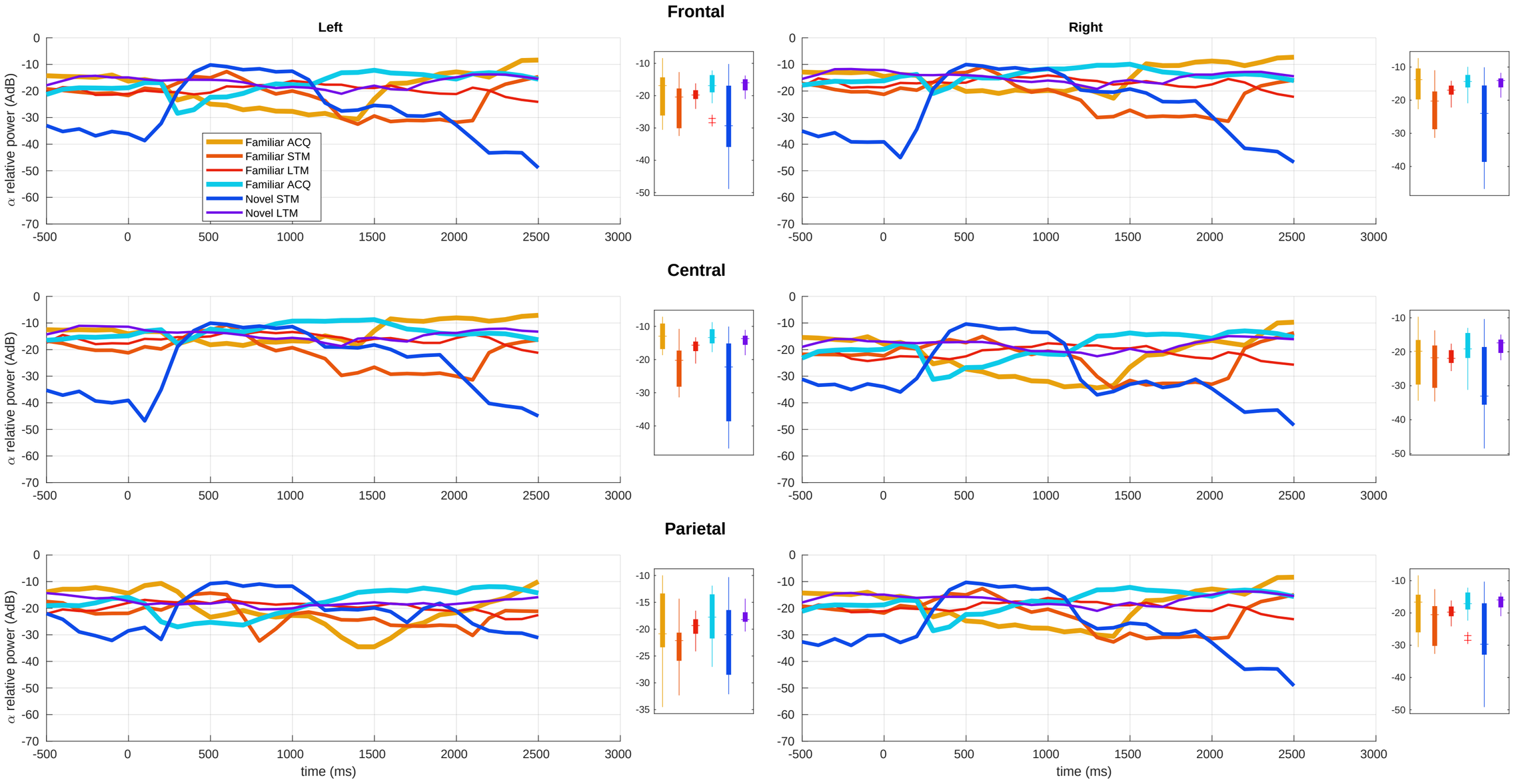

FIGURE 8

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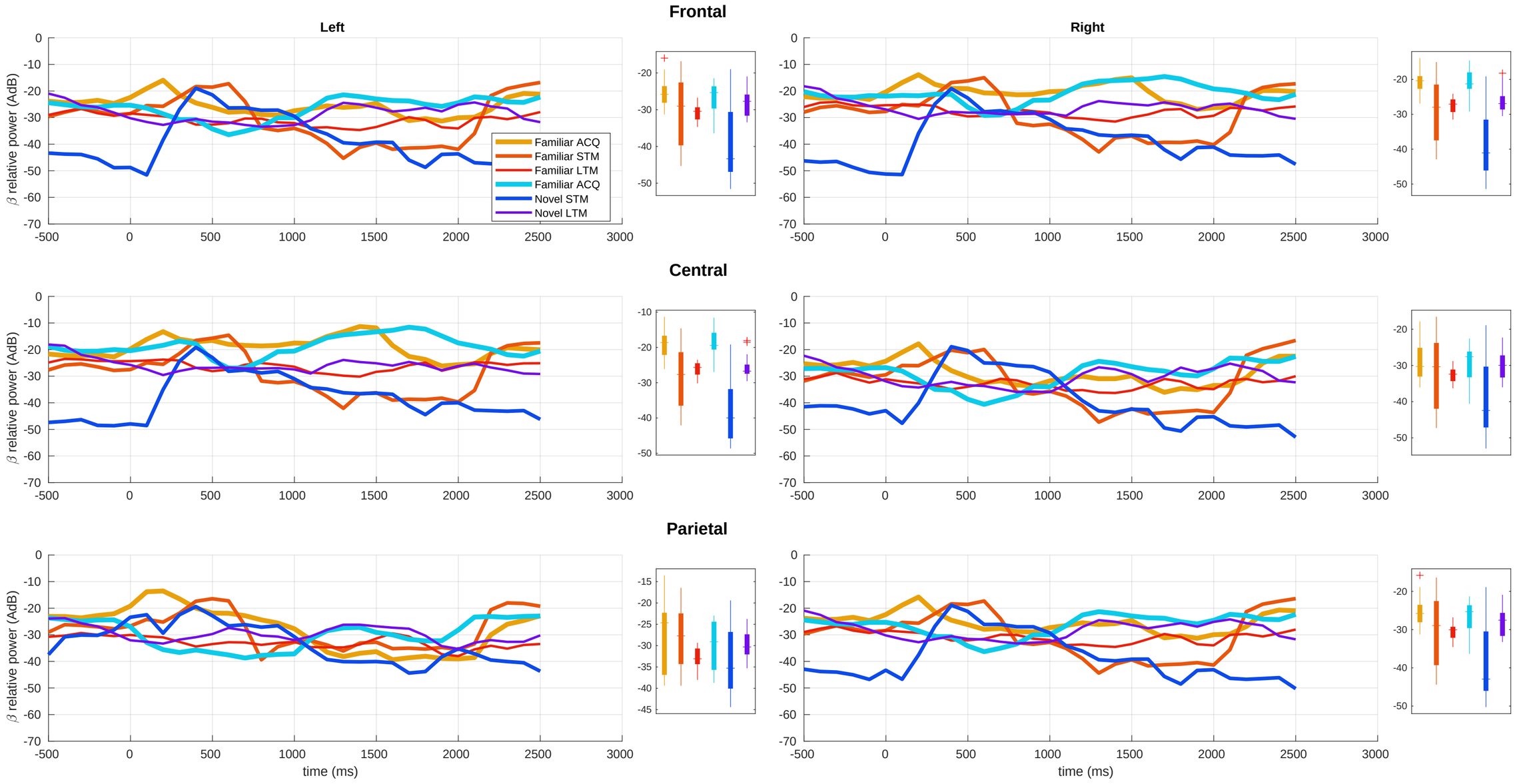

Video 1

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Video 2

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VIDEO 2.mp4

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| #2 Variable speed rotary tool tip | Reorder #310048, Lenght | SS White |
| #4 Scalpel and blade | | |
| 50 X 50 X 50 cm Open Field Black Mate Arena | | |
| 8 pin Receptacle Housing Female | Amphenol FCI | 10147606-00008LF |
| 8 pin Receptacle Housing Male | Amphenol FCI | 10147603-00008LF |
| Acrylic Resin | MDC Dental | NicTone |
| ANY-maze video tracking software | Stoelting, Co. | version 6.1 |
| benzalkonium chloride antiseptic solution | Benzal | Benzal |
| Bulldog clamps | Cientifica VelaQuin | |
| Camera | Logitech | c920 |
| Copper wire | | |
| Crimp contact | Amphenol FCI | 10147604-01LF |
| DELL PC | DELL | |
| Electrode | | |
| JAGA16 | Jinga-Hi, Inc. | JAGA16 |
| Ketamine | PiSA Agropecuaria | ANESKET |
| MATLAB | R2020a | MathWorks |
| Monomer | MDC Dental | NicTone |
| Neurophys software | Jinga-Hi, Inc./ Neurosys, LLC | Neurosys 3.0.0.7 |
| Screwdrive | | |
| Screws | | |
| Screws equiped with electrode | | |
| Stereotaxic instrument | KOPF | |
| Variable speed rotary tool | Dremel 3000 | Dremel |
| Voltmeter | PROAM | MUL-040 |
| Xilazine | PiSA Agropecuaria | PROCIN |
| | | |

| Comments/Description |
| --- |
| For making the holes where the screws will be |
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| For fixating the screws to the skull |
| http://www.anymaze.co.uk/) |
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| For retracting the skin |
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| For inserting the screws into the skull |
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| For making the holes where the screws will be |
| For confirming that the electrode conducts |
| For anesthesia |

Vineeta Bajaj, Ph.D.
Senior Review Editor
Journal of Visualized Experiments

1 July 2020

Dear Dr. Vineeta Bajaj,

Subject: Wireless electrophysiology (EEG) and memory behavioral test simultaneously monitored as a tool for the study of hippocampal neurogenesis. Manuscript No. JoVE61494.

Thank you for your email, enclosing the editor's comments. We have carefully reviewed the comments and have revised the manuscript accordingly. Our responses are given in a point-by-point manner below. The changes to the manuscript have been tracked to identify all of the manuscript edits. The corrections and suggestions provided by you helped improve the paper. We hope the revised version is now suitable for publication, and we look forward to hearing from you in due course.

Sincerely,
Mario Buenrostro-Jauregui, Ph. D.
Universidad Iberoamericana
México City
México
mario.buenrostro@ibero.mx

**General Statements about the Revised Manuscript**

We strived to cover most of the reviewers' comments. We addressed those comments that we felt were the most relevant and those who stuck to our article's focus. In other cases, we justify the reasons for noncompliance in the Point-by-Point Response to Comments section of this letter (see below). We also performed minor modifications throughout the article adds to those kindly recommended by reviewers to improve clarity and accuracy. Changes in the manuscript were agreed on by all authors. An academic English editor service was hired to revise and proofread the manuscript. All the changes in the manuscript by both the editor and us were tracked. You will find below a point-by-point response to yours' comments. Regular font style is used for featured comments, and italics have been used to show our responses.

**Point-by-Point Response to Editorial Comments**

The language in the manuscript is compromising the quality and not bringing out the clear message. Please proofread the manuscript well and/or employ professional copyediting services.

*Response: This issue was addressed. An academic English Editor service has edited the manuscript.*

Title reworded to make is concise: "Simultaneous Monitoring of Wireless Electrophysiology (EEG) and Memory Behavioral Test as a Tool to Study Hippocampal Neurogenesis."
*Response: We are agreed with the new title. (page 1, line 1).*

This is redundant as it is stated in the first line. Instead include clear reasons for doing so. "We will discuss the fabrication process of electrodes for EEG and simultaneous monitoring of EEG and behavior by video-tracking in real-time."
*Response: We changed the paragraph as "The protocol presented here provides information on the simultaneous electroencephalography (EEG) and behavioral assessment in real-time. We will discuss all processes involved for this purpose as an attractive solution for researchers in many fields of Neuroscience, particularly in learning and memory areas." (page 1, line 38).*

How are behavioral changes and EEG analysis related? "Therefore, the simultaneous monitoring of behavioral changes and EEG analysis are particularly interesting."
*Response: We reworded the paragraph as follows "Therefore, the simultaneous monitoring of behavioral changes and EEG is particularly interesting to correlate data between brain activity and task-related behaviors." (page 2, line 49).*

Please ensure the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
*Response: We reworded most parts of the Introduction section. (page 2, line 72).*

Citation? "In this regard, researchers have been widely using different paradigms of behavioral tests to analyze cognitive, motor, or emotional functions."
*Response: We reworded the paragraph as follows ". In this regard, researchers have been widely using different behavioral tests to analyze sensory-motor function, social interactions, anxiety-like and depressive-like behavior, substance dependence and various forms of cognitive functions." We added the next reference,* "Hånell, A., Marklund, N. Structured evaluation of rodent behavioral tests used in drug discovery research. *Frontiers in Behavioral Neuroscience*. **8** (JULY), 1–13, doi: 10.3389/fnbeh.2014.00252 (2014)."

Citation? “Video tracking is usually the most useful method for a quick and accurate behavior recording.”
*Response: We reworded the paragraph as follows “Video tracking is a valuable method for quick and accurate behavior recording used in a wide variety of applications.” We added the next reference* Noldus, L.P.J.J., Spink, A.J., Tegelenbosch, R.A.J. Ethovision Video Tracking System. *Behavior Research Methods, Instruments, & Computers*. **33** (3), 398–414, doi: 10.3758/BF03195394 (2001). *(page 2, line 80).*

This part needs clarity, please reword. “since it could reveal how the neuronal activity, from single cells to the major brain areas, correlates to specific behavioral patrons.”
*Response: We reworded the sentence as follows “Simultaneous recording of neuronal activity (from single cells to the major brain areas) and behavioral tasks could bring us light of how the brain generates specific behavioral patrons.” (page 2, line 85).*

Which activity is being recorded and in conjunction with what? “If this activity could be simultaneously recorded through multiple timescales.”
*Response: We reworded the sentence as follows “If neuronal activity and behavioral patrons could be simultaneously recorded through multiple timescales,.” (page 3, line 90).*

Video tracking behavior… ? “Behavioral videotracking.”
*Response: We reworded the paragraph as follows “Thus, synchronized recording of behavioral and neuronal activity at the desired scale (from neurons to large areas of the brain) is considered an extremely useful tool. There are several systems intended to integrate behavioral recordings with other measurements as neural activity.” (page 3, line 94).*

Unclear “Behavioral videotracking is a useful tool to synchronize recording electroencephalography (EEG) in real time. This has a specific feature that allows to record behavioral and neuronal activity at the desired scale (from neurons to large brain areas).”
*Response: We reworded the paragraph as follows “Thus, synchronized recording of behavioral and neuronal activity at the desired scale (from neurons to large areas of the brain) is considered an extremely useful tool. There are several systems intended to integrate behavioral recordings with other measurements as neural activity.” (page 3, line 94).*

Please reword for clarity. “The use of cables and swivel-devices that allows animals to move freely.”
*Response: We reworded the sentence as follows “Some solutions to this problem have been developed, as the use of cables and swivel-devices that allow animals to move freely in the arena.” (page 3, line 104).*

Signal on the animal or from the animal? “In this study, we used a commercially available wireless system that sends signals through a Wi-Fi connection on a freely-moving rodents system.”

*Response: We reworded the sentence as follows "In this study, we used a commercially available wireless system that sends EEG signals from the animal through a Wi-Fi connection with a freely-moving rodents system." (page 3, line 112).*

Citation? "This process is known to be associated with memory and learning improvement which increases adult neurogenesis in rodents through enriched environment (EE) conditions."
*Response: We added the next reference* Leal-Galicia, P., Romo-Parra, H., Rodríguez-Serrano, L.M., Buenrostro-Jáuregui, M. Regulation of adult hippocampal neurogenesis exerted by sexual, cognitive and physical activity: An update. *Journal of Chemical Neuroanatomy*. **101** (August), 101667, doi: 10.1016/j.jchemneu.2019.101667 (2019). *(page 3, line 126).*

Please reword for clarity. "It has been reported new granular cells (GC) in the hippocampal dentate gyrus (DG) from middle-aged mice exposed to EE just for 7 days."
*Response: We reworded the sentence as follows "It has been reported in middle-aged mice exposed to EE housing for seven days, the birth of new granular cells (GC) in the hippocampal dentate gyrus (DG)." (page 3, line 130).*

What kind of new cells? "Studies attempting to selectively ablate adult neurogenesis in adults rats have suggested that new cells of about 1 - 2 weeks of age are required in the learned response."
*Response: We added the "granular." word to the sentence "…suggested that new granular cells …."(page 4, line 133).*

Significance? Also please reword. ". It also enhances neurogenesis increasing the density of BrdU/NeuN DG granular cells in mice."
*Response: We reworded the sentence as follows ". Also, it has been reported that exposure to an enriched environment at 1–4 weeks after BrdU injections significantly increased the density of BrdU/NeuN cells in the DG granular layer in mice." (page 4, line 142).*

Please bring out clarity. "Authors suggest the existence of a critical period between the first and third weeks were a substantial increase in the number of new neurons observed19. Studies of adult hippocampal neurogenesis (AHN) in humans has been controversial since there is no direct evidence. However, recently Moreno-Jiménez et al. reported and described the developmental stages of AHN in the human adult brain, identifying thousands of immature neurons in the DG, thus demonstrating the persistence of AHN during both physiological and pathological aging in humans20. However, many researchers reply back to this finding. We argue that this result is due to their methodological procedures and how their research is framed, therefore, itis not a sign of evidential proof13. Based on the evidence mentioned previously, the study of AHN in animal models is more important than ever (for a more in-depth examination of AHN, see Leal-Galicia et al., 2019 review13)."
*Response: We reworded the paragraph as follows " These authors suggest a critical period between one and three weeks after EE exposure since a substantial increase in the number*

*of new neurons was observed. Studies of adult hippocampal neurogenesis (AHN) in humans has been controversial since there was no direct evidence. However, a recent report described the developmental stages of AHN in the human adult brain, identifying thousands of immature neurons in the DG, and thus demonstrating the persistence of AHN during aging in humans. Based on the evidence mentioned previously, the study of AHN in animal models is more important than ever (for a more in-depth examination of AHN, see Leal-Galicia et al., 2019 review).“ (page 4, line 145).*

Please reword. “. The present work will expose the protocol to monitor simultaneous behavioral changes (related to memory and learning) and electroencephalography using the wireless EEG and behavioral tracking software recording during the performance of novel object recognition task.”
*Response: We reworded the sentence as follows “The present work will expose all processes involved in the simultaneous behavioral video-tracking assessment (novel object recognition task) and wireless electroencephalography recording.” (page 5, line 186).*

Please move this to the table of materials “(http://www.anymaze.co.uk/).”
*Response: This issue was addressed.*

Please reword for clarity “This is an artisanal process that does not ensure a correct contact between both materials, so any improper adjustment could cause artifacts in EEG signals be affected in positioning during the analysis.”
*Response: We reworded the paragraph as follows “3.2.Roll one end of the copper wire to the head of a small-sized screw (electrodes) and make sure it is firmly fixed since this is a crucial step. Correct contact between both materials must be guaranteed to avoid artifacts in the EEG signals.” (page 6, line 258).*

From section 2 ( the one maintainedin enrichment cage?
*Response: We added a note before the 4.1 step as follows “NOTE: After 20 days of environmental enrichment treatment, the animals will undergo surgery following the procedure described below:.” (page 7, line 276).*

So you keep doing this for 5 min?
*Response: We reworded the paragraph as follows “4.6. Instill a drop of ophthalmic solution or saline to each animal’s eyes every 5-10 min to help them not dry out.” (page 7, line 303).*

In this figure eyes don’t have any ophthalmic ointment. Also the animal’s head is not shaved properly, Also do not see any iodine based scrub traces used on the surgical site.
*Response: We replace the figure with appropriate photos following the protocol's instructions. (page 8, line 340).*

Heating pad was not required for the experiment?

*Response: We added a note after the 4.3 step as follows "NOTE: Provide the animal with a heating pad for all the surgery since the anesthesia used in this procedure usually causes hypothermia and breathing problems." (page 7, line 294).*

Please bring out clarity… this is after drilling the screws right? "4.18. Keep the rats in single cages at the beginning of the surgery to seven days before conducting the tests for a full recovery."
*Response: We reworded the paragraph as follows "4.18. After the surgery, keep the rats in single cages for seven days before conducting the behavioral tests for a full recovery." (page 9, line 353).*

How do you manipulate? "4.19. Gently manipulate the animal on a periodical basis (at least once a day) to help to reduce the stress in future manipulations."
*Response: We reworded the paragraph as follows "4.19. Gently manipulate the animal on a periodical basis (at least once a day) to help to reduce the stress in future manipulations. While holding the rat with one hand, finger pressure is gently applied to the back of the animal, sliding the fingers through the fur." (page 9, line 357).*

After how many days of surgery is this process done? "NOTE: All behavioral procedures were performed between the 14:00-16:00 h which corresponds to the light cycle of the rat."
*Response: We reworded the NOTE as follows "NOTE: Seven days after surgery, we proceeded to behavioral tests. All behavioral procedures were performed between the 14:00-16:00 h, which corresponds to the rat's light cycle." (page 9, line 367).*

Are these same as in section 2? "5.3.Fix two identical objects to the floor center of the arena using double-sided tape (to prevent its displacement by the animals). Objects must be equidistant from each other and the arenas' walls."
*Response: We reworded the paragraph as follows "5.3. Fix two identical novel objects to the floor center of the arena using double-sided tape (to prevent its displacement by the animals). Objects must be equidistant from each other and the arena walls." (page 9, line 380).*

What previous manipulations were performed? Please refer to the step number. Also notes cannot be filmed. Please remove the highlight. "NOTE: Gentle previous manipulations might help to reduce the stress in animals during the connection procedure. Otherwise, the risk of damage on the device or the animals increases. Fully pre-charge the device battery using an USB-Port."
*Response: We removed the highlight and added the specific step number to the NOTE as follows "NOTE: Gentle previous manipulation of the animal (Step 4.19) might help to reduce the stress in animals during the connection procedure. Otherwise, the risk of damage to the device or the animals increases. Fully pre-charge the device battery using a USB-Port." (page 10, line 396).*

*Highlighted this because step 6.12 is highlighted. Steps 5.6.3. and 5.6.4.*

*Response: We highlighted steps 5.6.3. and 5.6.4. (page 10, line 421).*

Please move this to the table of materials "6.1.Open the Behavioral Tracking Software (version 6.1)."
*Response: We moved the version number to the table of materials. (page 10, line 434).*

How? "6.5.Go to "Apparatus." Define the arena area. Determine the object's region."
*Response: We reworded the paragraph as follows "6.5. Go to "Apparatus." Define the arena area, moving the orange rectangle to the out limits of the arena projected from the camera on the screen. Determine the object's region, moving the orange circles to the out limits of the objects inside the arena projected from the camera on the screen." (page 11, line 447).*

How? "6.6. Setup the scale. Move the ruler line to a position along the known length of the image (the arena). Enter the length of the object in millimeters in the option "The length of the ruler line is." of the Settings panel. In this case, the arena measures 500 x 500 mm."
*Response: We reworded the paragraph as follows "6.6.Setup the scale moving ruler line to a position along the known length of the image (the arena)." (page 10, line 452).*

Is this for video production purpose only? " (see Supplemental video 1)."
*Response: Supplemental Video 1 shows step-by-step instruction video for EEG device connection to NeuroPhys EEG and Any-Maze software. It can be used for production purposes. We want the video to be available as a supplemental video for users to use. (page 12, line 496).*

Added this, please check. "The files are provided by the manufacturer."
*Response: We reworded the sentence as follows "The converting functions are provided by the manufacturer and must be added to MATLAB's path." (page 13, line 544).*

If these steps needs filming please include all the scripts associated with it. There are scripts uploaded with the submission. Please include in the steps which one is used when? "Steps 9.7 - 9.9 "
*Response: These steps are not necessary to be filmed. (page 13, line 564).*

Not required. Please remove. This can be added to the materials table. "NOTE: Script was develop ped in collaboration with Jinga-Hi, Inc. "
*Response: We removed this and added to table of materials.*

If this needs to be in the protocol section, please make substeps. Please follow JoVE numbering pattern 1 followed by 1.1 followed by 1.1.1.. Please use imperative tense to describe how the actions are performed. Please refer to the steps above and format accordingly.
*Response: We reworded the paragraph as follows "9.10. Filter Each EEG signal with a 4th order butterworth passband filter at [5-40] Hz, using phase correction. 9.11.Visually inspect*

*Signals before to the following analysis, and those channels with artifacts derived from a defective electrodes placement or misadjustment by animal movements were excluded from further analysis. 9.12. Reference signals to common average to alleviate motion artifacts. 9.13. Segment EEG signals to form epochs of 4 s length synced by timestamps derived from BTS. The target events were the exploration of the object marked by the distance of the animal to objects border. These events are marked on the BTS timestamps and were used as identifiers fix the windows' positions. So, EEG epochs are delimited by 1 s before exploration begins to 3 s after. At this point, no validation about exploration length was used, but it will be considered for future researches. 9.14.Estimate Power spectral density on those epochs by using Welch's periodogram method using 1 s window length, an overlap of 90%, Hanning window prior to Fourier transform estimation, with these parameters a resolution of 1 Hz was achieved. 9.15.Assess Power spectral on each band by evaluating area under periodogram, and the values presented corresponds to relative energy, it means that the energy of each EEG band was divided by the epoch's total energy. This procedure also reduces erroneous estimations due to artifacts on EEG signals." (page 14, line 582).*

Please include reasons for performing BrdU staining in this case. Are the animals euthanized …. After how long, etc? "We present BrdU positive cell count data from animals under EE and animals housed under standard conditions, as evidence of increased neurogenesis of the EE protocol used (see Figure 5)."
*Response: We reworded the paragraph as follows "As evidence that the 20 days environmental enrichment housing protocol used stimulates the adult neurogenesis, we present BrdU positive cell count data from animals under EE and animals housed under standard conditions from unpublished data from our lab. We used three-month-old male Wistar rats. They were injected three times with BrdU with 12 h between each other. Animals were anesthetized (pentobarbital (50 mg/kg, i.p.) and euthanized by transcardial perfusion (see Figure 5)." (page 15, line 640).*

What are the three assessed parameters in this case? "In the acquisition trial, there are no distinctions between objects in the three assessed parameters."
*Response: We reworded the paragraph as follows "In the acquisition trial, there are no distinctions between objects in the three assessed parameters: head time in the object zone for the three trials, time moving towards the objects for the three trials, and time per visit in each object." (page 16, line 668).*

What does alpha and beta band represent?." Figures 7 and 8, show the changes in EEG relative power over alpha and beta bands."
*Response: We reworded the sentence as follows "Figures 7 and 8 show the changes in EEG relative Power over alpha and beta bands. These are related to motor control, concentration, and memory, suggesting that exploration is just related to these functions." (page 16, line 677).*

Please include a one liner title for all the panels combined. "Figure 5."

*Response: We added a liner title for all the panels combined as follows "Figure 5: Evidence of movement capability, and adult neurogenesis stimulation by EE protocol." (page 17, line 722).*

Please include a one liner title. "Figure 7."
*Response: We added a liner title for all the panels combined as follows "Figure 7: Changes over alpha EEG band Power associated with exploration." (page 17, line 741).*

Please include a one liner title. "Figure 8."
*Response: We added a liner title for all the panels combined as follows "Figure 8: Changes over beta EEG band Power associated with exploration." (page 18, line 748).*

Are these for production? In that case, it will be used for video production. "Supplemental video 1. Supplemental video 2."
*Response: Supplemental Video 1 shows step-by-step instruction video for EEG device connection to NeuroPhys EEG and Any-Maze software. Supplemental Video 2 shows step-by-step instruction video for the integration between NeuroPhys EEG and Any-Maze software's files. They can be used for production purposes. We want the videos to be available as supplemental videos for users to use. (page 18, line 763).*

Please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
*Response: We reworded most parts of the Discussion section. (page 18, line 772).*

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The undersigned, Angelica Dennisse Alvarez Moreno, legal representative of the translation agency Tekamolo, S.C., validates that the revision of this document written by PhD. Mario Buenrostro Jáuregui and entitled as "*Simultaneous Monitoring of Wireless Electrophysiology (EEG) and Memory Behavioral Test as a Tool to Study Hippocampal Neurogenesis*", delivered on July 3, 2020 was carried out and reviewed by Yeshwanthi Kamalraj, English and Spanish translator and reviewer of the United States nationality, who is also part of the team of the highly experienced translators of the company.

Mexico City, July 6, 2020, 2020

Angélica Dennisse Alvarez Moreno
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