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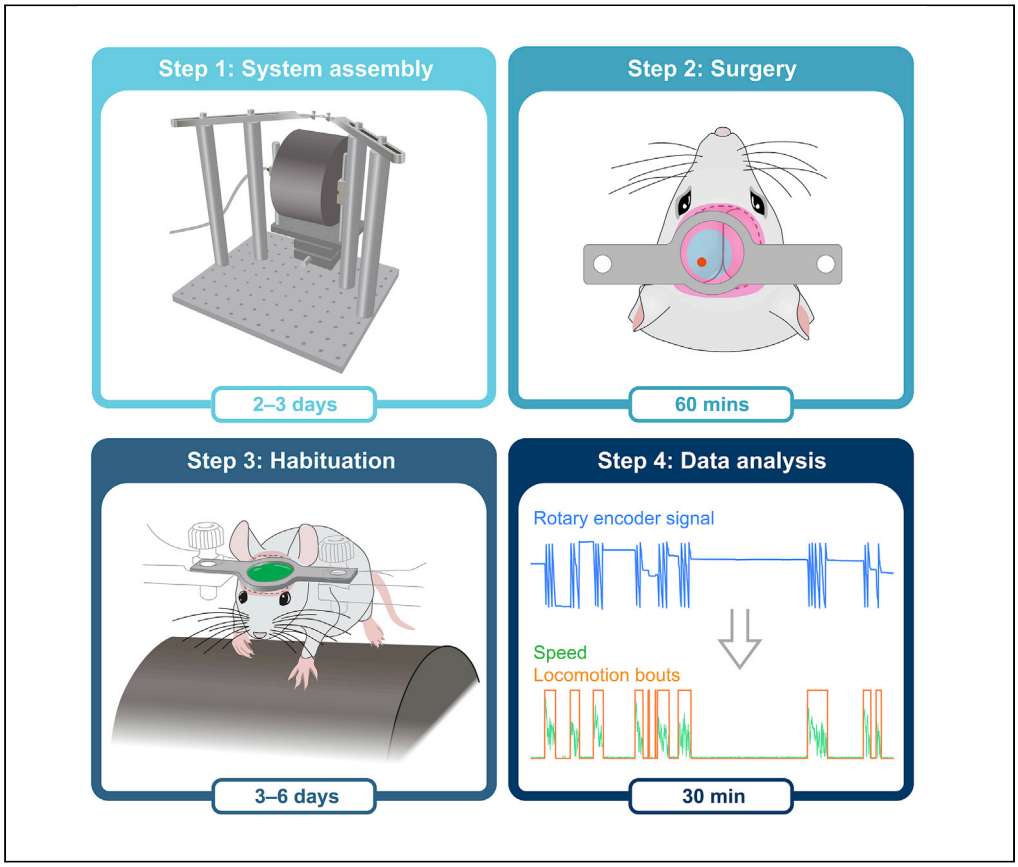
# Versatile treadmill system for measuring locomotion and neural activity in head-fixed mice




Here, we present a protocol for using a versatile treadmill system to measure locomotion and neural activity at high temporal resolution in head-fixed mice. We first describe the assembly of the treadmill system. We then detail surgical implantation of the headplate on the mouse skull, followed by habituation of mice to locomotion on the treadmill system. The system is compact, movable, and simple to synchronize with other data streams, making it ideal for monitoring brain activity in diverse behavioral frameworks.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Step-by-step protocol for building and using a treadmill system for head-fixed mice

Stable surgical implantation of head-fixation headplate

Gradual habituation of mice to locomotion on the treadmill system

Open-source analysis code for extracting quantitative locomotion metrics

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# Versatile treadmill system for measuring locomotion and neural activity in head-fixed mice

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## SUMMARY

**Here, we present a protocol for using a versatile treadmill system to measure locomotion and neural activity at high temporal resolution in head-fixed mice. We first describe the assembly of the treadmill system. We then detail surgical implantation of the headplate on the mouse skull, followed by habituation of mice to locomotion on the treadmill system. The system is compact, movable, and simple to synchronize with other data streams, making it ideal for monitoring brain activity in diverse behavioral frameworks.**

**For complete details on the use and execution of this protocol, please refer to Rasmussen et al. (2019).**

## BEFORE YOU BEGIN

This protocol describes acquiring, building, and utilizing a flexible and easy-to-use treadmill system for measuring locomotion (i.e., walking and running) in head-fixed mice (Rasmussen et al., 2019) (Figure 1). The system is assembled on a single breadboard, which allows it to be easily moved between different recording rigs. To ensure that the system accommodates mice of different sizes, the position of the mouse on the treadmill is adjustable along all three axes (i.e., forward-backward, left-right, and up-down). To measure locomotion at a high temporal resolution — required for aligning neural activity and locomotion in time — a magnetic rotary encoder is attached to the treadmill and connects to the data acquisition device with a BNC cable, a feature that furthermore makes it easy to synchronize treadmill activity to other data streams recorded in parallel. The design of the head-fixation apparatus was motivated by the need for maximal stability, essential for whole-cell patch-clamp recordings and subcellular imaging in awake behaving mice. Finally, we provide MATLAB analysis code for extracting and characterizing the treadmill-derived data.

The protocol consists of the following major steps: 1) *manufacturing of custom parts*, 2) *assembling the treadmill system*, 3) *surgical headplate implantation*, 4) *habituation*, and 5) *data analysis*.

### Institutional permissions

All procedures explained and performed in this protocol were approved by the Danish National Animal Experiment Committee.

### Manufacture custom parts

Timing: ~2–3 weeks

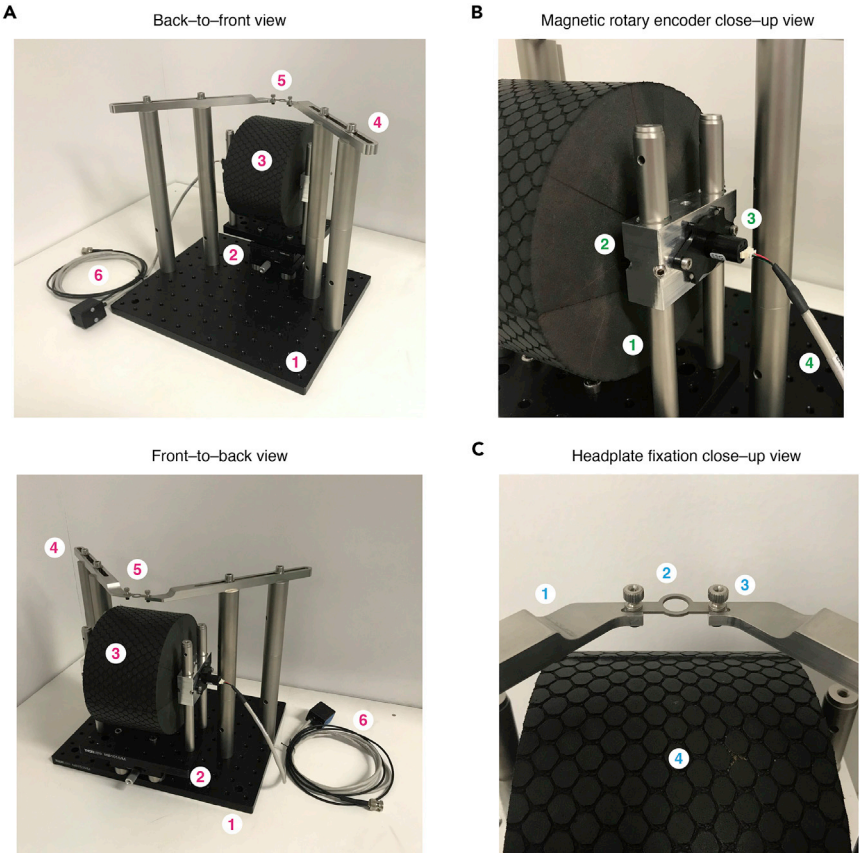


**Figure 1. Treadmill system for head-fixed mice**

(A) Photos showing the assembled treadmill system from the back (top) and front (bottom). 1: breadboard, 2: translation stage, 3: foam roller-based treadmill, 4: head-fixation apparatus, 5: head-fixation headplate, and 6: magnetic rotary encoder and accompanying electrical circuit.

(B) Photo highlighting the assembly of the treadmill and magnetic rotary encoder. 1: optical post, 2: ball-bearing block, 3: magnetic rotary encoder base and body, and 4: magnetic rotary encoder cable.

(C) Photo highlighting the fixation of the headplate on the treadmill system. 1: headplate-fixation arm, 2: head-fixation headplate, 3: thumb nut for securing the headplate, and 4: foam roller-based treadmill.

The treadmill system is designed to consist as much as possible of commercially available off-the-shelf parts (see key resources table). However, few parts need to be custom made (listed below). Blueprints and photos for these are available on GitHub: https://github.com/Neurune/TreadmillSystem.

1. Foam roller cut to a width of 9 cm (Ø15 cm, weight = 61 g) with a 6 mm diameter drilled center hole.

   *Note:* The foam roller constitutes the surface upon which the mouse rests and locomotes. The rationale behind this material solution is that it is lightweight, ensuring that the mouse can easily move the treadmill, furthermore, it is easy to clean between animals and sessions, and finally, is soft, providing the surface some "give" such that the mouse cannot exert strong forces against the headplate implant (Jordan, 2021).

2. Aluminum shaft with a length of 18 cm and a diameter of 6 mm.

   *Note:* The shaft constitutes the base upon which the foam roller, shaft collars, washers, and ball bearings are attached. Furthermore, on one end of the shaft, the magnetic hub — from the magnetic rotary encoder kit (see key resources table) — is attached.

3. Aluminum ball-bearing blocks.

   *Note:* The blocks provide the scaffold for the ball bearings attached on each end of the shaft passing through the foam roller, allowing the treadmill to rotate smoothly, and being mounted on standard optical posts at different heights.

4. Stainless steel headplate-fixation arms.

   *Note:* The metal arms provide the base for securing and stabilizing the headplate implant of the mouse while being on the treadmill. The design of these arms was inspired by previous work (Nestvogel and McCormick, 2022), with maximal stability as the critical parameter. Moreover, the 45° angle of the arms allows the mouse's visual field to be largely unobstructed. In case the length of the arms interferes with other equipment on the rig, this can be reduced.

5. Titanium headplate.

   *Note:* The headplate, when implanted on the skull of the mouse, allows fixation of the head during habituation and experiments via two screws on the headplate-fixation arms. The design of the headplate is simple, and biocompatibility, strength and light weight is obtained by producing it in Grade 2 titanium (thickness = 1 mm).

6. 3D-printed plastic enclosure for magnetic rotary encoder interface.

   *Note:* The enclosure, consisting of a box and a lid, secures and shields the small electrical circuit needed for powering the magnetic rotary encoder, detecting the rotational angle of the treadmill, and for connecting it to the data acquisition device. Note that when printing the enclosure, we advise to print with at least four layers to accommodate cutting the M3 threads later in the protocol.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Isoflurane | Zoetis | Cat# 199112 |
| Carprofen (Rimadyl) | Local pharmacy | N/A |
| Lidocaine | Local pharmacy | N/A |
| Buprenorphine (Temgesic) | Local pharmacy | N/A |
| Hydrogen peroxide | Apopro | Cat# 221379 |
| Deposited data | | |
| Example treadmill data | Nedergaard Lab | https://github.com/Neurune/TreadmillSystem |
| Experimental models: Organisms/strains | | |
| Female and male C57BL/6 mice (8–14 weeks) | Janvier | Strain name: C57BL/6JRj |
| Software and algorithms | | |
| Axon Clampex data acquisition software | Molecular Devices | https://support.moleculardevices.com/s/article/Axon-pCLAMP-11-Electrophysiology-Data-Acquisition-Analysis-Software-Download-Page |
| MATLAB 2017a and 2021b | MathWorks | https://se.mathworks.com/products/matlab.html |
| Custom code for treadmill data analysis | (Rasmussen et al., 2019) | https://github.com/Neurune/TreadmillSystem |
| Other | | |
| EVA Foam roller (Ø15 mm, length = 30 cm, weight = 204 g) | Yogaudstyr | https://yogaudstyr.dk/product.php?id=899&style=g |
| Aluminum shaft (Ø6 mm, length = 18 cm) | Local machine shop | https://github.com/Neurune/TreadmillSystem |
| Shaft collars | Ruland | Cat# MCL-6-A |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Ball bearings | SKF | Cat# 628/6-2Z |
| M6 washers | Thorlabs | Cat# W25S050 |
| Optical posts (Ø12.7 mm) | Thorlabs | Cat# TR150/M |
| M6 set screw kit | Thorlabs | Cat# HW-KIT4/M |
| M6 cap screw kit | Thorlabs | Cat# HW-KIT2/M |
| M4 set screw kit | Thorlabs | Cat# HW-KIT3/M |
| M3 cap screw kit | Thorlabs | Cat# HW-KIT5/M |
| M5 set screws | RS PRO | Cat# 137-809 |
| M3 countersunk nylon screws | RS PRO | Cat# 232-6931 |
| Small breadboard (width = 15 cm, length = 10 cm, thickness = 12.7 mm) | Thorlabs | Cat# MB1015/M |
| Large breadboard (width = 30 cm, length = 25 cm, thickness = 12.7 mm) | Thorlabs | Cat# MB2530/M |
| Dovetail translation stage | Thorlabs | Cat# DT25/M |
| Post spacer | Thorlabs | Cat# RS10M |
| Magnetic rotary encoder kit | US Digital | Cat# MAE3-A10-236-500-18-B |
| Magnetic rotary encoder connector cable | US Digital | Cat# CA-MIC3-SH-NC-5 |
| 5 V power supply | RS PRo | Cat# 121-7115 |
| Custom aluminum ball-bearing block | Local machine shop | https://github.com/Neurune/TreadmillSystem |
| 3D-printed enclosure for rotary encoder interface | Local machine shop | https://github.com/Neurune/TreadmillSystem |
| DC power connector plug | RS PRO | Cat# 448-382 |
| RG178 coaxial cable with BNC male plug | Pasternack | Cat# PE33130 |
| Rubber grommets (dimensions = 5 × 8 × 1.5 mm) | RS PRO | Cat# 187-9576 |
| Cable ties (width = 2.5 mm) | RS PRO | Cat# 213-2993 |
| Heat shrinking tube | RS PRO | Cat# 700-4535 |
| Optical posts (Ø25 mm, length = 15 cm) | Thorlabs | Cat# RS150/M |
| Optical post (Ø25 mm, length = 10 cm) | Thorlabs | Cat# RS100/M |
| Custom stainless steel head plate-fixation arms | Local machine shop | https://github.com/Neurune/TreadmillSystem |
| M2.5 cap screws (length = 8 mm) | McMaster-Carr | Cat# 91290A100 |
| Custom titanium head plates | Local machine shop | https://github.com/Neurune/TreadmillSystem |
| M2.5 thumb nuts | McMaster-Carr | Cat# 96445A320 |
| M2.5 hex nuts | McMaster-Carr | Cat# 94159A310 |
| Axon Digidata 1550B4 data acquisition system | Molecular Devices | https://www.moleculardevices.com/products/axon-patch-clamp-system/digitizers/axon-digidata-1550b-plus-humsilencer |
| Surgical tools: scissor, forceps, scalpel | Fine Science Tools | https://www.finescience.com/en-US/ |
| Isoflurane anesthesia system | Dameca | Cat# 11547 |
| Viscotears eye ointment | Webapoteket | Cat# 464110 |
| Feedback-controlled heating pad | World Precision Instruments | Cat# BF100-50-10 |
| Stereotaxic apparatus | World Precision Instruments | Cat# 505313 |
| Cotton swabs | Webapoteket | Cat# 221318 |
| Ethanol wipes | Webapoteket | Cat# 224765 |
| Iodine swabs | Fisher Scientific | Cat# 19-061617 |
| Insulin syringes | BD | Cat# 324921 |
| Dental drill | AgnThos | Cat# 1474w/o1464 |
| Vetbond tissue adhesive | Fisher Scientific | Cat# 13204619 |
| Paladur dental cement | AgnThos | Cat# 203097 |
| Kwik-Cast silicone sealant | World Precision Instruments | Cat# KWIK-CAST |
| Standard surgical microscope | Leica | https://www.leica-microsystems.com/ |

## STEP-BY-STEP METHOD DETAILS

### Assemble the treadmill system

Timing: ~2–3 days

The assembly of the treadmill system consists of three main steps: 1) *assembling the treadmill*, 2) *assembling the magnetic rotary encoder interface*, and 3) *assembling the head-fixation apparatus*.

1. Assemble the treadmill.
   a. Affix the foam roller to the ball bearing blocks.
      i. Push the aluminum shaft through the pre-drilled center hole (Ø6 mm) in the foam roller and slide shaft collars and washers onto the shaft from each side.
      ii. Slide the ball bearings onto the shaft and affix them within the ball-bearing blocks on each side of the foam roller by securing the ball bearings in place within the groove of the blocks.

   ***Note:*** The aluminum shaft should not be centered through the foam roller, rather 20 mm of shaft needs to extend on one side for the attachment of the magnetic rotary encoder.

   b. Build base for the treadmill.
      i. Position four post spacers on top of the large breadboard (width = 30 cm, length = 25 cm, thickness = 12.7 mm) and secure the dovetail translation stage on top using cap screws. Choose the position on the breadboard based on where you want the treadmill positioned.
      ii. Secure the small breadboard (width = 15 cm, length = 10 cm, thickness = 12.7 mm) on top of the translation stage using cap screws and attach four optical posts (Ø12.7 mm) using set screws. Validate that the translation stage can move within the full XY range.

   ***Note:*** In case the small breadboard is not perfectly stable on the translation stage, four contact points of power tack adhesive can be added between the board and the stage.

   c. Connect the treadmill to the base.
      i. Slide the ball-bearing blocks onto the four optical posts and secure them with set screws. Make sure that the foam roller is level and rotates unhindered.
      ii. Push the magnetic hub — from the magnetic rotary encoder kit (see key resources table) — onto the exposed end of the aluminum shaft and secure the base and encoder body using cap screws.
2. Assemble the magnetic rotary encoder interface.
   a. Join the rotary encoder interface circuit.

   ***Note:*** While not strictly required, we advise the researcher to perform this step with someone who has prior experience with assembling electrical wiring circuits, or alternatively, outsource this part to the local electrical workshop. For detailed schematics and photos on how to join the rotary encoder interface circuit see files available on GitHub: https://github.com/Neurune/TreadmillSystem.
      i. Drill two holes (Ø2.5 mm) in the 3D-printed plastic interface box, used for cutting M3 threads to hold the screws securing the box lid, according to the "*Rotary encoder interface box.pdf*" file.
      ii. Cut M3 threads in the two drilled holes using a M3 tap drill.
      iii. To protect the cables, fit rubber grommets to the two larger holes (Ø8.2) in the interface box, which will hold the rotary encoder connector cable and the coaxial cable.
      iv. Cut the rotary encoder connector cable and the coaxial cable to the desired length and pull them through the rubber grommets.
      v. Add a piece of heat shrinking tube to the exposed wires of the rotary encoder connector cable and the coaxial cable.
      vi. Solder the wires of the rotary encoder connector cable and the coaxial cable to the DC power connector plug according to the "*Rotary encoder interface diagram.pdf*" file. That is, for the rotary encoder connector cable: red wire (+5 V DC) soldered to the DC power socket pin 1 (pin A), black wire (ground) soldered to pin 2 (pin C), and green wire

(analog output) soldered to the center core of the coaxial cable. For the coaxial cable, the braid (ground) is soldered to the DC power socket pin 2 (pin C).

vii. Shrink the two pieces of heat shrinking tube and secure the cables inside the interface box using cable strips.

viii. Click the DC power socket into the recess in the interface box and secure the lid using two counter sunken nylon screws.

*Note:* The length of the rotary encoder connector cable and the coaxial cable will depend on the geometry of the experimental setup (i.e., distance to the data acquisition device which samples the treadmill output). In our setup, the rotary encoder connector cable and the coaxial cable is 155 cm and 110 cm, respectively.

b. Connect and test the rotary encoder interface.

i. Connect the rotary encoder connector cable to the encoder body, the coaxial cable (with a BNC male plug) to the data acquisition device, and power the rotary encoder interface circuit with the 5 V power supply.

ii. Test that the rotary encoder is working by turning the treadmill at different speeds while visualizing the analog output voltage signal (0–5 V) on a PC or an oscilloscope. The encoder should sense absolute shaft position information over 360° of rotation with no stops or gaps.

⚠ CRITICAL: When connecting the rotary encoder to the data acquisition device, it is very important to configure the input port to be working in high-impedance mode; if the port is terminated with, for example, 50 Ohm the output signal of the rotary encoder will be severely distorted.

3. Assemble the headplate-fixation apparatus.

a. Affix headplate-fixation arms to breadboard.

i. Attach four optical posts (each composed of two optical posts stacked; height = 15 cm and 10 cm, respectively, Ø25 mm) to the breadboard using set screws.

ii. Screw, from the bottom, small cap screws through the threads in the custom stainless steel headplate-fixation arms and secure these onto the optical posts by cap screws and washers using the extended groove.

b. Test headplate fixation and adjust treadmill height.

i. Slide the two small holes at the ends of the headplate down onto the cap screws and fit the headplate ends into the small groove on each of the arms.

ii. Lock the headplate by either thumb nuts or hex nuts.

*Note:* We prefer to use the thumb nuts for habituation sessions, as these are easy to maneuver and tighten by hand, and the hex nuts for experiments as these have a lower profile (an advantage for immersion objectives with short working distance) and can be tightened with a nut driver.

iii. Adjust the height of the treadmill relative to the headplate.

*Note:* The optimal position will depend on the size of the mouse, but a starting point is to have 4 cm between the headplate and the top of the foam roller, when this is centered along the forward-backward axis relative to the circular opening of the headplate.

### Headplate implantation

Timing: ~60 min

Here, we describe how to surgically implant the headplate on the skull of the mouse for subsequent head fixation (Figure 2). Note that the position of the headplate on the skull dictates which brain

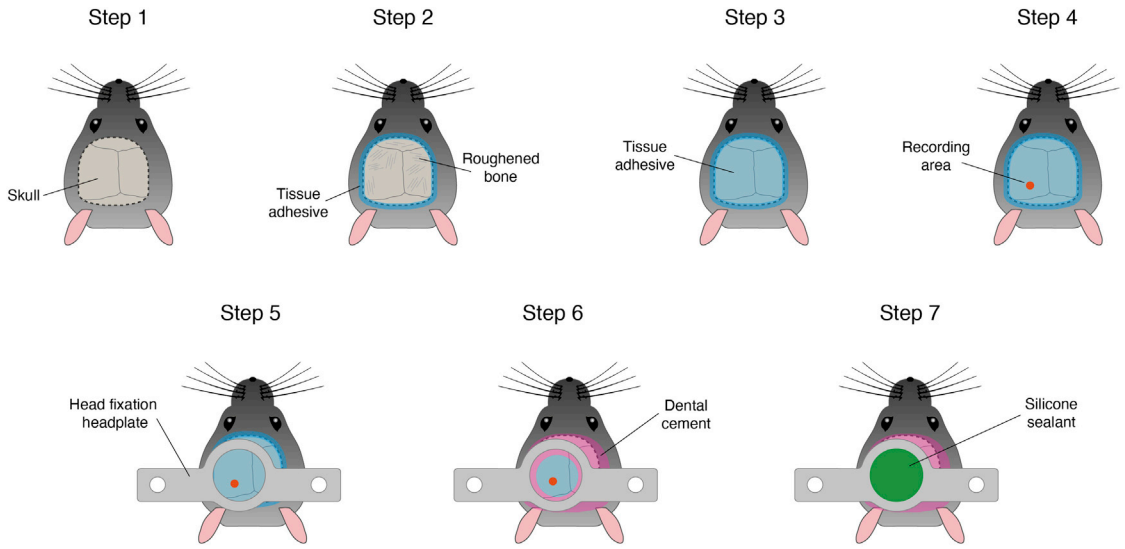


**Figure 2. Headplate implantation procedure**

Diagrams outline key steps for the surgical implantation of a head-fixation headplate. Step 1: cut the skin to expose the skull. Step 2: seal the skin edges with tissue adhesive and roughen bone. Step 3: apply a thin layer of tissue adhesive to the skull surface. Step 4: mark the recording area coordinate. Step 5: affix the headplate to the skull using tissue adhesive. Step 6: apply dental cement. Step 7: cover the skull with silicone sealant.

regions can be studied (in this protocol, we implant above the primary visual cortex), and researchers should update the stereotaxic coordinates of headplate positioning to suit their needs. Additionally, the shape and dimensions of the headplate can be changed to accommodate recordings from specific brain regions. We have used mice ranging from 8 to 14 weeks and both males and females have been used successfully. The two major tools for monitoring neural activity in head fixed mice are through chronic cranial windows, used for microscopy, or through an acute craniotomy, used for electrophysiology. Protocols for these procedures are very well described elsewhere (Augustinaite and Kuhn, 2020; Jordan, 2021).

4. Prepare the mouse for surgery.
   a. Anesthetize the mouse with isoflurane (3% induction, 1.5% for maintenance). Throughout the surgery, make sure to monitor the depth of anesthesia by the pinch withdrawal reflex and the respiratory rate.

   *Note:* Alternatively, injectable anesthesia can be given intraperitoneally, such as a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) or fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg).

   b. Apply a generous drop of eye ointment to prevent dehydration of the eyes. Similar, to further protect the eyes, whenever possible during the surgery, try to reduce the light source intensity as much as possible.
   c. To suppress inflammation, subcutaneously inject carprofen (5 mg/kg).
   d. Maintain core body temperature at 37°C–38°C by inserting a rectal probe and using a feedback-controlled heating pad.
   e. Secure the head of the mouse within the stereotaxic apparatus.

   ⚠ CRITICAL: It is important that the head is firmly fixed and stable. Probe this by gently pushing down on the dorsal part of the head with a cotton swab; there should be no vertical head movement.

5. Prepare the surgical field.

a. Using a small-animal clipper or a scissor, remove all fur from the dorsal part of the head. To gently remove loose hair from the skin surface, a small piece of painter's tape can be used.
b. Thoroughly disinfect the skin surface using ethanol wipes and iodine swabs.
c. To provide local analgesia, inject lidocaine (10 mg/kg) subcutaneously around the exposed skin surface and where the subsequent skin incision will be made. Wait 10 min for the drug to act.
d. With a scalpel or a scissor and a forceps, make a longitudinal skin incision of about 5–6 mm and pull the skin to the sides to expose the skull surface.

*Note:* If the headplate needs to be positioned very laterally (e.g., for recording from lateral higher-order visual areas) it might be necessary to remove parts of the remaining skin and retract the temporal muscle on the hemisphere side that will be recorded from.

6. Prepare the skull surface.
   a. Carefully remove all periosteal soft tissue from the exposed skull by gently brushing the surface with cotton swabs soaked in hydrogen peroxide (1% solution).
   b. To prevent infections, seal the skin edges with tissue adhesive (e.g., Vetbond).
   c. Once tissue adhesive is fully dry, with a dental drill or a needle, gently scrape the skull surface — omitting the area above the recorded site — to roughen the bone and increase surface area. It is essential to remove all soft tissue from the skull surface to obtain a stable implant.
   d. Apply a thin layer of tissue adhesive to skull surface to protect the bone overlaying the recording area and to create a base for affixing the headplate.
   e. *Optional:* mark the coordinate of the recording area (e.g., primary visual cortex: 2.5 mm lateral and 0.5 mm anterior relative to lambda).
7. Affix the headplate to the skull.
   a. Position and affix the headplate to the skull surface using tissue adhesive. If a recording area was marked, use the mark as a reference for centering the headplate opening.
   b. Once the tissue adhesive is dry, mix dental cement and apply to all exposed skull surfaces except the recording area. Make sure to apply the cement both on the inside and outside of the headplate implant. To apply the dental cement, the wooden end of a cotton swab, cut to a 45° angle can be used.
   c. Once the dental cement is fully dry, cover the skull above the recording area with Kwik-Cast silicone sealant.
8. Provide postoperative care.
   a. To provide pain management, subcutaneously inject a general analgesic such as buprenorphine (1.5 mg/kg) and place the animal in a heated recovery cage for 20–30 min until the mouse is mobile.
   b. In the subsequent 2–3 days, provide subcutaneous injections of carprofen (or similar anti-inflammatory drug) and buprenorphine (or similar analgesic) according to the animal experimentation license. We advise one daily injection of carprofen and three daily injections of buprenorphine (with maximally 8 h between injections).

*Note:* Depending on the geometry of the installed headplate (i.e., the length of the fixation arms) and the home cage of the mice, it might be an advantage to remove the food tray, if this is made of metal bars, and instead provide food on the floor of the cage to reduce the chance of mice losing their headplate implant due to it getting caught in the food tray.

### Habituation to treadmill system

Timing: 3–6 days

To reduce the stress level of the mouse during actual experiments, it is essential to first habituate the mouse to head fixation and voluntary locomotion on the treadmill. Optimally, it is vital that that the

same experimenter handles the mice on each day. Furthermore, the duration of head fixation on the treadmill should be gradually increased. The number of days required for mice to habituate and walk and run smoothly on the treadmill varies across mice, but in our hands, three to six days is usually sufficient for this if all parameters are optimized.

9. Habituate the mouse to the treadmill system.
   a. Day 1:
      i. At least 3 days after headplate implantation, head fix the mouse to the treadmill system by affixing the headplate to the cap screws using thumbs nuts.
      ii. Optimize the position of the mouse on the treadmill by the adjusting the height (slide the ball-bearing blocks up and down) and position (turn the knobs on the translation stage) of the treadmill relative to the headplate-fixation arms.

*Note:* The mouse should be able to sit comfortably on the treadmill (i.e., body position not too far back) and locomote unhindered (i.e., not sitting too high nor being squeezed toward the treadmill surface).

      iii. Let the mouse habituate and acclimatize on the treadmill, ideally without interference from the experimenter, for a duration of 10–15 min while sampling and recording the locomotor activity by means of the rotary encoder signal.

*Note:* If the mouse at any time during the session notably contorts its body and turns sideways on the treadmill (see troubleshooting), the experimenter can gently nudge the side of the mouse's trunk with their fingers to support re-alignment on the treadmill.

*Note:* If subsequent experiments will be carried out in darkness, habituate the mice in darkness and vice versa for experiments in light (see troubleshooting for considerations).

*Note:* Recording the rotary encoder signals during habituation sessions is not strictly required, but it provides a good output measure for assessing the habituation process of each mouse (e.g., percentage of time spent locomoting, maximal speed, bout durations, and distance covered).

   b. Day 2–6:
      i. On the subsequent days, expose the mouse to gradually increasing durations of head fixation on the treadmill system (e.g., Day 2: 20–25 min, Day 3: 30–35 min etc.). On the final day of habituation, aim to have the mouse on the treadmill system for the duration of the planned experiment.
10. Assess habituation process.

To determine if mice are habituated, use the locomotor activity pattern (Figures 3 and 4), body trunk posture (should be straight and not contorted), and tail posture (while stationary, the tail should be relaxed and resting on the treadmill surface) as a guide. Optionally, if needed, a further measure to gauge the stress level of the mice could be to track the pupil size dynamics — used as a proxy of arousal level (McGinley et al., 2015; Privitera et al., 2020) — during habituation sessions. After habituation, mice should exhibit dynamic fluctuations in pupil size that correlates with locomotion (McGinley et al., 2015) (see troubleshooting).

*Note:* While most mice will habituate and show intermittent locomotion on the treadmill and reduced stress levels after 4–6 days of habituation, some mice might not achieve this and in that case, we advise that those are not included in further experimental recording sessions. To guide this process, researchers can choose to define objective output measures for setting threshold criteria for when mice a habituated (e.g., maximal speed > 30 cm/s and/or locomotion time > 30% of session duration or similar).

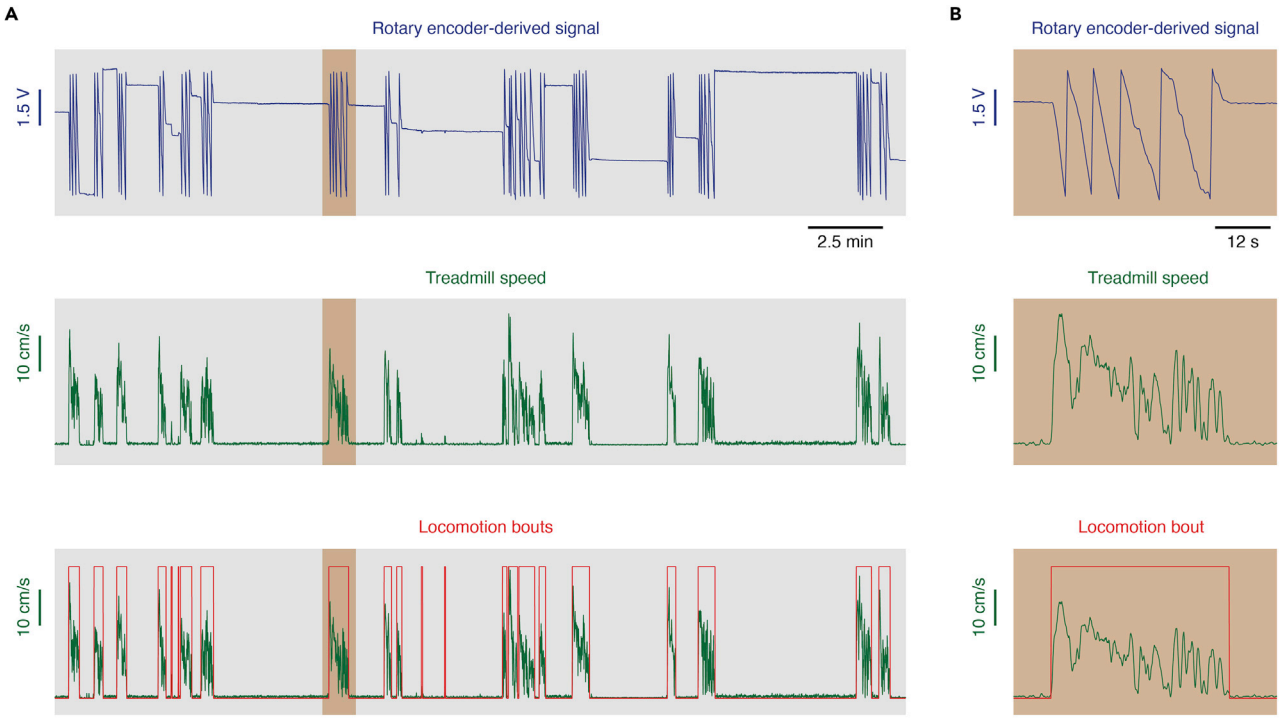


**Figure 3. Converting voltage trace from rotary encoder to locomotor activity**

(A) Upper: example rotary encoder-derived voltage time course. Middle: treadmill speed time course obtained by computing the approximative derivate of the rotary encoder signal above. Lower: treadmill speed time course (green) with overlayed binary locomotion time course (red).
(B) Close-up time courses for the temporal window highlighted in (A).

At the end of the habituation protocol, mice are now ready to enter experimental recordings sessions, such as longitudinal two-photon microscopy (Augustinaite and Kuhn, 2020), whole-cell patch-clamp recordings (Jordan, 2021), or extracellular potassium recordings (Rasmussen et al., 2019) (Figure 5).

## EXPECTED OUTCOMES

By the end of this protocol, researchers should have acquired an easy-to-use and adjustable treadmill system that seamlessly can be integrated with electrophysiological measurements such as local field potential and extracellular potassium recordings (Figure 5) as well as with microscopy (e.g., two-photon imaging or widefield imaging). Furthermore, the system is designed to be versatile and can thus easily be expanded to include electromyography recordings, pupil or face tracking, reward delivery, or virtual reality paradigms etc.

The recorded raw data from the treadmill contains the angular position-dependent voltage signal from the magnetic rotary encoder (sampled at a desired frequency) as a function of time (Figure 3). By utilizing the dimensions of the treadmill (i.e., circumference ~47 cm), this data can then be converted into treadmill speed. From this, a wide variety of quantifications can be extracted, depending on the experimental needs, such as: distance covered, maximal speed, percentage of time spent locomoting, and locomotion bout duration etc. (Figure 4). Example treadmill data and code for analyzing it are available on GitHub: https://github.com/Neurune/TreadmillSystem.

## QUANTIFICATION AND STATISTICAL ANALYSIS

The analysis code is written in MATLAB (tested on versions 2017a and 2021b) and organized into a master script (*"TreadmillMaster.m"*) which calls three main functions (*"getABFfiles.m"*, *"getLocSpeed.m"*,

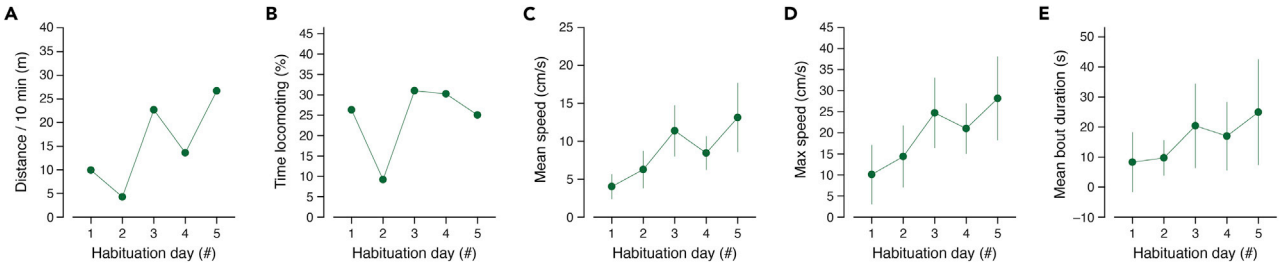


**Figure 4. Locomotion-related metrics over the course of habituation for one example mouse**

(A) Distance covered during first 10 min of the session.

(B) Percentage of time spent locomoting during the session.

(C) Mean speed during locomotion bouts.

(D) Maximal speed during locomotion bouts.

(E) Mean locomotion bout duration. Error bars in (C), (D), and (E) are mean ± standard deviation.

and *"getLocQuant.m"*) for the analysis of the treadmill-derived data. The first function extracts the voltage signal and sampling rate of the magnetic rotary encoder from *N* files (e.g., habituation sessions) recorded with Axon Clampex software (i.e., ABF file format). The second function converts the voltage signal into speed by computing the approximative derivate, removing the voltage setpoints (i.e., when voltage passes from 5 V to 0 V), and computing the speed at each time point. The last function computes a range of quantitative metrics from the speed signal, such as locomotion bout durations, average and maximal speed during locomotion bouts etc. For this, a few input criteria need to be given: 1) speed threshold (e.g., 1.5 cm/s), 2) minimum interval between locomotion bouts (e.g., 2 s), and 3) minimum locomotion bout duration (e.g., 1 s). The specific values for these criteria will depend on the experimental setting and what other physiological signals are recorded and their temporal kinetics, but the values provided here should serve as a good reference point for starting out. The final part of the master script generates figures and saves a file containing the computed locomotion metrics.

## LIMITATIONS

There are several limitations to this treadmill system. One, mice can only locomote along a single axis (i.e., forward walking/running) which prohibits the study of neural mechanisms underlying other motor behaviors, such as turning or rearing, as well as precluding the use of virtual reality-based tasks in which the mouse needs to turn left or right to express its decision-making process. Second, mice only seem to be able to engage in walking and trotting types of locomotor gait (i.e., locomotion speeds up to around 40 cm/s), thus precluding the possibility of studying gallop and bound gaits (Bellardita and Kiehn, 2015). Finally, mice need to be head fixed while on the treadmill which precludes any head movements and thus prevents studying the impact of proprioception signaling on neural activity in various brain areas such as the visual cortex.

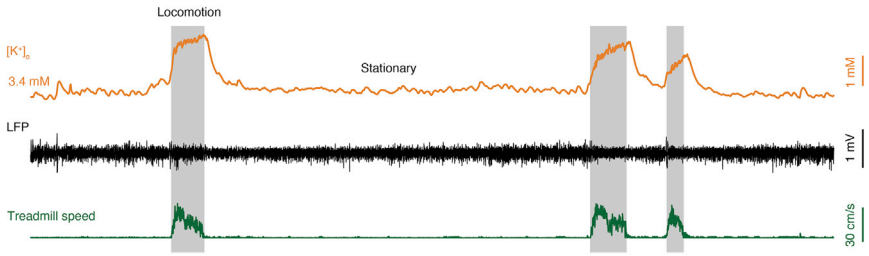


**Figure 5. Example application of treadmill system to study state-dependent brain dynamics**

Example extracellular potassium concentration ($[K^+]_o$, top trace) and local field potential recordings (LFP, middle trace) obtained from layer 5 of the visual cortex during bouts of intermittent spontaneous locomotion (bottom trace) on the treadmill system. Figure is adapted with permission from Rasmussen et al. (2019).

## TROUBLESHOOTING

### Problem 1

Mice do not locomote smoothly but rather rotate their trunk, so their hindpaws grab and cling onto the edge of the treadmill (step 9).

### Potential solution

Early in the habituation process (i.e., days 1 and 2) this is quite common and usually goes away with more habituation to the treadmill system. To facilitate this process, the experimenter can gently nudge the side of the mouse's trunk with their fingers to encourage re-alignment on the treadmill. However, this may also be a sign that the mouse is positioned too far back or too forward on the treadmill, which makes it difficult for the mouse to keep its balance and rest comfortably while attempting to be stationary on the treadmill. To resolve this, try changing the positioning of the mouse either more forward or more backward on the treadmill by adjusting the translation stage along the forward-backward axis.

### Problem 2

Mice constantly locomote while on the treadmill (step 9).

### Potential solution

For many experiments, intermittent bouts of both locomotion and rest are needed for comparing neural activity between these two states. Hence, if mice constantly locomote this can interfere with the purpose of the experiment. Rarely, the researcher might encounter mice that simple prefers to locomote almost all the time while on the treadmill, but more likely this problem is caused by the mouse being positioned too far back on the treadmill which makes it difficult for the mouse to stop locomoting once the treadmill is rotating and has built up momentum. Hence, to solve this problem, try positioning the mouse more forward on the treadmill by adjusting the translation stage along the forward-backward axis.

### Problem 3

Mice never locomote while on the treadmill (step 9).

### Potential solution

Although this may simply be because the mouse prefers to rest rather than to locomote, it is more likely that its position on the treadmill can be improved. Try to move the mouse slightly more backwards on the treadmill and adjust the height between the headplate and the top of the treadmill; the mouse should neither be sitting to high (forepaws cannot carry out a proper gait cycle) nor to low (forepaws are squeezed below its body).

*General considerations*: It is important to highlight, that all three of the above-described problems can also be related to, or stem from, the overall stress level of the mouse. That is, if the mouse is feeling overly stressed in the experimental setting, this alone could cause suboptimal locomotor behavior. It is thus essential to minimize the stress level of the mouse while it is head fixed on the treadmill system. As described, this is achieved by gradual and careful habituation sessions, but the sensory context (e.g., light, sound, and odor) to which the mouse is exposed is also important. For example, the brightness of the ambient light is known to modulate the anxiety level and locomotor behavior of rodents (Barrot et al., 2002; Valle, 1970). Furthermore, odors from stressed mice can affect the stress and vigilance level of other mice (Zalaquett and Thiessen, 1991). Hence, while some of the parameters of the sensory context may be constrained by the scientific question explored (e.g., visual or auditory stimulation to characterize neuronal responses), other parameters can be tweaked to reduce the overall stress of the mouse, such as always cleaning the foam roller in between mice to reduce the smell of excreta, and we therefore highly encourage the experimenter to critically evaluate such stress-modulating sensory sources in their specific experimental setup. Optionally, to gauge the stress level of the mouse, we propose measuring the pupil size as an

objective readout; a technique for which detailed protocols already exist (Privitera et al., 2020). If the pupil of the mouse is constantly dilated — independent of the momentary locomotor activity — this could suggest that the mouse is feeling overly stressed, and if this is the case, we advise to either habituate the mouse more or exclude it from the experiment.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rune Nguyen Rasmussen (rune.nguyen.rasmussen@sund.ku.dk).

### Materials availability

Blueprints for the custom foam roller, ball-bearing blocks, headplate-fixation arms, headplate, and magnetic rotary encoder interface and electrical circuit are available at GitHub: https://github.com/Neurune/TreadmillSystem (https://doi.org/10.5281/zenodo.6992230).

### Data and code availability

Example treadmill-derived data and associated analysis code generated during this study are available at GitHub: https://github.com/Neurune/TreadmillSystem (https://doi.org/10.5281/zenodo.6992230).

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## AUTHOR CONTRIBUTIONS

E.M.M.C. and R.N.R. wrote the protocol with guidance and inputs from M.N.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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