Supplementary Methods

Experimental animals and data quantification:

Mice expressing YFP in Layer V pyramidal neurons (H-line) were purchased from the Jackson Laboratory and used in this study\(^1\textsuperscript{-3}\). Mice were group-housed and bred in the Skirball animal facilities. All experiments were done in accordance with animal protocols approved by the institutional animal committee.

The procedures of transcranial and open-skull surgery and imaging were done as previously described\(^4\textsuperscript{-6}\). The detailed protocols are also provided below. In order to compare data obtained from open-skull and thinned-skull imaging, we only quantified dynamics of spines and filopodia that were within 100 \(\mu\)m of the pial surface in the barrel cortex. The percentage of spines eliminated or formed is defined as the number of spines eliminated or formed/pre-existing number of spines. Spine turnover is defined as the average of spine elimination and formation. Data on spine dynamics throughout the text is presented as mean \(\pm\) standard deviation (s.d.) according to previous studies\(^4\textsuperscript{-6}\). Animal ages, imaging intervals and the ratio of microglial density between cortical regions are presented as mean \(\pm\) standard error of the mean (s.e.). P-values were calculated using the student’s \(t\)-test.

Labeling of microglia and astrocytes in the mouse cortex:

Mice were first perfused with 4% paraformaldehyde. The imaged regions (barrel cortex) under open-skull or thinned-skull windows were identified based on the CCD images of blood vasculature under a dissecting microscope and subsequently marked by injecting 100 kDa rhodamine dextran with a fine needle. The brains were removed, post-fixed, and sectioned using a vibratome into 150 \(\mu\)m slices. To visualize microglia in the mouse cortex, we either used heterozygous \(C\text{X}_{12}CR1^{GFP}\) mice in which microglia are labeled with GFP\(^7\) or performed immunostaining with antibodies against ionizing calcium-binding adaptor
molecule 1 (Iba1) (Wako Chemicals USA Inc.) to fluorescently label microglia in fixed brain slices. Labeling of astrocytes was performed with antibodies against glial fibrillary acidic protein (GFAP) (Sigma). The sections were stained for Iba1 or GFAP using a standard immunostaining protocol.8

Protocols and procedures for surgery and imaging:

The following protocols for “open-skull” and “thinned-skull” surgery are essentially the same as described previously.6

Thinned-skull preparation:

1. Anesthetize transgenic mice by intraperitoneal injection (0.2 ml/20 g body weight) of 20 mg/ml Ketamine and 3 mg/ml Xylazine in 0.9% NaCl.

2. Thoroughly shave the hair over most of the scalp with a conventional razor blade. Perform a midline scalp incision using microsurgical tools. Incision should extend approximately from the neck region to the frontal area. The brain area to be imaged can be localized based on stereotactic coordinates.9

3. Remove soft tissue attached to the skull over the area to be imaged with fine forceps. High-speed micro-drill is used to thin a circular area of skull (typically ~1 mm in diameter) over the region of interest under a dissection microscope. Drilling should be done intermittently and the drill bit can be immersed periodically in cold artificial cerebrospinal fluid (ACSF) solution to minimize heat induced tissue injury.

4. The mouse skull consists of two thin layers of compact bone, sandwiching a thick layer of spongy tissue. This spongy bone contains tiny cavities arranged in concentric circles and multiple canaliculi that carry blood vessels. After removing the external compact bone, the middle layer of spongy bone is carefully thinned to about 75% of its original thickness. Some bleeding from the blood vessels running through the canaliculi may occur during the thinning.
process. This bleeding will usually stop spontaneously.

5. After removing the majority of the spongy bone, concentric cavities within the bone can usually be seen under the dissecting microscope, indicating the drill is close to the internal compact bone layer. At this stage, skull thickness should be more than 50 µm and the edge of the thinned region is thicker.

6. The mouse skull is then immobilized by gluing it to a metal plate made by gluing 3 razor blades together. Place a small amount of cyanoacrylate glue around the edges of the internal opening of the blade and press the blade against the skull leaving the area to be imaged uncovered and surrounded by the internal blade opening. Pay special attention to avoid contamination with glue of the thin skull area.

7. Wait for 10–15 minutes until the metal plate is firmly glued to the skull and place the mouse face down with the blades supported by the acrylic blocks. Tighten up the screws to completely immobilize the blades. Wash the area to be imaged extensively with ACSF to remove remnants of non-polymerized glue. We have observed that the microscope objectives retain trace amounts of glue remnants if the preparation is not washed thoroughly. We recommend keeping the objectives immersed in water after imaging and cleaning them periodically with 100% ethanol.

8. After the immobilization of the mouse skull, a microsurgical blade (Surgistar, Cat# 38-6961) is used to continue thinning a smaller area (~200 µm in diameter) to ~20 µm in thickness. The bone is shaved by using the blade at ~45 degree angle, taking great precaution not to push the skull downwards against the brain surface or to break through the bone, as minor brain trauma or bleeding may potentially cause inflammation and disruption of neuronal structures. The thinning continues until a very thin (~20 µm) and smooth preparation (~200 µm in diameter) is achieved. The optimal degree of skull thinning is ultimately determined by looking at the preparation with a conventional fluorescence microscope. Dendrites and spines in the area of interest should be clearly visualized at this
stage.

***** Key steps and criteria for a properly performed "thinned-skull" surgery:

A. A small region of the skull (~1 mm in diameter) is first thinned to > 50–100 μm in thickness with a drill. It is important not to thin a large region (> 1.5 mm) to a thin layer (< 50 μm) with the drill as it may cause damage to the cortex. B. The above thinned region is then thinned further with a surgical blade so that a small area of the skull (~200 μm in diameter) now has a thickness of ~ 20 μm. Avoid pushing the skull downwards against the brain surface or breaking through the bone. It is important not to thin a large area (> 300 μm in diameter) to less than 15 μm in thickness as minor brain trauma may cause inflammation and disruption of neuronal structures as indicated by growth of connective tissues and neurite blebbing underneath the thinned skull window. C. After the surgery, the area with the skull thickness ~20 μm is scanned with a 60x water immersion objective under an epi-fluorescent microscope to make sure that no neurite blebbing has occurred, as such blebbing is concurrent with high spine turnover.1-3.

Open-skull preparation:

1. Anesthetize transgenic mice and shave the hair over most of the scalp according to steps 1–2 as described in thinned-skull preparation.

2. Administer dexamethasone (0.02 ml at 4 mg/ml) by intramuscular injection and remove soft tissue attached to the skull over the area to be imaged with fine forceps.

3. Use a high-speed micro-drill (1/4 bit, Fine Science Tools) to thin a circumference of a 5 x 5 mm region of the skull over the region of interest under a dissection microscope. Drilling should be done intermittently and the drill bit can be immersed periodically in cold ACSF solution to minimize heat induced tissue injury.

4. Take great caution to lift up the island of bone within the drilled circle with a pair of sharp
forceps. Immediately after removing 5 x 5 mm region of the skull, bleeding above dura may occur in 2–3 locations as such tiny bleeding is presumably from small blood vessels attached to the removed skull. But such bleeding should stop spontaneously within 10–20 seconds. Rinse the cortex with room temperature ACSF once or twice if necessary. At this step, make sure there is absolutely no bleeding under the dura.

5. Cover the clear dura with a thin layer of low-melting-point agarose (Sigma) (1.2% in ACSF). Make sure that 1.2% freshly-made agarose is cool than 37 °C before applying it to the cortex.

6. A custom-made circular coverslip (5–7 mm diameter, No. 1 thickness) is gently lowered to cover the open-skull region. Take great caution not to push the cortex underneath and remove any excess agarose with sterile cotton applicators.

7. Seal the edge of the optical window to the skull with a thin layer of cyanoacrylate glue and then with dental acrylic. The dental acrylic will also cover all the exposed areas of skull. A small custom-made steel bar will be embedded into the acrylic to stabilize the animal during subsequent imaging.

8. Allow the mice to recover 7–14 days before chronic imaging starts.

****** Key steps and criteria for a properly performed “open-skull” surgery:

A. Throughout the open-skull surgery, there must not be any bleeding under the dura. Immediately after removing a 5 by 5 mm region of the skull, a very small amount of bleeding above the dura may occur in 2–3 locations, presumably from small blood vessels attached to the removed skull. This bleeding should stop spontaneously within 10–20 seconds. B. After cover-glass implantation, one should be able to obtain good images of dendrites without signs of neurite blebbing within the first 2 days after surgery. This can be done quickly by scanning open-skull windows under an epi-fluorescence microscope with a 40X water immersion objective. C. Open-skull windows generally become opaque 2–3 days after
surgery but some of them should become transparent again 7–14 days after surgery. In our hands, ~30% of animals had clear optical windows for imaging 10–14 days after surgery.

**Mapping the imaging area for future relocation:**

In order to identify the same imaged area at a later time point, a high quality picture of the meningeal blood vessels is first obtained as landmark for relocation. This can be done with a CCD camera attached to a stereo dissecting microscope. Subsequently, the mouse is placed under an epi-fluorescence microscope and a specific area is selected for two-photon imaging. The selected area is then carefully identified in the CCD camera map by observing on the epi-fluorescence microscope the pattern of brain vasculature adjacent to it and then marking it on the map.

**Two-photon imaging of neuronal structures.**

1. Tune the TPM to the appropriate wavelength (e.g. 920 nm for YFP). Imaging is done by using a 60x water-immersion objective (Olympus, N.A. 0.9). ACSF should be used at all times during imaging for objective immersion.

2. Obtain a stack of fluorescently labeled neuronal processes at a digital zoom of 1x. These low magnification images are useful as a more precise map for relocation of the same area at a later time point in conjunction with the CCD camera picture of meningeal blood vessels.

3. Without changing the position of the stage, take a zoomed image of the same area (e.g. 3x zoom). The stack depth is typically 100 μm below the pial surface. Additional zoomed images can be taken around the central image by electronically moving the imaging position 360° around the initial imaged area. We typically use laser intensities in the range of 10 to 20 milliwatts (measured at the sample) to minimize phototoxicity\(^\text{10}\). So far, our studies have limited to spines within the first 100 μm from the pial surface. It is worth to point out that we found that the open-skull window is somewhat better for imaging structures > 200 μm.
Deeper under the pial surface than the thinned-skull window. Presumably this is because the coverglass and thinned-skull induce different degrees of spherical aberration and spherical aberration has a larger effect on imaging of deeper tissues.

4. Following imaging, the head immobilization device is gently detached from the skull with thinned-skull preparations. The scalp is sutured with 6-0 silk (LOOK, Reading, PA) and the mouse is kept in a separate cage until fully awake then put back in the original cage until the next viewing. For open skull preparations, simply dismount the steel bar from a fixing post.

5. Image stacks can be viewed and analyzed with ImageJ software obtained from the NIH.

References