Supplementary Information

Microglial *Cx3cr1* knockout prevents neuron loss in an Alzheimer's disease mouse model

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Supplementary Methods, Supplementary Figures 1-5 and Supplementary References

Supplementary Methods

Transgenic mice

Homozygous triple transgenic mice (3xTg-AD) harboring a $PS1_{M146V}$ knockin as well as APP_{Swe} and tau_{P301L} transgenes[1], were crossed with heterozygous mice of the YFP-H line[2] and heterozygous *Cx3cr1*-$^{GFP/+}$ mice[3] (The Jackson Laboratory, Bar Harbor, USA). The offspring was intercrossed to yield homozygous 3xTg-AD mice, heterozygous for thy1-YFP and either homo- or heterozygous for *Cx3cr1* knockout. Littermates of the F1 generation of a crossing of 3xTg-AD$^{+/+}$ - thy1-YFP$^{+/-}$ - $CX_3CR1^{GFP/+}$ with 3xTg-AD$^{+/+}$ - thy1-YFP$^{+/-}$ - $CX_3CR1^{GFP/GFP}$ were used for the experiments. As controls age-matched littermates with the genotypes thy1-YFP$^{+/-}$ - $CX_3CR1^{GFP/+}$ or thy1-YFP$^{+/-}$ - $CX_3CR1^{GFP/GFP}$ were used. The mice were of mixed gender at an age of 4-6 months. Mice were group housed under pathogen-free conditions until surgery, after which they were singly housed. All procedures were in accordance with an animal protocol approved by the University of Munich and the government of Upper Bavaria.

Cranial window surgery

A cranial window over the right cortical hemisphere was surgically implanted as previously described[4,5]. The mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (0.13/0.01 mg/g body weight). Additionally, dexamethasone (0.02 ml at 4 mg/ml) was intraperitoneally administered immediately before surgery. A circular piece of the skull over the somatosensory cortex (4 mm in diameter) was removed using a dental drill (Schick-Technikmaster C1; Pluradent; Offenbach, Germany). Special care was taken to leave the underlying dura intact. The dura was covered with PBS. Immediately, a round coverslip (5 mm diameter) was glued to the

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skull using dental acrylic (Cyano-Veneer fast; Heinrich Schein Dental Depot, Munich, Germany) to close the craniotomy. A small metal bar, containing a screw thread, was glued next to the coverslip to allow repositioning of the mouse during subsequent imaging sessions. After surgery, mice received a subcutaneous analgesic dose of carprophen (Rimadyl; Pfizer, New York, USA) for 3 days (5 mg/kg). Imaging began following a 21 day rest period after surgery for the window to become stable.

Two-photon *in vivo* imaging

Two-photon imaging was performed using an upright Zeiss (Jena, Germany) Axioskop 2Fs mot LSM5MP setup, equipped with a MaiTai HP two-photon laser (Spectra Physics, Darmstadt, Germany). All fluorophores were excited at 880 nm and the fluorescence emission was detected by two non-descanned detectors with large aperture detection optics. For the simultaneous detection of YFP and GFP a Filterset with a dichroic (long pass 510) and two bandbass filters (480/40; 535/30) was used (AHF Analysentechnik AG, Tübingen, Germany). For the detection of Texas Red dextran (70000 MW, D1830, Invitrogen, Karlsruhe, Germany) a dichroic (LP 560) combined with a bandpass filter (575-640) was used. The standard microscopy table was removed and replaced by a custom-made table to allow repeated positioning of a living mouse under the microscope. Prior to each imaging session, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (0.13/0.01 mg/g body weight). To lable the blood vessels a tail vein injection with 5 mg/kg body weight of Texas Red dextran in sterile 0.9% Saline was carried out. Imaging sessions lasted for no longer than 60 minutes. Image acquisition was performed with the Zeiss LSM510 Rel3.5 software. To follow the fate of individual cortical layer 3 neurons and surrounding microglia a Zeiss 20x water-immersion objective (1.0 NA) was used to acquire image stacks of 300 µm depth with 3 µm z-resolution and 1024 x 1024 pixels per image frame(0.41 µm/pixel). Image stacks were acquired every 7 days for a period of four weeks. To measure the microglia surveying capacity (turnover rate TOR) time-lapse image stacks (140 x 140 x 40 μm^3; 2 µm z-resolution; Δt: 5 min) with 512 x 512 pixels per image frame (0.27 µm/pixel) were generated. Care was taken to ensure similar fluorescence levels in space and time. Repositioning over time was achieved by orienting to the vascular pattern and unique branch points of vessels, neurons and dendrites.

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Relocation of individual neurons in fixed tissue after *in vivo* imaging

The histochemical staining protocol was adapted from Gogolla et al.[6]. Briefly, mice anesthetized with an intraperitoneal injection of ketamine/xylazine (0.13/0.01 mg/g body weight) were transcardially perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). To relocate previously imaged neurons the brains were left in the intact mouse head with the fixation metal plate attached to the skull. Only the circular glas coverslip of the cranial window was carefully removed to give access to the 4% PFA solution for post fixation at 4° C overnight. On the next day the mouse head was fixed to the metal bar of the mouse holder and the skull above the right cortical hemisphere was removed using a dental drill. Subsequently, the metal bar with the fixed mouse head was attached to a vibratome (VT 1000S from Leica, Wetzlar, Germany) in a way that the cutting direction was exactly the same as the previous imaging direction. Five to eight 100 µm thick brain slices were cut and directly mounted on one objective slide subsequently descending in depth. Care was taken that the brain slices kept their bottom and top orientation. The sections were permeabilized with 2% Triton X-100 in a moist chamber at 4°C overnight. Then a fluorescent Nissl (NeuroTrace^{TM} 530/615; 1:20 in PBS; Invitrogen, Karlsruhe, Germany) and DAPI (10 µg/ml, 4,6-Diamidino-2-phenylindole dihydrochloride, Sigma, Germany) staining was carried out. At the end the stained brain slices were mounted with coverslips and fluoromount to the objective slide. Previously imaged positions were relocated by eye comparing the distribution of neuronal somata in the *in vivo* images with the fixed brain slice epi-fluorescence images. Fluorescence images were acquired with a confocal laser scanning microscope mounted on an inverted microscope support (LSM 510 and AxioVert 200, Carl Zeiss MicroImaging GmbH, Jena, Germany). Three different lasers were used for excitation: Ar ion laser at 488 nm for GFP/YFP, HeNe laser at 543 nm for Nissl NeuroTrace^{TM} 530/615, and a Ti-Sapphire laser (Mai Tai DeepSee, Spectra-Physics Lasers Division, Newport Corporation, Mountain View, CA, USA) at 780 nm for DAPI. A 40x immersion oil objective was used (Plan-Apochromat NA 1.3) (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Determination of Aβ levels in the mouse brain

Mouse brains were weighed and each homogenised in 1 ml ice-cold carbonate buffer (100mM Na_2CO_3, 50mM NaCl, pH 11.5 + protease inhibitors) before centrifugation at

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20,000 x *g* for 30 min at 4°C. The resulting supernatant was removed for the extraction of soluble Aβ and the pellet was stored on ice to be used in the extraction of insoluble Aβ. Aliquots of 1ml of supernatant were mixed with 1 ml Guanidinium buffer 1 (8.2M Guanidinium HCl, 82mM Tris HCl) and vortexed thoroughly. After centrifugation at 20,000 x *g* for 30 min at 4°C, the supernatant was removed and stored at ¯20°C. To extract insoluble Aβ, the original pellet was re-suspended by vortexing in 1ml Guanidinium buffer 2 (5M Guanidinium HCl, 50mM Tris HCl). The samples were incubated with gentle mixing on a horizontal shaker for 3 hrs at room temperature. Samples were then centrifuged at 20,000 x *g* for 30 min at 4°C and supernatants stored at -20°C. For use in the Aβ immunoassay, samples had to be diluted so as to reach a tolerable guanidinium concentration. Samples were diluted 1:100 in Block buffer (1% BSA, 0.1% Tween in PBS) and then vortexed thoroughly. Aβ peptides were quantified by a Meso Scale Discovery (MSD) sandwich immunoassay using the MSD Sector Imager 2400. 96-well Multi-SPOT plates pre-coated with anti-$A\beta_{40}$ or anti-$A\beta_{42}$ antibodies were incubated in Block buffer for 1 hr at room temperature and were then washed twice in Wash buffer (0.1% Tween in PBS). Samples and Aβ peptide standards (MSD) were added together with ruthenylated 6E10 detection antibody (1 µg/ml) diluted in Block buffer. Plates were incubated at room temperature for two hours before washing three times with Wash buffer. For detection, 150 µl of 2x MSD Read buffer was added and the light emission at 620 nm after electrochemical stimulation was measured using the MSD Sector Imager 2400 reader. The corresponding concentrations of Aβ peptides were calculated using the MSD Discovery Workbench software.

Image processing and data analysis

All images were median filtered and maximum intensity projected for illustrations. For image processing the LSM510 Rel. 3.5 (Zeiss, Jena, Germany) and the open source software ImageJ were used (NIH, USA). The presence of individual YFP expressing cortical layer III neurons over a time-period of four weeks was determined by manually scrolling through z-stacks aligned in space and time dimensions marking present and lost neurons from one time point to the other. To measure the microglia migration and number in cortical layer III, maximum intensity projections of 40 µm z-stacks acquired weekly over a period of four weeks were aligned in space and time

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using the “stackreg” plugin of ImagJ. Individual microglia were counted and their migration was tracked over this time period with the “MTrackJ” plugin of ImageJ (Supplementary Video 2). The velocity [µm/day] of individual microglial cell somata was calculated as the traveled distance [µm] divided by the observation period of 28 days.

To quantify the microglia density before and after a neuron loss event, the timepoint of the removal was set to day zero. The TOR of microglial fine processes (Supplementary Video 3) was determined by overlaying subsequent maximum intensity projected z-stack-images spanning 40 µm in z-direction. These were pseudocolored in green and red allowing to measure the area of gained processes in green, lost in red and stable in yellow (Supplementary Video 4). The TOR was calculated as the sum area of gained and lost processes, divided by the whole area occupied by the microglial cells in percent. The TOR per cell was divided by the number of detected microglia in the imaging field. All analysis was performed in a blind manner by an experimenter without knowledge of the experimental conditions. All results are reported as mean ±s.e.m. and statistical significance was set at $P < 0.05$. Statistical differences in measurements over time were determined using repeated measure ANOVA. Statistical differences between multiple different experimental groups were carried out by Holm-Sidak ANOVA or Kruskal-Wallis ANOVA on ranks.

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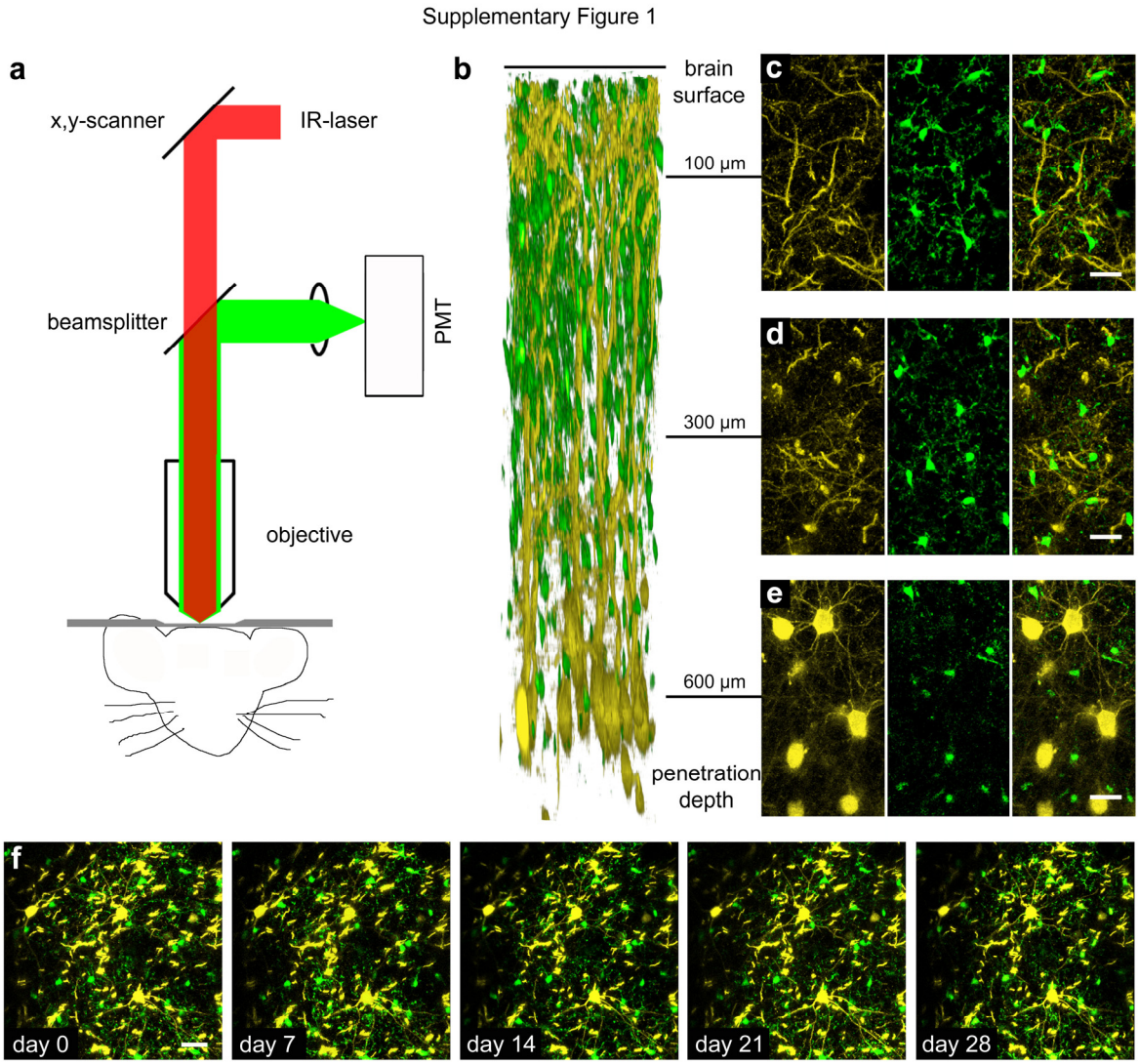
Supplementary Figures



Supplementary Figure 1. Repetitive long-term two-photon *in vivo* imaging of microglia and neurons.

(a) Schematic of a mouse fixed under a two-photon laser scanning microscope. **(b)** 3D-reconstruction of a 650 µm z-stack with neurons labeled in yellow and microglia in green. **(c-e)** Optical slices from various depth (100, 300, 600 µm) illustrating neurons (yellow) and microglia (green). **(f)** Time-lapse of cortical layer III neurons and surrounding microglia. Scale bars: (c-e) 10 µm, (f) 20 µm.

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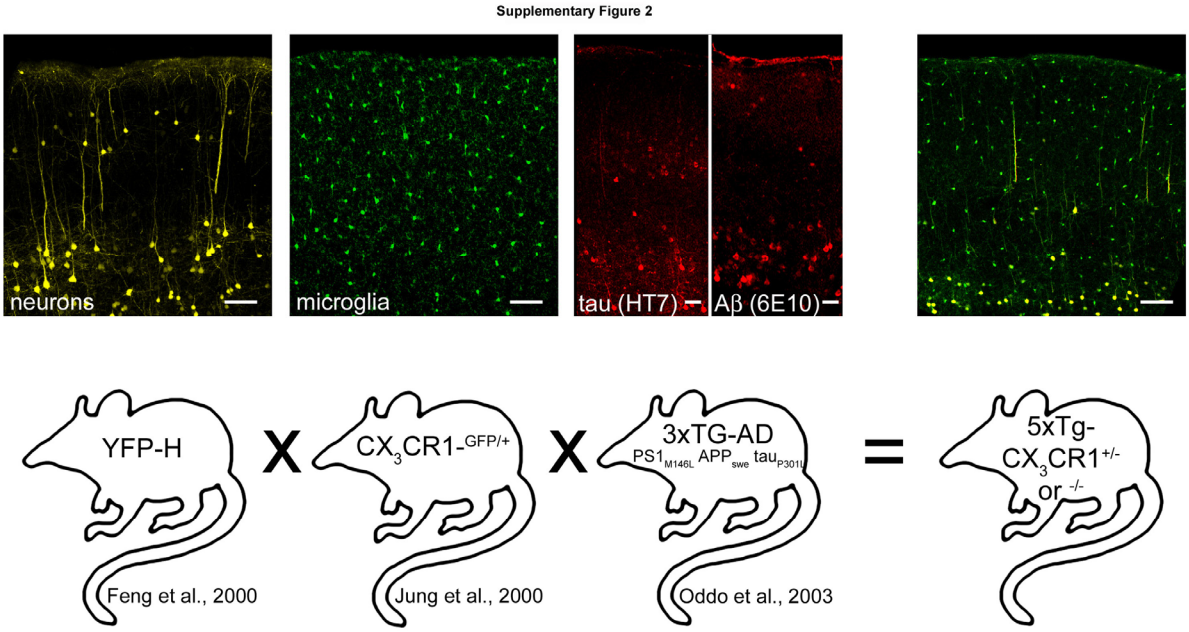

Supplementary Figure 2. Genertion of *5xTg-Cx3cr1* mouse model.

5xTg-Cx3cr1$^{+/-}$ or *5xTg-Cx3cr1*$^{-/-}$ were heterozygous for *thy1-YFP*$^{+/-}$ and homozygous for *PS1*$_{M146V}$, *APP*$_{Swe}$ and *tau*$_{P301L}$. The YFP-H line exhibits YFP-expression in subsets of cortical layer III and V neurons. *Cx3cr1* mice carry a *GFP* knock-in in the *cx3cr1*-locus. 3xTg-AD mice are characterized by a *PS1*$_{M146V}$ knock-in in combination with *APP*$_{Swe}$ and *tau*$_{P301L}$ transgenes on a single construct. Fluorescence images of the cortex illustrate the neuronal YFP-expression, the microglial GFP-staining, as well as tau (antibody: HT7) and Aβ (antibody: 6E10) localization. Scale bars: 60 µm, 30 µm (tau, Aβ)

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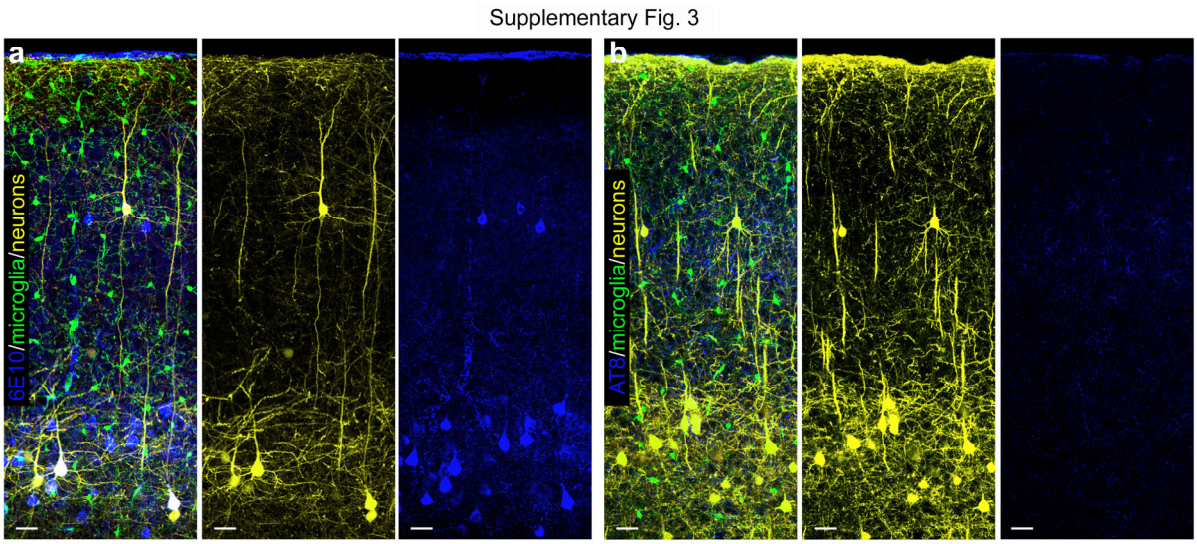

Supplementary Figure 3. AD-pathology of cortical layer III neurons

(a) Layer III neurons of 4-6 months old *5xTg-Cx3cr1* mice are immunoreactive for amyloid beta specific antibody 6E10. **(b)** No hyperphosphorylated tau was detected using monoclonal antibody AT8. Scale bars: 20 µm.

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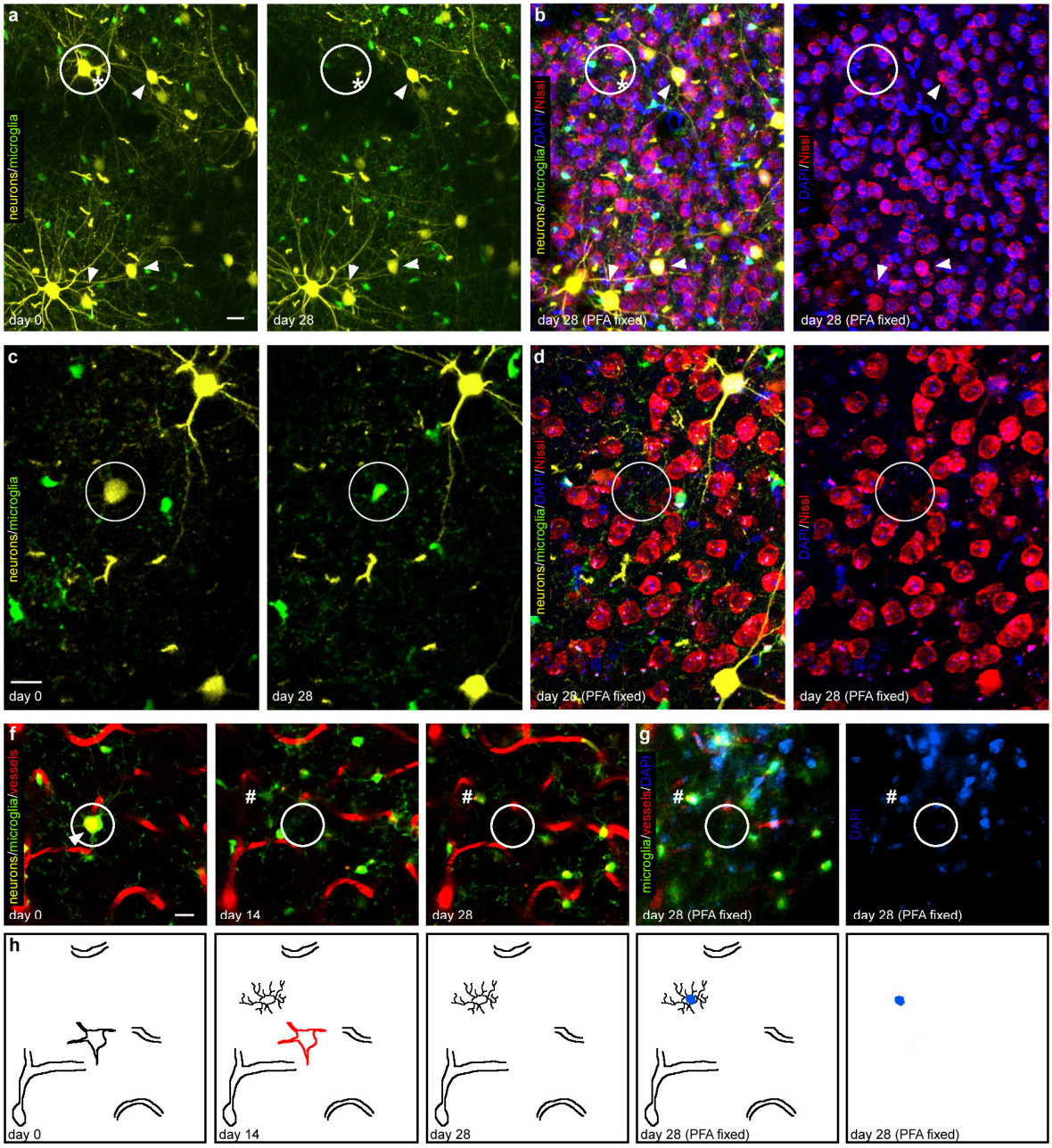
Supplementary Fig. 4



Supplementary Figure 4. Validation of neuron loss by post mortem DAPI and Nissl staining.

(a, c, f) Examples of three lost cortical layer III neurons (circles) first imaged by two-photon *in vivo* imaging. **(b, d, g)** Subsequent relocation (see Supplementary methods for relocation procedure) of positions with lost neurons after 4% PFA fixation and DAPI and Nissl-red staining. Arrows indicate YFP-expressing neurons, circles indicate lost neurons. * marks an YFP-labeled dendrite. # indicates a microglial cell. **(h)** Schematic of the eliminated neuron illustrated in (f, g). Scale bars: 10 µm.

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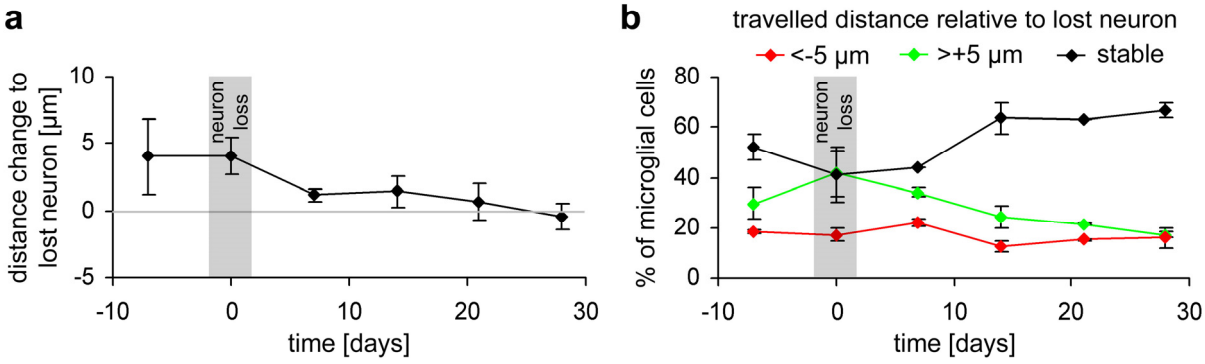
Supplementary Fig. 5



Supplementary Figure 5. Directed migration of microglia towards lost neurons

(a) Mean distance change of microglial cells to a lost neuron before, at and after the elimination (n = 4 mice). **(b)** Percent distribution of microglial cells (n = 351) in three different groups: towards (relative distance change to lost neuron Δd >+5 µm), away (Δd <-5 µm) and stable (Δd $\leq$+5 and $\geq$-5 µm).

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Supplementary Videos

Supplementary Video 1:

Example of a z-stack acquired by two-photon in vivo imaging. Z-stack from 650 µm depth to the surface in a living mouse brain. Neurons are labelled with YFP and microglia with GFP.

Supplementary Video 2:

Tracking of microglia migration in vivo. Five week time-lapse example of microglia migration around a neuron. Coloured lines representing the tracks of the microglia are superimposed.

Supplementary Video 3:

Screening behavior of microglia with extension and retraction of fine processes. The video consists of z-stack projections (40 µm) of fluorescence images recorded with a time interval of 5 minutes 150 µm below the brain surface.

Supplementary Video 4:

Turnover rate (TOR) of microglia. Red/green overlay of subsequent time points to visualize gained (green) and lost (red) as well as stable (yellow) areas of microglial processes.

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