Supporting Information

In vivo delivery of a fluorescent FPR2/ALX-targeted probe using focused ultrasound and microbubbles to image activated microglia

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General synthetic procedures for Rho-pip-C1. Commercially available reagents were bought from Sigma Aldrich (St-Louis, MO, USA) or Fisher Scientific. Deuterated solvents were bought from Goss Scientific. Thin-layer chromatography was conducted using pre-coated Silica gel 60, F254 plates with a thickness of 0.2 nm. UV-vis absorption spectra were recorded on a Perkin Elmer 650 spectrometer and fluorescence spectra were obtained using a Varian Cary Eclipse spectrophotometer. For pH measurements a Jenway model 3510 pH/mV/temperature meter was used calibrated against pH 10.00, pH 7.00 and pH 4.00 buffers. Column chromatography was performed using silica gel and laboratory grade solvents, under mild pressure. Synthetic and spectral details for preparation of Rho-pip-C1 have been described previously [1].

Characterisation of Rho-pip-C1. Data obtained by NMR-spectroscopy, high resolution mass spectrometry and an LCMS chromatogram have been described previously. Optical properties in methanol (emission λ_{max} = 582 nm upon excitation at 350 nm, and excitation λ_{max} = 561 nm (λ_{em} = 580 nm)) were found to be compatible with *in vivo* imaging.

Focused ultrasound setup. Ultrasound pulses were emitted from a single-element spherical-segment focused ultrasound transducer (centre frequency: 1 MHz, active diameter: 90 mm, geometric focus: 77 mm; Sonic Concepts, Bothell, Wash). A function generator (33500B Series; Agilent Technologies, Santa Clara, Calif) was used to generate the pulses which were then passed through a 50-dB power amplifier (Precision Acoustics, Dorchester, England) before reaching the transducer. A cone was

mounted onto the transducer, filled with distilled water and then covered with an acoustically transparent parafilm membrane. Targeting of the desired brain region was facilitated by the transducer being mounted onto a 3D positioning system. Prior to *in vivo* experiments, the pressure amplitude was measured with a needle hydrophone (diameter: 0.2 mm; Precision Acoustics) in a degassed water tank. The lateral, elevational and axial full width at half maximum (FWHM) of the ultrasound focus were 2, 1 and 20 mm respectively. Pressure values were attenuated by 11% to account for the mouse skull based on measurements taken with and without the top layer of the mouse skull (n = 4) in between the transducer and the hydrophone.

In vivo experimental procedure. All experimental protocols were approved by the UK Home Office and Imperial College London's animal facility committee. Three female C57BL/6 wild-type mice (8-10 weeks old, 19.3 ± 0.8 g; Envigo, Huntingdon, England) were anesthetised with 1.5-2 % vaporized isoflurane (Zoetis UK, London, England) mixed with oxygen (0.8 L/min). The fur was first removed from the head using an electric clipper and depilatory cream. The mouse head was then placed inside a stereotaxic frame (World Precision Instruments, Hertfordshire, England) and ultrasound gel applied on top of the head. A container filled with degassed water was then lowered onto the gel and a metal grid placed in alignment with the sutures of the skull. Using a previously reported technique [1], a 2D raster scan of the cross was generated to position the transducer above the left hippocampus (0.5 mm above the lambdoid suture and 3 mm lateral of the sagittal suture).

Rho-pip-C1 was diluted in phosphate buffered saline (PBS) and 5% ethanol and was delivered intravenously through the tail vein using a 30-gauge catheter following SonoVue microbubbles (concentration 5 µL/g; Bracco, Milan, Italy). Ultrasound pulses at 1 MHz were applied for 10 ms at a pulse repetition frequency of 0.5 Hz at 535 kPa peak-negative pressure. Microbubbles were administered after 5 pulses and the probe was administered 30 pulses into the sonication, given a total of 126 pulses. During the ultrasound treatment, a passive cavitation detector (PCD, centre frequency: 7.5 MHz, focal length: 76.2 mm; Olympus Industrial, Essex, UK) was used to detect microbubbles signals to verify their presence within the targeted brain region. The detector was placed through a central opening in the therapeutic transducer and the acoustic emissions were filtered by a 3-30 MHz band-pass filter (Mini circuits, Brooklyn, NY, USA) and then amplified by a 28-dB pre-amplifier (Stanford Research Systems, Sunnyvale, CA, USA). An 8-bit oscilloscope was then used to record the data at 250 MHz sample frequency (Picoscope 3205A; Pico Technology, Cambridgeshire, UK).

Following the treatment, mice were sacrificed and transcardially perfused with 20 mL ice-cold phosphate-buffered saline with heparin (10 units/mL) to reduce blood coagulation. Brains were extracted and stored in 10% formalin at 4 °C overnight and then cryoprotected in 30% sucrose overnight. Brains were then cryosectioned into 30 µm slices. Fluorescence microscopy was used to capture images of the left (treated) and right (untreated control) hippocampus (10x; Zeiss Axio

Observer Inverted Widefield Microscope; Oberkochen, Germany). Rhodamine B was excited at 562/40 nm and emissions filtered at 624/40 nm. Five sections from each brain were used to calculate the normalised optical density (NOD), a measure of the amount of Rho-pip-C1 delivered. Matlab (2018a, The Mathworks, Natick, MA, USA) was used for this quantification. Immunostaining to detect microglial cells was performed using a goat anti-Iba1 primary antibody (1:500 overnight; Ab5076; Abcam, Cambridge, England) and a donkey anti-goat IgG H&L Alexa Fluor 488 secondary antibody (1:1000 for 2 h; Ab150129; Abcam). Neuron staining was performed using a primary recombinant anti-NeuN antibody (1:500 overnight; Ab177487; Abcam) and a secondary donkey anti-goat IgG H&L Alexa Fluor 488 antibody (1:500 for 2 h; Ab150129; Abcam). Astrocyte staining was performed using a primary GFAP monoclonal antibody (1:50 overnight; 13-0300; ThermoFisher) and a secondary mouse anti-rat IgG2a FITC antibody (1:500 for 2 h; 11-4817-82; ThermoFisher).

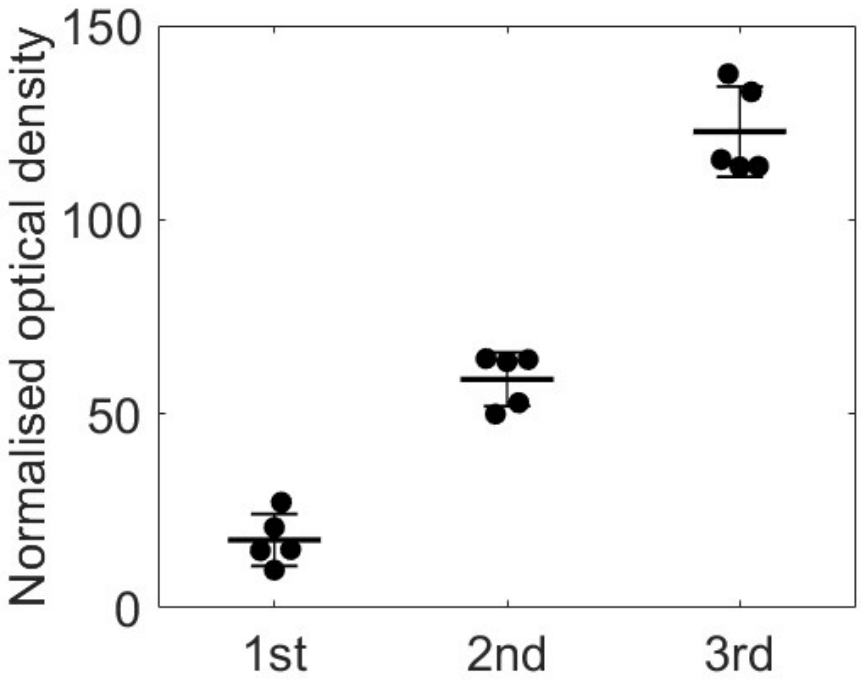

Figure 1s. Normalised optical density (NOD) calculated from fluorescence images from the three brains where Rho-pip-C1 was delivered. The NOD gives an indication of how much higher the pixel intensities are in the targeted region compared to the control region (NOD = 0). Delivery of the probe was detected in all five slices for all three of the mouse brains (1st, 2nd and 3rd).

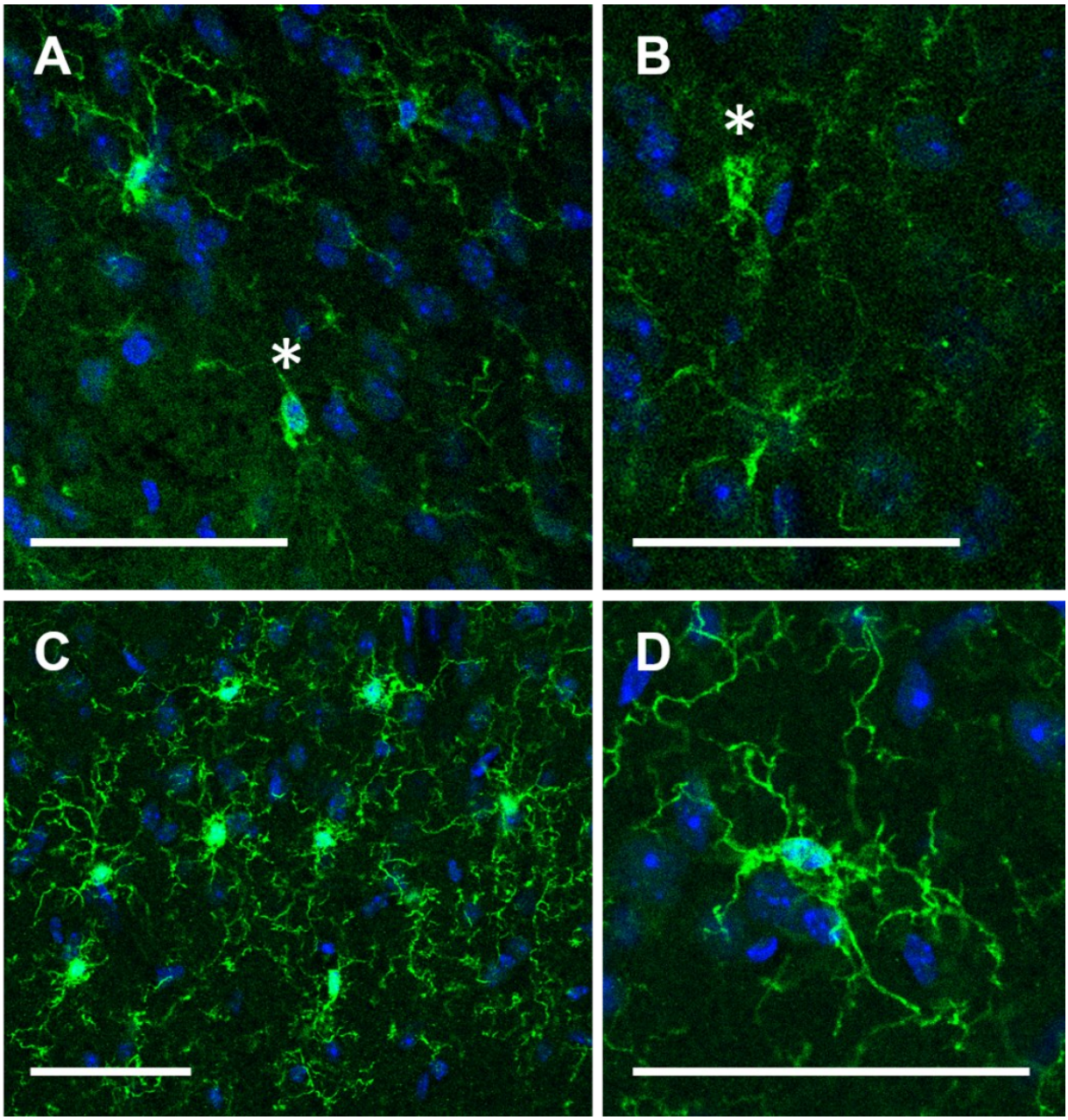

Figure 2s. Confocal images (20x) highlight regions in the ultrasound targeted areas of the brain where both (A-B) rounded microglia are present (white asterisks) and (C-D) regions with resting microglia with long processes. The fluorescence channels are shown both for the microglia stained with Iba1 (green) and nuclei stained with DAPI (blue). The scale bars represent 50 µm.

References

1 T. Boltersforf, J. Ansari, E. J. Senchenkova, J. Groeper, D. Pajonczyk, S. A. Vital, G. Kaur, S. J. Alexander, T. Vogl, U. Rescher, N. J. Long and F. N. E. Gavins, *Theranostics*, 2020, **10**, 6599–6614.