

Supplementary Materials for

Localized calcium accumulations prime synapses for phagocyte removal in cortical neuroinflammation

Mehrnoosh Jafari^{1,2†}, Adrian-Minh Schumacher^{1,2†}, Nicolas Snaidero^{1,3,4†}, Tradite Neziraj^{1,2}, Emily M. Ullrich Gavilanes^{1,2}, Tanja Jürgens⁵, Juan Daniel Flórez Weidinger^{6,7,8,9}, Stephanie S. Schmidt¹, Eduardo Beltrán^{1,2}, Nellwyn Hagan¹⁰, Lisa Woodworth¹⁰, Dmitry Ofengeim¹⁰, Joseph Gans¹¹, Fred Wolf^{6,7,8,9}, Mario Kreutzfeldt^{5,12}, Ruben Portugues^{13,14}, Doron Merkler^{5,12*#}, Thomas Misgeld^{3,4,14*#} and Martin Kerschensteiner^{1,2,14*#§}

¹ Institute of Clinical Neuroimmunology, University Hospital, Ludwig-Maximilians Universität München, Munich, Germany

² Biomedical Center (BMC), Faculty of Medicine, Ludwig-Maximilians Universität München, Martinsried, Germany

³ Institute of Neuronal Cell Biology, Technische Universität München, Munich, Germany

⁴ German Center for Neurodegenerative Diseases (DZNE), Munich, Germany

⁵ Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland

⁶ Theoretical Neurophysics, Max Planck Institute for Dynamics and Self-Organization, Göttingen, Germany

⁷ Bernstein Center for Computational Neuroscience, University of Göttingen, Göttingen, Germany

⁸ Max Planck Institute for Experimental Medicine, Göttingen, Germany.

⁹ Center for Biostructural Imaging of Neurodegeneration, Göttingen, Germany.

¹⁰ Sanofi Neuroscience, Framingham, MA, USA

¹¹ Translational Sciences Genomics, Sanofi, Framingham, MA, USA

¹² Division of Clinical Pathology, Geneva University Hospital, Geneva, Switzerland

¹³ Sensorimotor Control, Max Planck Institute of Neurobiology, Martinsried, Germany

¹⁴ Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

† These authors contributed equally to this work.

Co-senior authors

*Correspondence to:

M.K. (martin.kerschensteiner@med.uni-muenchen.de)

D.M. (doron.merkler@unige.ch)

T.M. (thomas.misgeld@tum.de)

This PDF file includes:

fig. S1 to S8
Captions for Movie S1

Other Supplementary Materials for this manuscript include the following:

Movie S1

Figure S1 - Histopathological characterization of a mouse model of cortical multiple sclerosis. Related to Figure 1.

(A) Schematic diagram of the experimental design to study histopathological changes in the cortex of age-matched naive (black), EAE (brown), only cytokine injected (violet) and cortical MS model (c-MS d3, orange) mice. **(B)** Bielschowsky's staining was used to analyze axonal density in each layer. Representative images depict Bielschowsky's staining in layer I of naive and c-MS d3 mice (**left, middle**, respectively), axonal density was normalized to average density of the naive group (**right**). **(C)** MBP staining was used to analyze myelin density in each layer. Representative images depict MBP staining in layer I of naive and c-MS d3 mice (**left, middle**, respectively), MBP density was normalized to average density of the naive group (**right**). **(D)** CD3 staining was used to analyze T cells density in each layer. Representative images depict CD3 staining in layer I of naive and c-MS d3 mice (**left, middle**, respectively), CD3 density was normalized to average density of the naive group (**right**). **(E)** Mac-3 staining was used to analyze mononuclear phagocytes density in each layer. Representative images depict Mac-3 staining in layer I of naive and c-MS d3 mice (**left, middle**, respectively), Mac-3 density was normalized to average density of the naive group (**right**). Data shown as mean \pm SEM (n= 5, 5, 6 and 6 mice in each group). Scale bars in **B**, 15 μ m; **C**, 45 μ m **D** and **E**, 25 μ m. Two-way RM ANOVA followed by Bonferroni's multiple comparisons test has been performed in **B-E**. *** P<0.001, **P<0.01, *P<0.05.

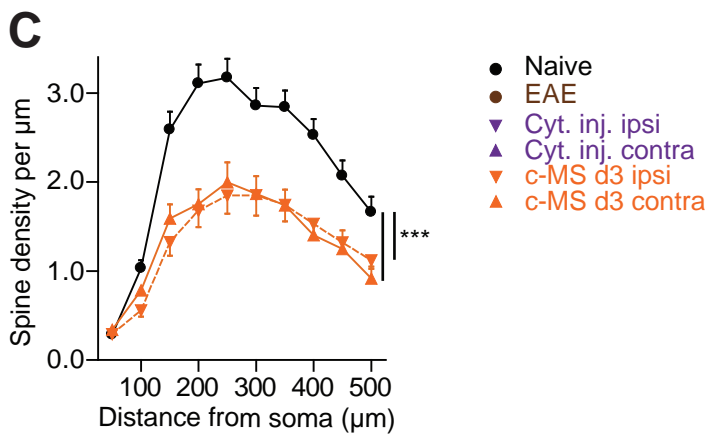
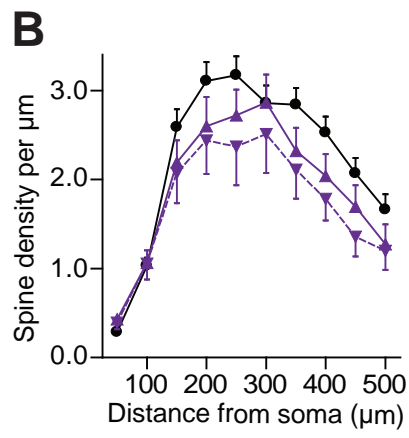
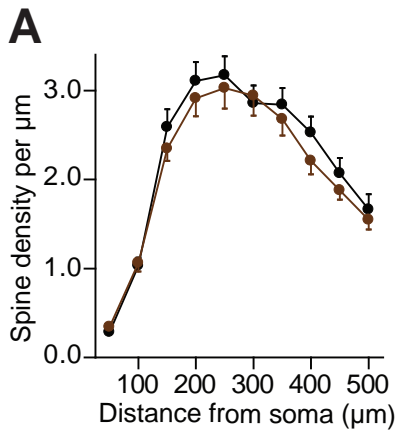


Figure S2 – Induction and spread of spine loss c-MS mice compared to mice with only EAE or sole cytokine injection. Related to Figure 1.

(A) Spine density along the apical dendrite of layer V pyramidal neurons in naive mice and EAE animals without cytokine injection (n=21 neurons from n=7 and 8 mice in each group, respectively; mean \pm SEM). (B) Spine density along the apical dendrite of layer V pyramidal neurons in naive mice and healthy animals 3d after cytokine injection, ipsi- and contralateral to the injection site (n=21, 8 and 13 neurons from n=7 and 10 mice in each group, respectively; mean \pm SEM). (C) Spine density along the apical dendrite of layer V pyramidal neurons in naive and c-MS d3 animals, ipsi- and contralateral to the injection site (n=21, 17 and 11 neurons from n=7 and 8 mice in each group, respectively; mean \pm SEM). Two-way RM ANOVA followed by Bonferroni's multiple comparisons test has been performed in A-C. *** P<0.001.

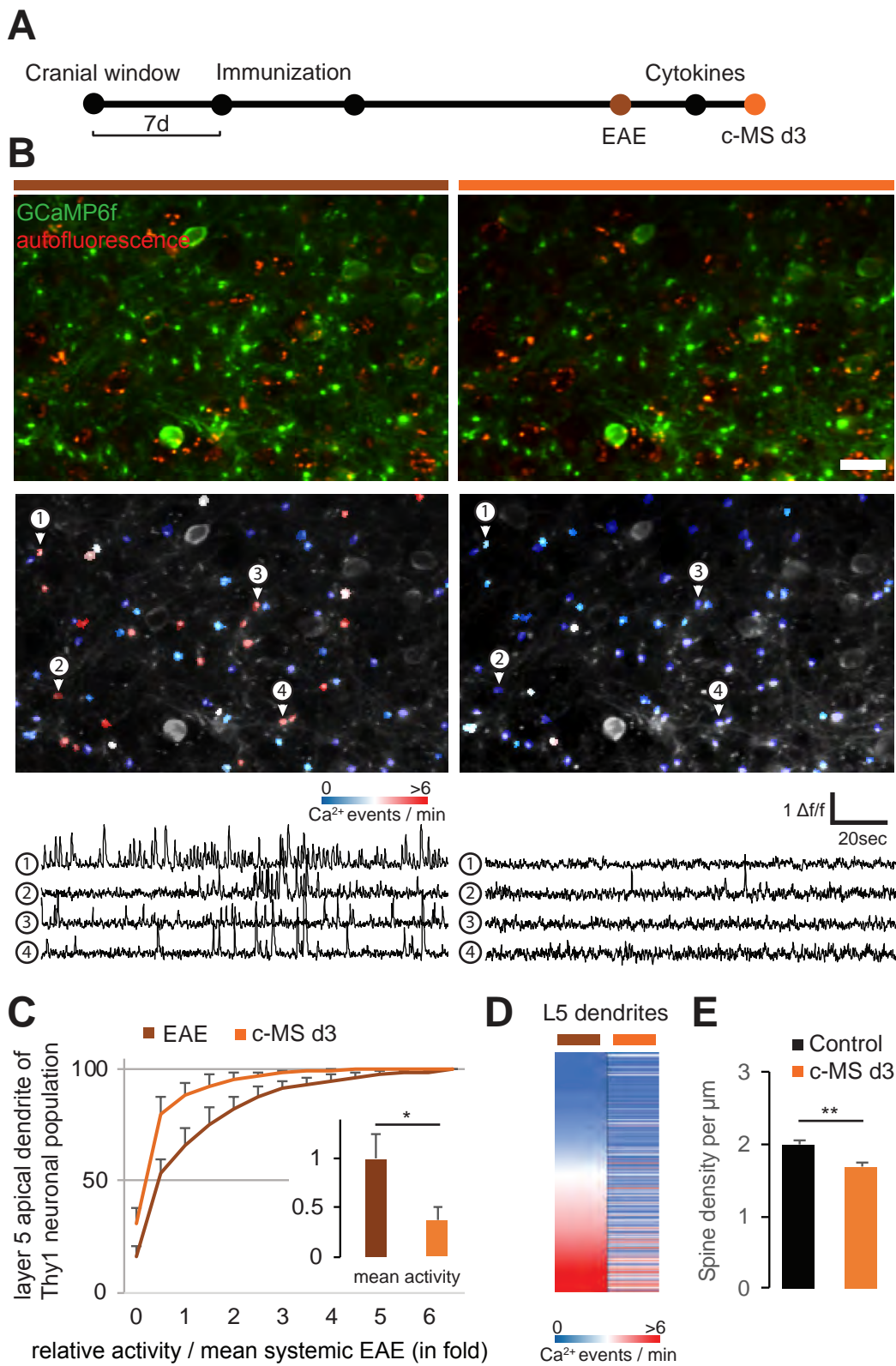


Figure S3 – Layer 5 projection neurons are silenced in a model of cortical multiple sclerosis. Related to Figure 2.

(A) Schematic diagram of experimental timeline to study longitudinal activity in apical dendrites from somatosensory layer 5 projection neurons in cortical MS model (c-MS), using Thy1-GCaMP6f transgenic animals and *in vivo* imaging. (B) *In vivo* multiphoton image of layer 5 apical dendrites over the course of c-MS through a cranial window; **left**, before cytokine injection (EAE) and **right** 3 days (c-MS d3) post cytokine injection. **Top**: Merged GCaMP6f and autofluorescence channels allowing the identification of apical dendrites (green only). **Middle**: GCaMP6f channel masked and color-coded for cytoplasmic Ca²⁺ events per minute. **Bottom**: Representative calcium activity traces displayed as delta f/f for the four apical dendrites marked in the middle panel. (C) Cumulative plots representing activity for the entire population of apical dendrites for each selected time point over the course of the cortical MS model. **Inserts**: Mean activity normalized to baseline for EAE and c-MS d3 shown as mean ± SEM (n=3 c-MS mice, Shapiro-Wilk and paired t-test). (D) Heat map representation of ranked activities at a single dendrite resolution between EAE and c-MS d3. (E) Spine density of dendritic stretches of pyramidal layer V neurons from c-MS animals used in B-D (imaged area was used for quantification) and in control animals (n=30 and n=40 dendritic stretches from n=3 and n=4 mice, respectively; mean ± SEM, unpaired t-test). Scale bar in **B**, 20µm. **P < 0.01, *P<0.05.

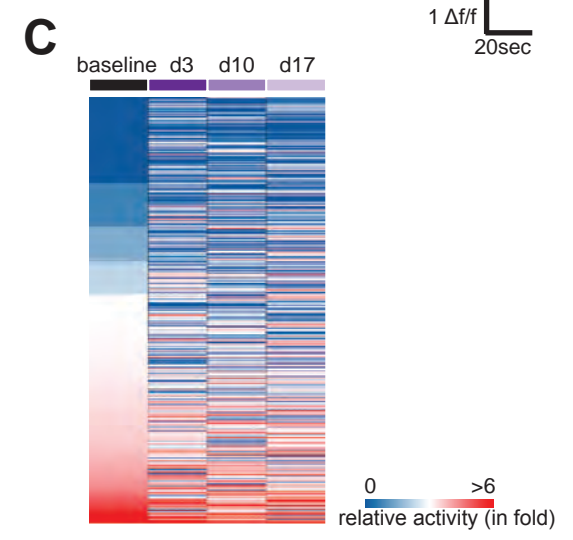
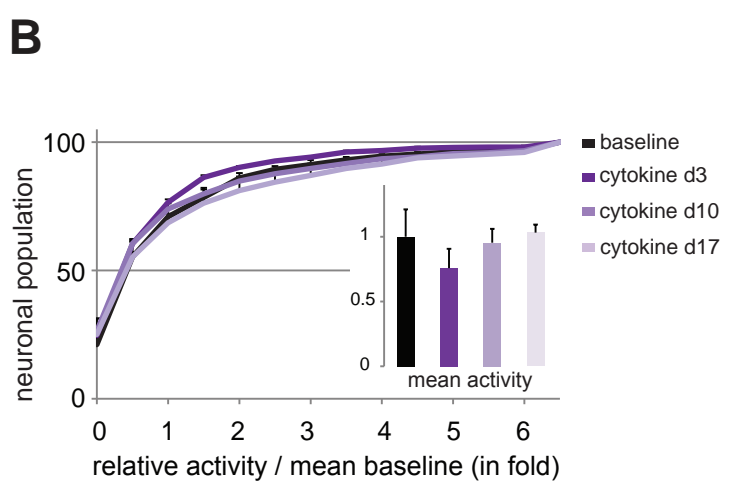
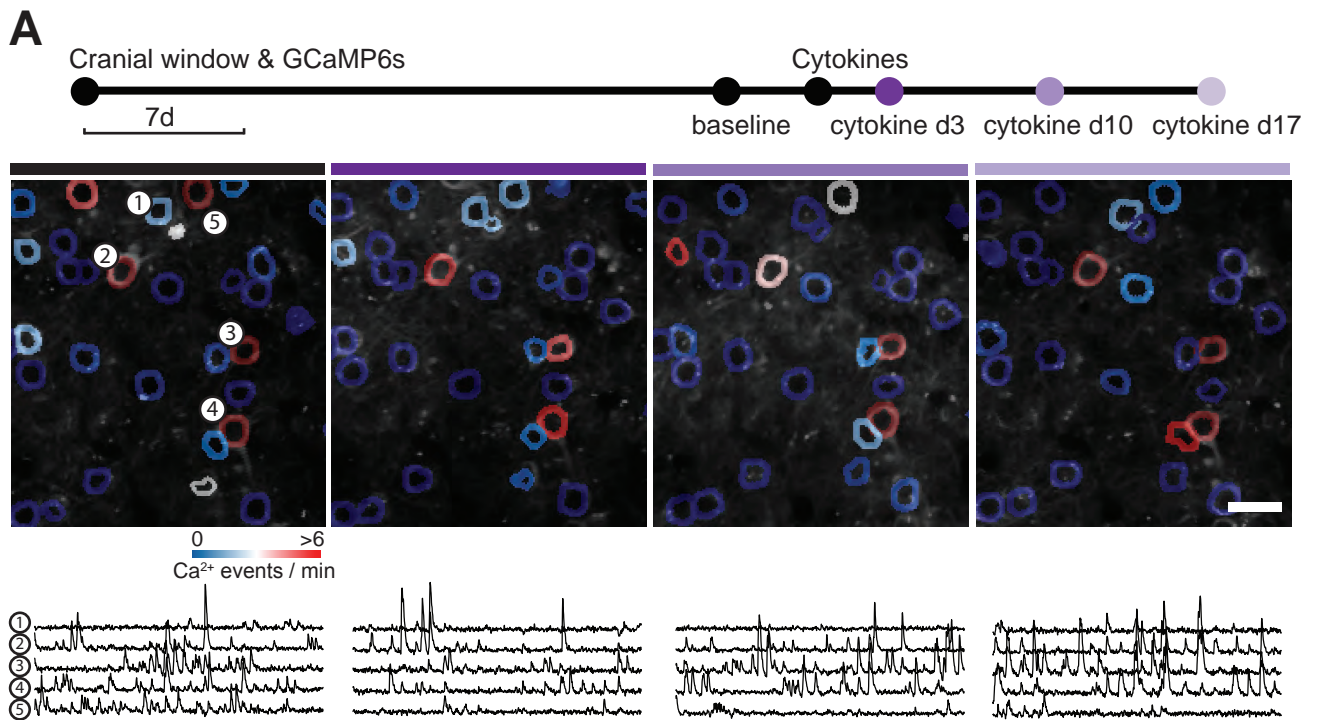


Figure S4 - Recording the activity of single cortical neurons over time in the intact and inflamed gray matter. Related to Figure 2.

(A) Top: Schematic diagram showing longitudinal *in vivo* imaging of Ca^{2+} transients in layer 2/3 somatosensory neurons upon injection of cytokines in healthy animals, using GCaMP6s encoded calcium indicator delivered via viral vector (AAV1.hSyn1.mRuby2.GCaMP6s).

Middle, Bottom: Longitudinal *in vivo* multiphoton images of the same layer 2/3 neuronal population before and after cytokine injection; from **left** to **right** respectively: baseline, 3 days, 10 days and 17 days post cytokine injection. **Middle:** Grayscale images of GCaMP6s channel masked and color-coded for cytoplasmic Ca^{2+} events per minute. **Bottom:**

Representative Ca^{2+} activity traces displayed as delta f/f for the neurons marked in the left panel. **(B)** Cumulative plots of the neuronal activity for the entire population of neurons investigated over the course of the cytokine injection in healthy animals. **Insert:** Mean neuronal activity normalized to baseline for each time point shown as mean \pm SEM (n=4 cytokine injected mice, tested with Shapiro-Wilk and one-way ANOVA). **(C)** Heat map representation of the single neurons' activities before and after cytokine injection. Scale bar in **A**, 20 μm .

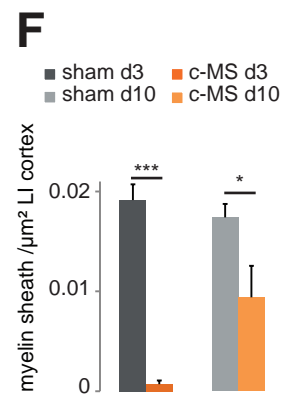
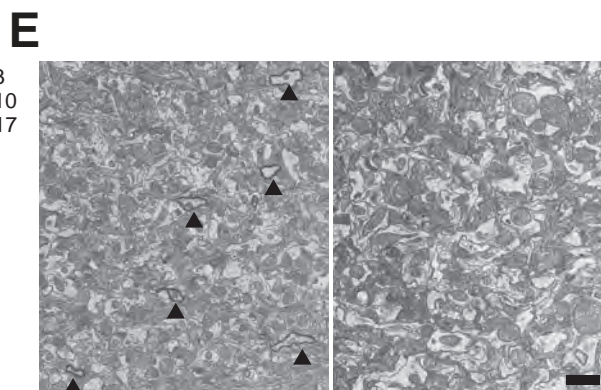
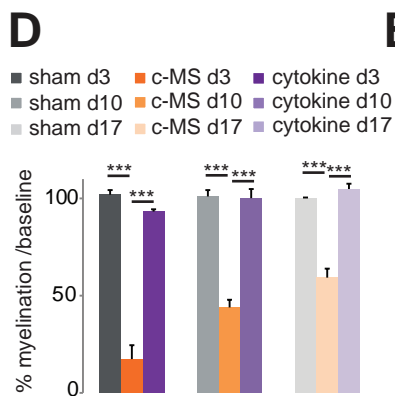
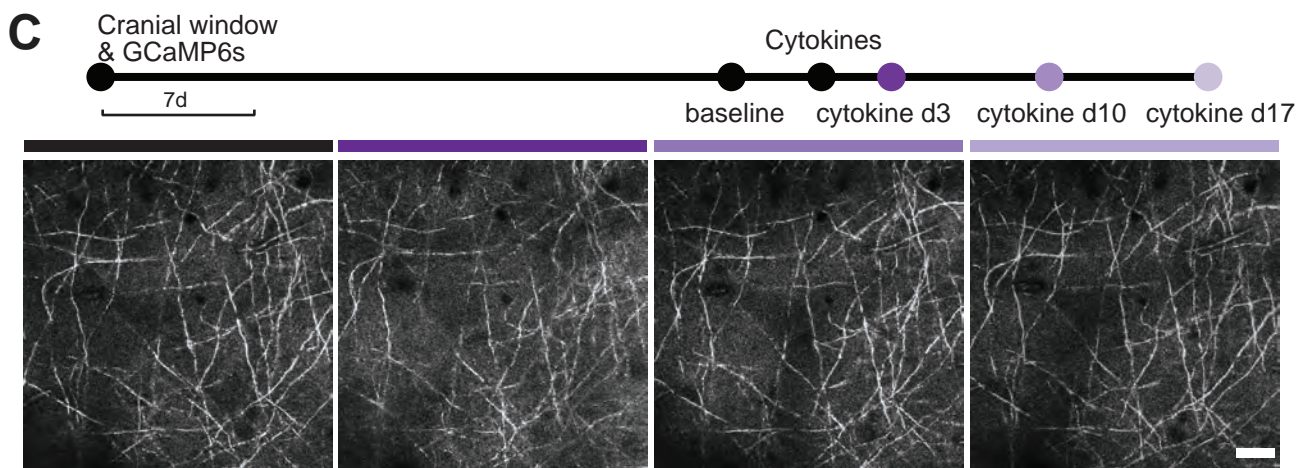
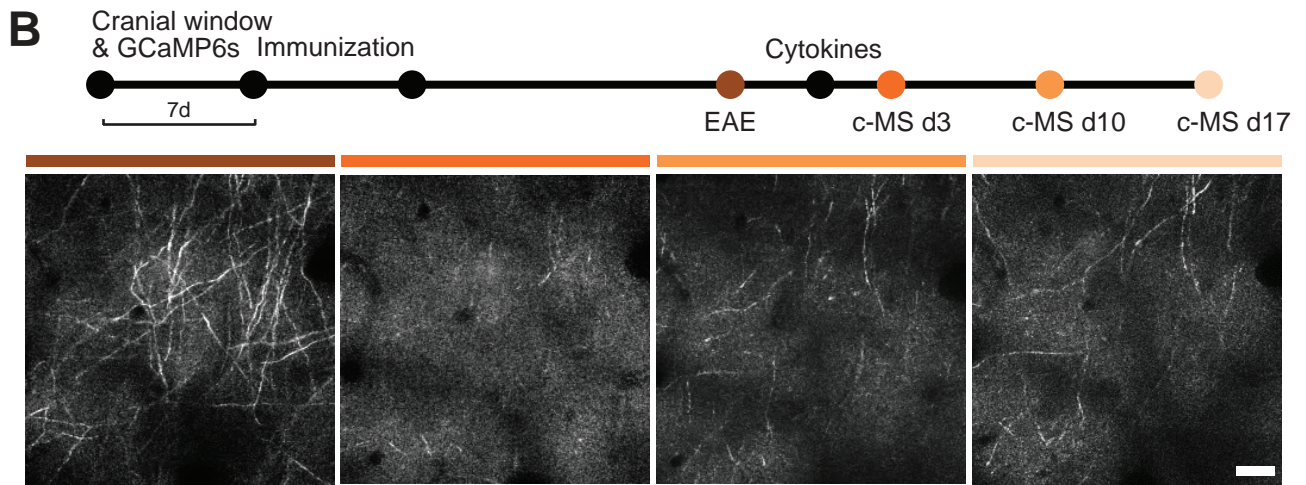
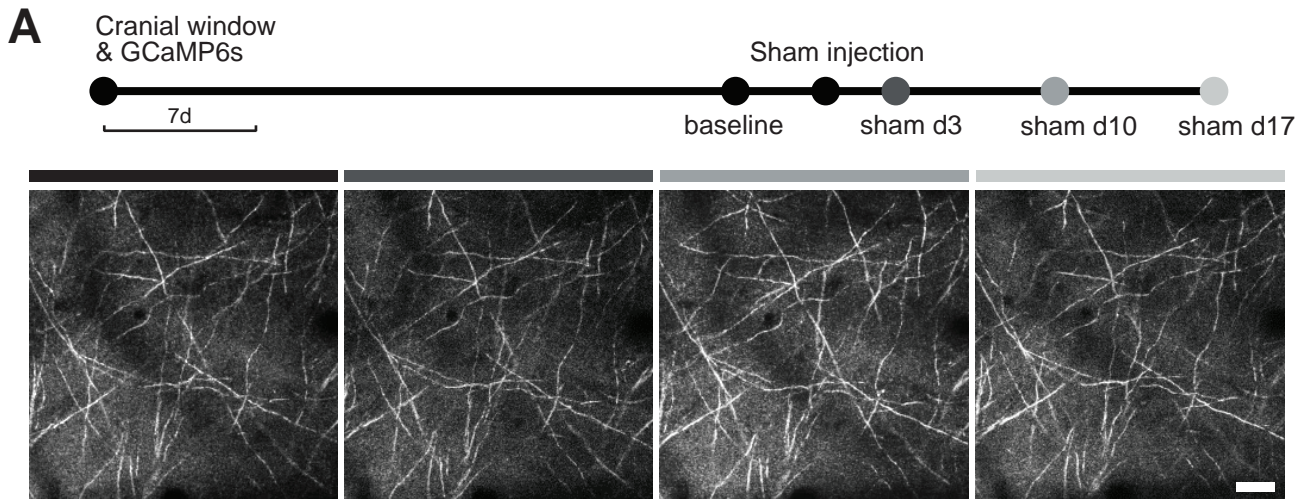


Figure S5 – Myelination recovers slower than neuronal activity. Related to Figure 2.

(A-C) Top: Schematic diagram showing longitudinal *in vivo* SCoRe imaging of myelin within the top 50 μm of layer 1 somatosensory cortex in sham injected animals **(A)**, animals with cortical MS model **(B)** and in cytokine injected healthy animals **(C)**. **(A-C) Bottom:** Longitudinal *in vivo* SCoRe images of the same layer 1 area. From **left to right** respectively: **(A)** baseline, 3 days, 10 days and 17 days post sham injection. **(B)** EAE baseline, 3 days, 10 days and 17 days post cytokine injection. **(C)** baseline, 3 days, 10 days and 17 days post cytokines injection. **(D)** Percentage of SCoRe signal compared to baselines for sham, cytokine only injection and systemic EAE for the c-MS model, 3 days, 10 days and 17 days post injection shown as mean \pm SEM (n=3 for sham, cytokine only and c-MS mice, Shapiro-Wilk and One-way ANOVA followed by Bonferroni's multiple comparisons test has been performed). **(E)** Electron micrographs of sham injected (**left**) and c-MS model (**right**) cortex layer 1 showing myelin sheath (black arrowheads) 3 days post injection. **(F)** Myelin sheath density in layer 1 of somatosensory cortex in sham injected and c-MS animals at 3 days and 10 days post injection shown as mean \pm SEM (tested in n=4 sham injected and n=4 c-MS mice for day 3 and n=5 sham injected and n=3 c-MS mice for day 10, Shapiro-Wilk and One-way ANOVA followed by Bonferroni's multiple comparisons test). Scale bar in **A, B, C**, 20 μm ; **E**, 1 μm . ***P<0.001, *P<0.05

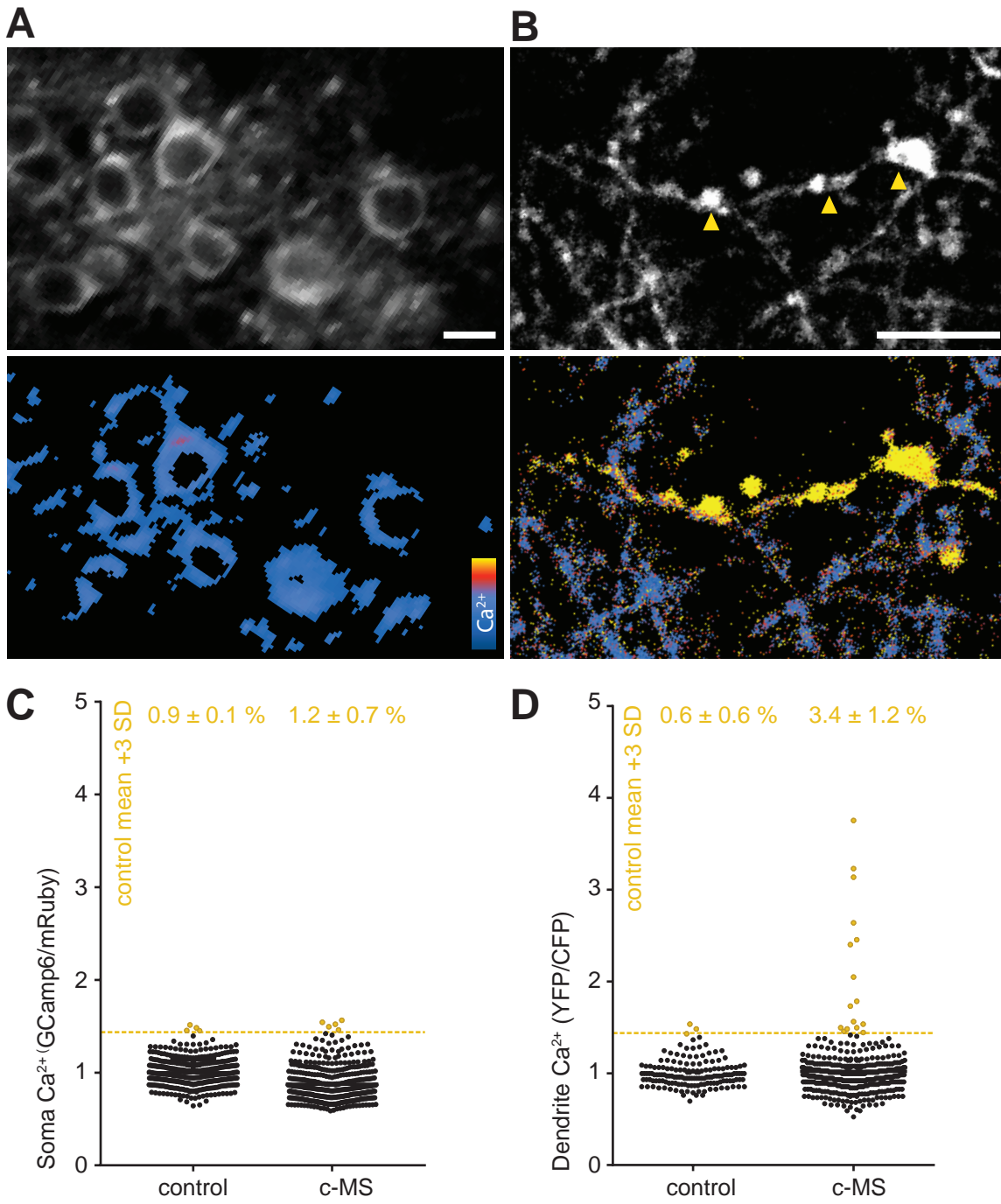


Figure S6 – Ratiometric imaging of calcium levels in the somata and dendrites of cortical neurons. Related to Figure 4.

(A) *In vivo* multiphoton projection images of layer 2-3 neuronal somata and their Ca^{2+} levels in acute cortical MS model. Neurons were labelled using the viral vector AAV1.hSyn.mRuby2.Gcamp6s (see Methods), allowing for ratiometric readout (GCamp6s/mRuby) of baseline neuronal Ca^{2+} concentration. Grayscale images of GCamp6s channel (**top**), ratiometric (GCamp6s/mRuby) images masked and color-coded for cytoplasmic Ca^{2+} (**bottom**). **(B)** *In vivo* multiphoton projection images of apical tuft dendrites and their Ca^{2+} levels in acute c-MS model, labelled with AAV1.hSyn1.Twitch2b. Grayscale images of YFP channel (**top**), ratiometric (YFP/CFP) images masked and color-coded for cytoplasmic Ca^{2+} (**bottom**). Example of a high Ca^{2+} dendrite exhibiting swellings (**arrow heads**). **(C)** Ca^{2+} concentration of single somata in control (sham injection) and c-MS (d3) animals, plotted as GCamp6s/mRuby channel ratios normalized to control mean. **Top:** Percentage of somata per animal with Ca^{2+} concentration $> 3\text{SD}$ above control mean, shown as mean \pm SEM (tested per animal in $n=2$ control and $n=3$ c-MS mice, Mann-Whitney U test). **(D)** Ca^{2+} concentration of single dendrites in healthy control and c-MS d3 animals, plotted as YFP/CFP channel ratios normalized to control mean. **Top:** Percentage of dendrites per animal with Ca^{2+} concentration $>3\text{SD}$ above control mean, shown as mean \pm SEM (tested per animal in $n=8$ control and $n=17$ c-MS mice, Mann-Whitney U test). Scale bars in **A, B**, 10 μm .

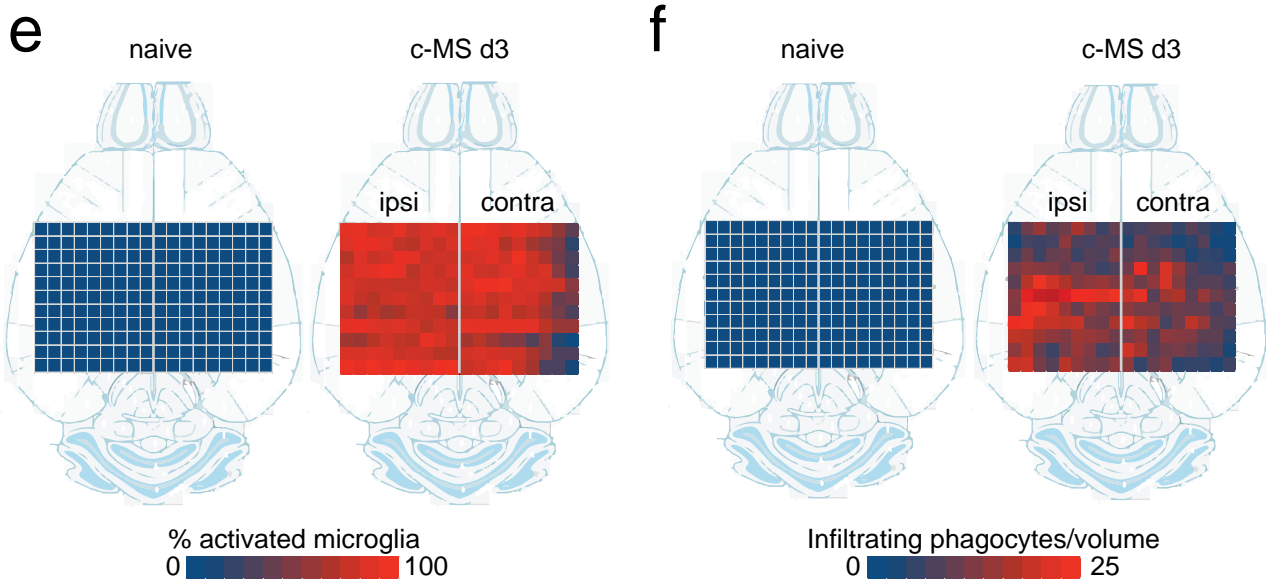
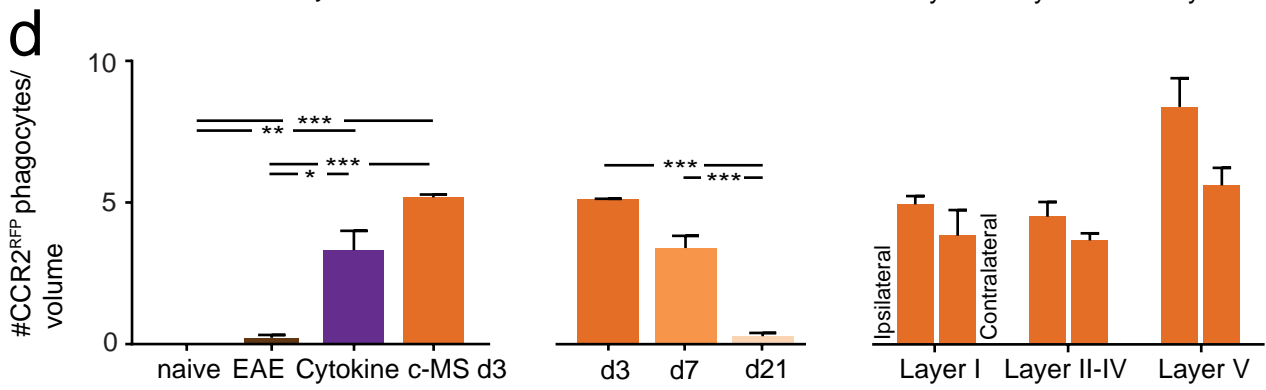
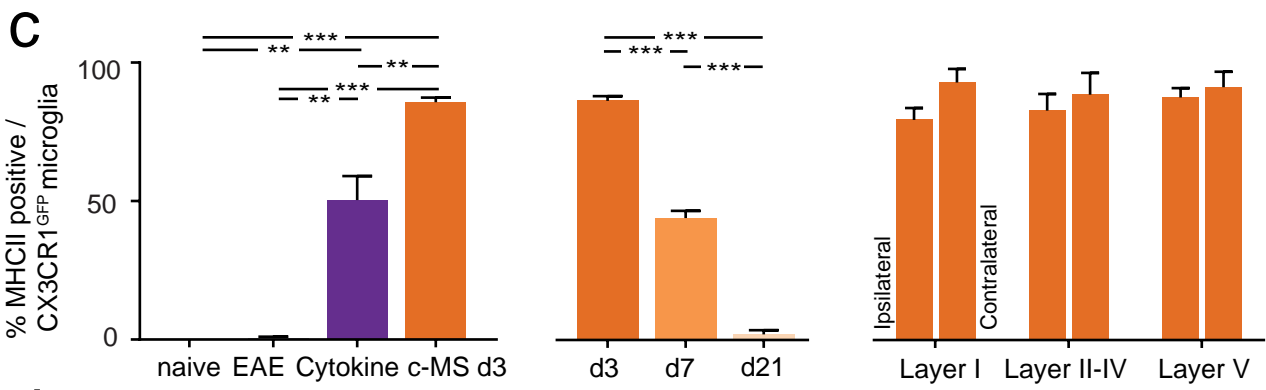
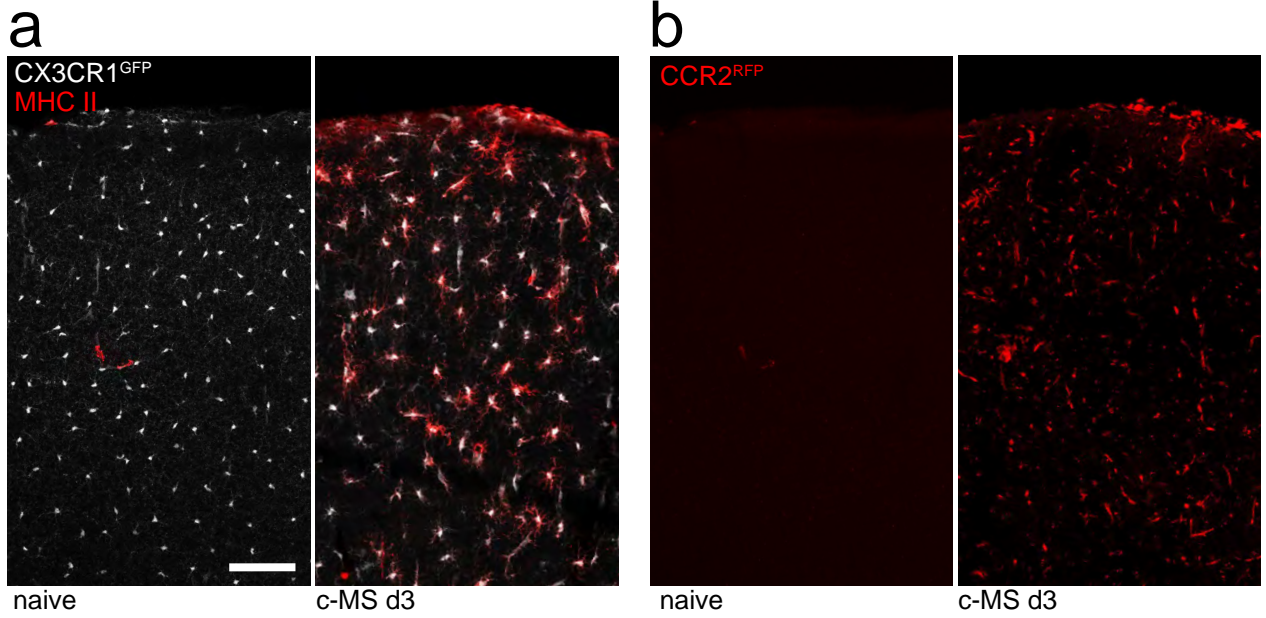


Figure S7 – Temporal and spatial characteristics of monocyte infiltration and microglial activation in the c-MS model. Related to Figure 5.

(A, B) Confocal projection images of a cortical column in healthy control (naive, **left**) and acute cortical MS model (c-MS d3, **right**) *BiozziABH CX3CR1^{GFP} x CCR2^{RFP}* mice. **(A)** Immuno-staining for MHCII (red), as a marker of microglial activation, overlaid on the GFP signal of resident microglia (greyscale). **(B)** RFP signal of blood-born infiltrating phagocytes shown in red. **(C) Left:** Percentages of activated microglia (% MHCII positive/all CX3CR1^{GFP} cells) in healthy control (naive), only immunized (EAE), only cytokine injected (cytokine) and acute cortical MS model (c-MS d3) mice. **Middle:** Timecourse of microglial activation (in %) 3d, 7d and 21d after induction of cortical MS model (d3, d7, d21). **Right:** Distribution of microglial activation (in %) within the layers of the cortical column (Layer1, Layer 2-4 and Layer 5) and between injected (ipsilateral) and non-injected hemisphere (contralateral). c-MS d3 data plotted in all 3 panels. Shown as mean ± SEM (tested per animal in n=2 naive, n=2 EAE, n=3 Cytokine, n=3 c-MS d3; 1way ANOVA). **(D) Left:** Numbers of infiltrating phagocytes (CCR2^{RFP} cells) per standardized volume in healthy control (naive), only immunized (EAE), only cytokine injected (cytokine) and acute cortical MS model (c-MS d3) mice. **Middle:** Timecourse of phagocyte infiltration 3d, 7d and 21d after induction of cortical MS model (d3, d7, d21). **Right:** Distribution of phagocyte infiltration within the layers of the cortical column (Layer1, Layer 2-4 and Layer 5) and between injected (ipsilateral) and non-injected hemisphere (contralateral). c-MS d3 data plotted in all 3 panels. Shown as mean ± SEM (tested per animal in n=2 naive, n=2 EAE, n=3 Cytokine, n=3 c-MS d3; 1way ANOVA). **(E)** Color-coded spatial matrix representing microglial activation (in %) of healthy control (naive, n=3, **left**) and acute cortical MS model (c-MS d3, n=3, **right**) mice, quantified in 11x8 cortical segments ipsi- and contralateral to the cytokine injection site. **(F)** Color-coded matrix showing numbers infiltrating phagocytes (Iba1 immunolabelling positive, CX3CR1^{GFP} negative cells) in healthy control (naive, n=3, **left**) and acute cortical MS model (c-MS d3, n=3, **right**) mice per cortical segment ipsi- and contralateral to injection site. Scale bar in **A** (applies to **B**) 100 μm. ***P<0.001, **P<0.01, *P<0.05.

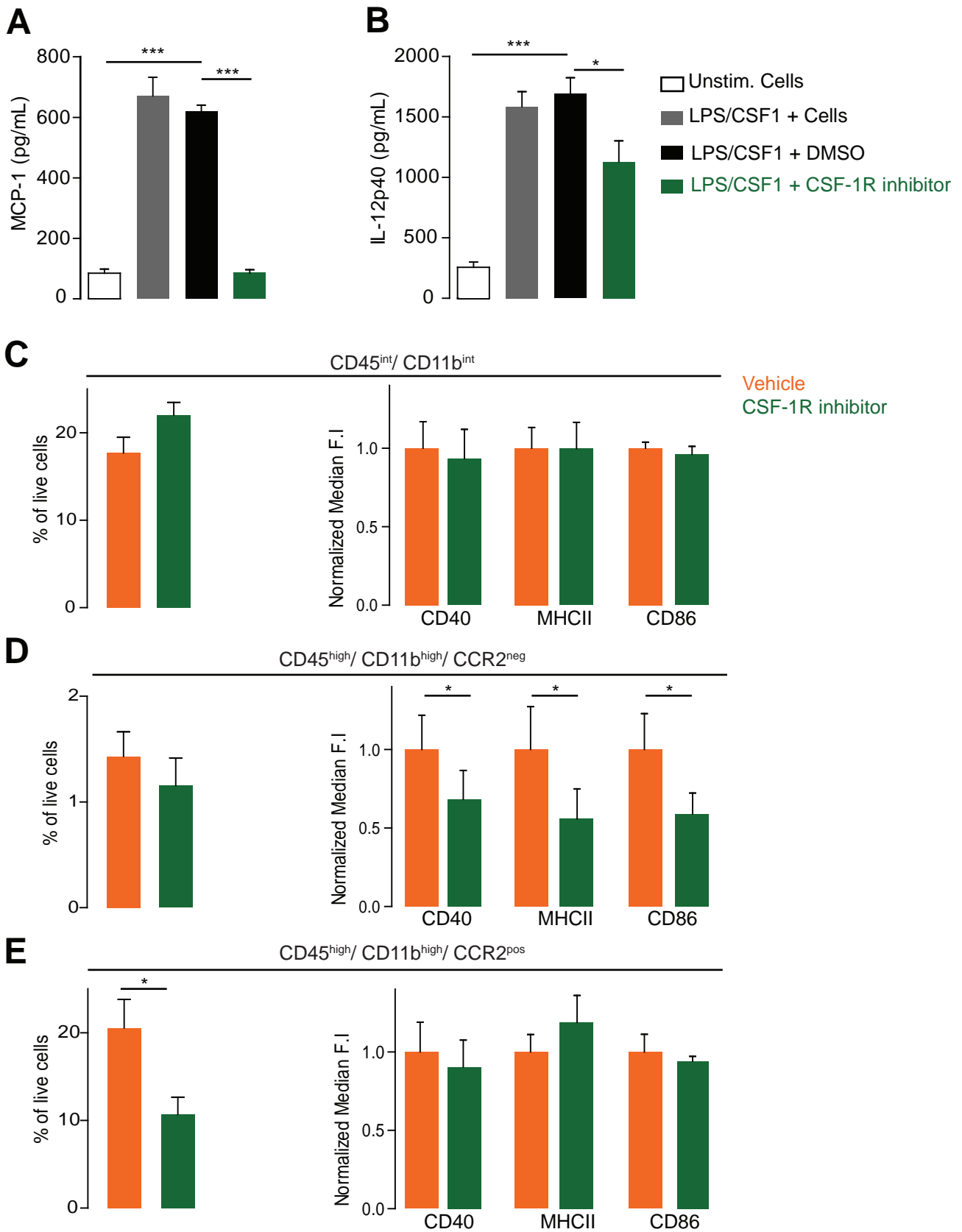


Figure S8 – *In vitro* and *in vivo* effects of CSF-1R inhibitor on inflammation. Related to Figure 6.

(A) Isolated primary murine microglia (untreated) show elevated MCP-1 (CCL2) after treatment with CSF-1. While adding DMSO does not change the levels of MCP-1, CSF-1R inhibitor compound decreases the levels of MCP-1 in primary murine cells (n=3 in all groups, one-way ANOVA). (B) Isolated primary murine microglia (untreated) show elevated Il-12p40 after treatment with LPS. While adding DMSO does not change the levels of Il-12p40, CSF-1R inhibitor decreases the levels of Il-12p40 in primary murine cells (n=6 for untreated cells and n=5 for the other groups, one-way ANOVA). (C-E) FACS analysis of cell populations in brains of acute c-MS model mice. (C) The proportion of CD45 int/ CD11b int cells to all the live cells remained unchanged following CSF-1R inhibitor treatment in the c-MS model compared to vehicle-treated c-MS model (**left**). CD40, MHCII and CD86 markers showed unchanged levels of median fluorescence intensity (**right**). (D) The proportion of CD45 high/ CD11b high/ CCR2^{RFP} negative cells to all the live cells remained unchanged following CSF-1R inhibitor treatment in the c-MS model compared to vehicle-treated c-MS model (**left**). CD40, MHCII and CD86 markers revealed differences between the levels of median fluorescence intensity of the two groups (**right**). (E) The proportion of CD45 high/ CD11b high/ CCR2 positive cells to all the live cells was decreased following CSF-1R inhibitor treatment in the c-MS model compared to vehicle-treated c-MS model (**left**). CD40, MHCII and CD86 markers showed unchanged levels of median fluorescence intensity (**right**) (n=10 mice were analyzed in each group. Unpaired two-tailed t-test and two-way ANOVA in left panels and right panels of C-E, respectively). *** P<0.001, *P<0.05.

Captions for Movie S1 - Activity of single cortical neurons over the course of cortical MS model.

Movie generated using FIJI software from 15 Hz recordings and displayed at 100 frames per second with a walking average of 3 (60 second recordings per time point).