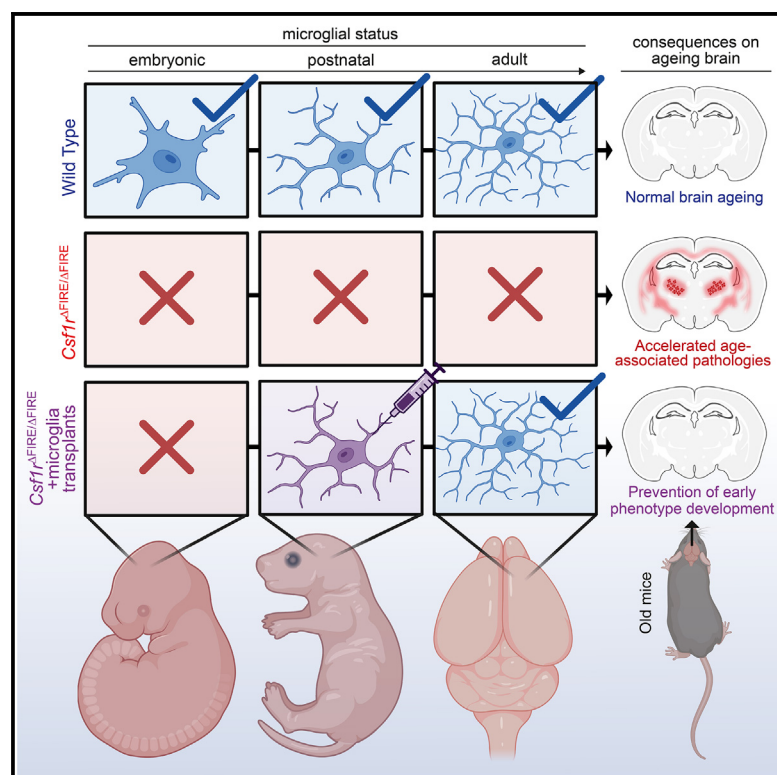


# Microglia protect against age-associated brain pathologies

## Graphical abstract



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## In brief

Microglia are resident immune cells in the brain. Munro et al. explore the consequences of the permanent absence of microglia on the maturation and maintenance of the brain. They show accumulating cellular disruption and damage to the brain as it ages without microglia.

## Highlights

- Lack of microglia leads to progressive brain calcifications and macroglial reactivity
- scRNA-seq reveals brain cell profiles across the lifespan in the absence of microglia
- The thalamus is particularly sensitive to the absence of microglia with aging
- Transplanting microglia protects against pathology development



Article

# Microglia protect against age-associated brain pathologies

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## SUMMARY

Microglia are brain-resident macrophages that contribute to central nervous system (CNS) development, maturation, and preservation. Here, we examine the consequences of permanent microglial deficiencies on brain aging using the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mouse model. In juvenile *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, we show that microglia are dispensable for the transcriptomic maturation of other brain cell types. By contrast, with advancing age, pathologies accumulate in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, macroglia become increasingly dysregulated, and white matter integrity declines, mimicking many pathological features of human CSF1R-related leukoencephalopathy. The thalamus is particularly vulnerable to neuropathological changes in the absence of microglia, with atrophy, neuron loss, vascular alterations, macroglial dysregulation, and severe tissue calcification. We show that populating *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains with wild-type microglia protects against many of these pathological changes. Together with the accompanying study by Chadarevian and colleagues<sup>1</sup>, our results indicate that the lifelong absence of microglia results in an age-related neurodegenerative condition that can be counteracted via transplantation of healthy microglia.

## INTRODUCTION

Microglia, the resident macrophages of the brain parenchyma, carry out diverse roles within the embryonic and adult central nervous system (CNS).<sup>2</sup> Their specialized signatures emerge progressively during development and are thought to be promoted and maintained in adulthood through the effects of other brain cells.<sup>3–5</sup> Reciprocally, microglia may promote the maturation and maintenance of other brain cells, such as macroglia and neuronal cells,<sup>6–9</sup> but this is incompletely understood.

To understand the role of microglia on the maturation and maintenance of brain cells, we investigated brain aging in a microglia-deficient mouse model. Colony stimulating factor 1 receptor (CSF1R) is essential for microglial survival, and its expression is controlled by the *fms*-intronic regulatory enhancer (FIRE), a highly conserved super-enhancer within intron 2 of the gene.<sup>10,11</sup> Mice generated to lack the FIRE element (*Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice) do not have microglia throughout embryonic or postnatal life.<sup>12,13</sup> As *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice reach adulthood, myelin abnormalities develop, but the mice are otherwise remarkably normal<sup>14,15</sup> and



without the confounding impacts of CSF1R antagonists or homozygous *Csf1r* knockouts.<sup>16,17</sup> With aging, we provide evidence for the development of a neurodegenerative condition in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice with severe brain calcifications, disruptions in oligodendroglial gene expression, impairments in white matter integrity, and neuronal loss. Notably, each of these pathologies also occurs in human leukoencephalopathy patients carrying compound heterozygous or homozygous mutations in *CSF1R*.<sup>18,19</sup> In the accompanying study, Chadarevian and colleagues independently demonstrate many of these findings within humanized immune-deficient *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, further showing that microglial transplantation using CRISPR-corrected induced pluripotent stem cell (iPSC)-microglia-like cells can prevent and reverse these pathologies.<sup>1</sup>

## RESULTS

### Non-microglial brain cells reach their mature transcriptomic states in the absence of microglia

Microglia are completely absent throughout *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains during embryonic and postnatal life,<sup>12,13</sup> and we hypothesized that this would result in transcriptomic disturbances to other brain cell types. To map mRNA changes to specific brain cell types, we performed single-cell RNA sequencing (scRNA-seq) on live cells isolated from juvenile (6–7 weeks old) *Csf1r*<sup>WT/WT</sup>, *Csf1r*<sup>WT/ΔFIRE</sup>, and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. Cells were dissociated and sorted from both hippocampi and, separately, from the remainder of the left hemisphere after hippocampal dissection (Figures S1A and S1B). After quality control steps (see STAR Methods, Figures S1C–S1F, and Table S1), we examined the transcriptomes of 16,912 *Csf1r*<sup>WT/WT</sup>, 15,133 *Csf1r*<sup>WT/ΔFIRE</sup>, and 15,493 *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain cells. Expected brain cell populations were captured, but not at normal proportions: this dataset is glia-enriched due to our dissociation and sorting protocol (Figures 1A and S1G–S1I). Mature/myelinating oligodendrocytes from this dataset were recently subsetted and compared between *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (see McNamara et al.<sup>14</sup>), but the remaining cell types have not yet been described. We provide an online resource to visualize transcriptomic changes in the different brain cell types in this dataset, as well as in the other datasets subsequently generated and described in this manuscript (link provided in the “data and code availability” section).

As clusters do not always capture continuous trajectories or provide a suitable resolution to understand cell state shifts, we performed “Milo” differential abundance testing.<sup>21</sup> Milo tests for significant abundance differences after assigning cells to small, partially overlapping cell “neighborhoods” on a k-nearest neighbor graph. Using this approach, we confirm the complete absence of microglia from *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figures 1B and 1C). Some border-associated macrophages (BAMs) were retained, but neighborhoods of these cells were significantly reduced in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figures 1B and 1C), consistent with previous reports describing reduced macrophage densities in certain CNS border compartments<sup>12,13</sup> (Figures 1B and 1C). For the other brain cell types, Milo differential abundance testing revealed only very minor differences in cellular composition (Figures 1B and 1C), except from a previously described subpopulation of *Serpina3n*<sup>high</sup>*C4b*<sup>high</sup> oligodendrocytes enriched in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> white matter.<sup>14</sup>

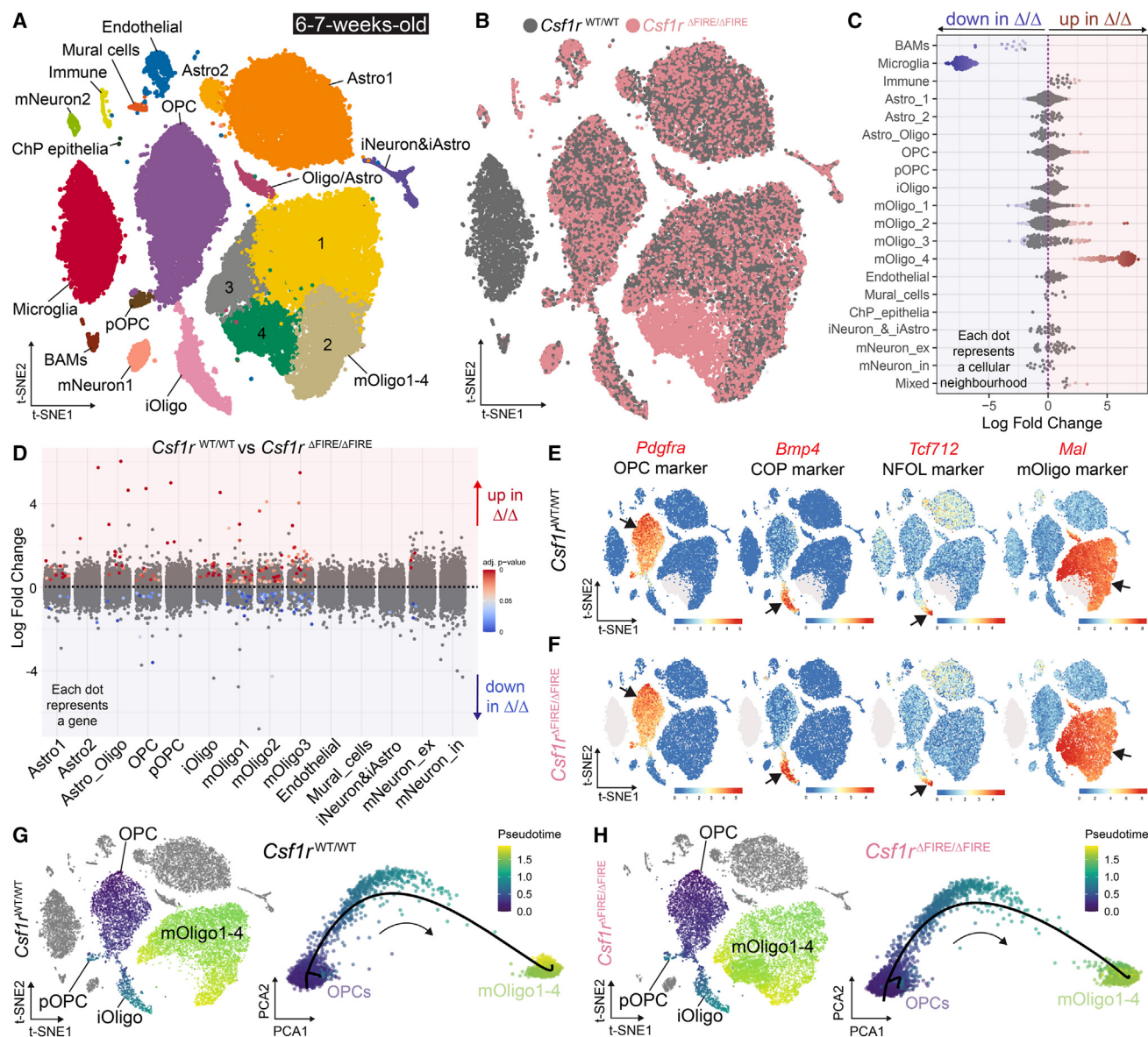
Although relative cell abundances were mostly similar in the different clusters, we wanted to determine whether cellular transcriptomic profiles differed between *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains by performing cluster-wise transcriptomic comparisons. We identified surprisingly few differentially expressed genes (DEGs) in the different brain cell clusters (using edgeR for pseudo-bulk transcriptomic comparisons and after removing predicted ambient RNAs, which are freely floating background transcripts captured alongside cells during the droplet-based encapsulation process; Figure 1D). The *Serpina3n*<sup>high</sup>*C4b*<sup>high</sup> mOligo4 cluster was not included in Figure 1D as too few of these cells are present in *Csf1r*<sup>WT/WT</sup> brains to make the comparison (Figures S2A and S2B), but we previously subsetted and described these cells.<sup>14</sup> Of the other clusters analyzed, mOligo1-3 were the most transcriptionally different in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, with enrichment of genes such as *Serpina3n*, *C4b*, *Cebpd*, and *Aldh2* indicating mild dysregulation of these cells. Other examples of DEGs in juvenile *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain cells include upregulation of aldehyde dehydrogenase 7 family member A1 (*Aldh7a1*) in Astro1, oligodendrocyte precursor cells (OPCs), and iOligos, and upregulation of interferon alpha-inducible protein 27 (*Ifi27*) in OPC and iOligo clusters. Similar findings (i.e., minor transcriptional disruption of most cell types) were also observed after separating and analyzing cell clusters based on the tissue region from which they were obtained (Figures S2C and S2D), and when comparing cell clusters from heterozygous *Csf1r*<sup>WT/ΔFIRE</sup> brains with the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain cells (Figure S2E; DEGs identified for the “Neuron&iAstro” cluster likely reflect the different ratios of heterogeneous cell types in this small cluster being captured between genotypes).

As previous studies suggested roles for microglia in promoting oligodendrocyte maturation,<sup>6,7,22–24</sup> we next took advantage of capturing *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> oligodendrocytes across their developmental lineage by exploring their maturation trajectories (namely, OPCs, differentiation-committed oligodendrocyte precursors [COPs], newly forming oligodendrocytes, and mature/myelinating oligodendrocytes<sup>20</sup>; Figures 1E and 1F). Notably, the developmental profiles and trajectories of these cells during oligodendrogenesis were comparable in *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figures 1G and 1H), indicating that microglia are dispensable for the normal transcriptomic maturation of oligodendrocytes in these mice. The finding that the other analyzed brain cell types also reached transcriptomic maturity in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice provides strong evidence that their mature profiles can also emerge independently from microglia (Figure 1D).

Together, these data indicate that the absence of microglia during embryonic and early postnatal life minimally impedes the transcriptomic maturation of other brain cell types. Moreover, we do not find evidence for a compensatory increase in the numbers of other macrophages/immune cell types during postnatal development when microglia are absent.

### Microglial *Csf1r* haploinsufficiency has minor consequences on brain maturation

Reduced CSF1R levels due to lack of its production from one dysfunctional *CSF1R* allele (also known as *CSF1R* haploinsufficiency) has been claimed to be a cause of some cases of



**Figure 1. Sensitivities of different brain cell types to the absence of microglia in the juvenile brain**

(A) Cells captured and analyzed for single-cell RNA sequencing from  $Csf1r^{WT/WT}$  and  $Csf1r^{AFIRE/AFIRE}$  brains ( $n = 4$  samples per genotype from 2  $Csf1r^{WT/WT}$  mice and 2  $Csf1r^{AFIRE/AFIRE}$  mice, all females).

(B) Cell identities by genotype (gray cells from  $Csf1r^{WT/WT}$  and pink cells from  $Csf1r^{AFIRE/AFIRE}$  brains).

(C) Differential abundance comparison of cellular neighborhoods based on genotype (red dots = significantly increased abundance of cells in this neighborhood in  $Csf1r^{AFIRE/AFIRE}$  brains; blue dots = significantly decreased abundance in  $Csf1r^{AFIRE/AFIRE}$  brains; gray dots = not significantly altered).

(D) Differentially expressed genes (DEGs) for different cell clusters from  $Csf1r^{WT/WT}$  and  $Csf1r^{AFIRE/AFIRE}$  brains.

(E and F) Expression of different markers across the process of oligodendrogenesis in  $Csf1r^{WT/WT}$  and  $Csf1r^{AFIRE/AFIRE}$  brains. *Pdgfra* = OPCs; *Bmp4* = differentiation-committed oligodendrocyte progenitors (COPs); *Tcf712* = newly forming oligodendrocytes; *Mal* = mature/myelinating oligodendrocytes (markers based on Marques et al.<sup>20</sup>).

(G and H) Principal-component analysis (PCA) plots show trajectory analysis of (G)  $Csf1r^{WT/WT}$  and (H)  $Csf1r^{AFIRE/AFIRE}$  oligodendrocyte-lineage cells (differentiation trajectory/pseudotime goes from left to right, as indicated by arrows). t-distributed stochastic neighbor embedding (t-SNE) plots show the cells included in the oligodendrocyte trajectory analysis colored based on their pseudotime scores, and cells not included in this analysis shown in gray.

CSF1R-related leukoencephalopathy.<sup>25,26</sup> By contrast, other studies support a dominant-negative effect of disease-associated *CSF1R* mutations.<sup>27,28</sup> Regardless of the precise mechanism that underlies CSF1R-related leukoencephalopathy, the

early life consequences of microglial *CSF1R* haploinsufficiency are incompletely understood.

Unlike mice with bi-allelic FIRE deletion, mice with single-allelic FIRE deletion ( $Csf1r^{WT/\Delta FIRE}$  mice) retain both microglia

and BAMs.<sup>12,13</sup> However, we demonstrate that CSF1R (CD115) surface protein levels are approximately halved on microglia and other brain myeloid cells in *Csf1r*<sup>WT/ΔFIRE</sup> brains compared with cells from control *Csf1r*<sup>WT/WT</sup> brains (Figures S3A–S3C). scRNA-seq substantiated this finding, as *Csf1r* mRNA levels were decreased in *Csf1r*<sup>WT/ΔFIRE</sup> microglia (Figure S3D). These data reveal that FIRE deletion, even from just one allele, markedly alters *Csf1r*/CSF1R production by CNS macrophages (i.e., there is no dosage compensation from the wild-type allele), making this a useful model to study the consequences of microglial CSF1R haploinsufficiency.

In juvenile *Csf1r*<sup>WT/ΔFIRE</sup> brains, the transcriptomic identities of haploinsufficient microglia were mildly disrupted (with 24 down-regulated and 18 upregulated transcripts compared with *Csf1r*<sup>WT/WT</sup> microglia, based on adjusted  $p < 0.05$ ), and we detected few differences in the abundances or transcriptomic profiles of other brain cell types compared with *Csf1r*<sup>WT/WT</sup> brain cells (Figures S3E–S3G). The developmental trajectory of oligodendrocyte-lineage cells (from OPCs to mature/myelinating oligodendrocytes) appeared normal in *Csf1r*<sup>WT/ΔFIRE</sup> brains (Figures S3H and S3I), as was observed for the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> oligodendrocyte-lineage cells. Furthermore, we did not observe behavioral changes in the open-field test or elevated plus maze test between haploinsufficient *Csf1r*<sup>WT/ΔFIRE</sup> and *Csf1r*<sup>WT/WT</sup> juvenile mice (for females or males; Figures S3J–S3M). These data indicate that there are only minor consequences of microglial *Csf1r* haploinsufficiency on brain cell status in juvenile mice.

### Progressive glial cell disruption with aging in the absence of microglia

We have shown that many cell types in the juvenile mouse brain can develop with remarkably few transcriptomic alterations in the absence of microglia (Figure 1D). However, less is known about the consequences of permanent microglial deficiencies as the brain ages. We therefore examined cohorts of old *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice. We observed an age-associated decline in the well-being of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, with 45% of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice reaching humane endpoints by 18 months of age (Figure 2A). Despite this, *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice that survived to 15.5–17 months of age without apparent health issues did not exhibit clear signs of reduced activity or gait disturbances when compared with age-matched *Csf1r*<sup>WT/WT</sup> mice (Figures S4A–S4I). Nor did they exhibit differences in their hematological profiles at this age (Figures S4J–S4R). We also examined macrophage populations in peripheral organs of 15.5- to 17-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, finding the organs lacking or retaining resident macrophages were consistent with those previously described in juvenile *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice<sup>13</sup> (Figure S5).

To examine whether brain cell profiles become increasingly dysregulated with aging in the absence of microglia, we performed scRNA-seq using 11- to 12-month-old brains (prior to apparent health issues in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice) and 16- to 18-month-old brains (when health issues are common in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice) (Figure 2A). Mixed single-cell suspensions were prepared from whole *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains using a similar protocol and approach as for Figure 1. Clusters from 11- to 12-month-old brains are labeled with a

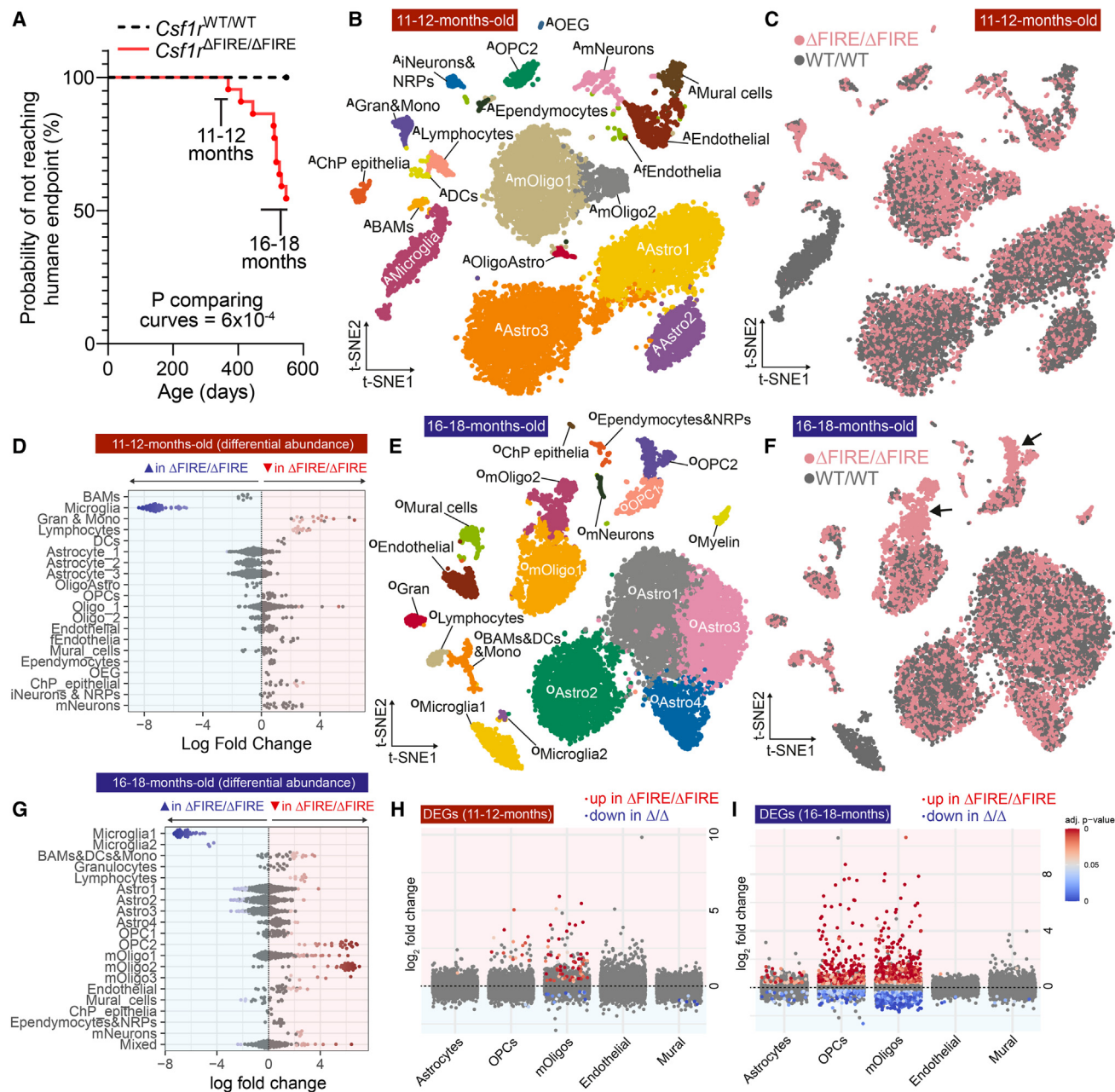
bold superscript “A” for adult (e.g., <sup>A</sup>Astro1), whereas clusters from 16- to 18-month-old brains are labeled with a bold superscript “O” for old (e.g., <sup>O</sup>Astro1) (Figures 2B and 2E).

As expected, microglia were still completely absent from *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains at both 11–12 and 16–18 months of age (Figures 2B–2G). In 11- to 12-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, the proportions of other cell types were largely equivalent to those of their *Csf1r*<sup>WT/WT</sup> counterparts, although a small enrichment of certain non-microglial myeloid cells and lymphocytes was detected (Figures 2C and 2D). At 16–18 months of age, however, specific neighborhoods of OPCs and myelinating oligodendrocytes were highly enriched in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, with 99% and 93% of cells in clusters <sup>O</sup>OPC2 and <sup>O</sup>mOligo2, respectively, being derived from *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> samples (Figures 2E–2G). Abundances of captured vascular cells and astrocytes were largely unchanged at all ages examined (Figures 1C and 2C–2G).

We next performed differential expression analyses to explore the transcriptomic profiles of the captured brain cell types in adult (11–12 months old) and old (16–18 months old) mice. In 11- to 12-month-old mice, the transcriptomic profiles of most cell types were minimally disrupted in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, except mature oligodendrocytes, which had upregulated markers previously described in “disease-associated” oligodendrocytes (DOLs), such as *Serpina3n* and *C4b*<sup>29,30</sup> (Figure 2H). In 16- to 18-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, transcriptomic dysregulation of astrocytes, OPCs, and mature oligodendrocytes was more striking, while we detected few DEGs for endothelia or mural cells at any age (the “mural cell” clusters in each dataset are predominantly comprised of pericytes; Figure 2I). Far fewer transcripts were altered at 11–12 months of age in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> OPCs, mature oligodendrocytes, and astrocytes when compared with 16–18 months of age. However, there was a high degree of overlap in the modified transcripts between both ages (Figure 3A), likely reflecting a progressive dysregulation of these cells with aging.

Astrocytes from 16- to 18-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains had several upregulated transcripts previously associated with “pan-reactive astrocytes” (such as *Gfap*, *Vim*, *Aspg*, *Cd44*, and *Osmr*),<sup>32</sup> as well as mRNAs involved in cellular defense (e.g., *Aldh7a1*<sup>33</sup> and *Nrro3*<sup>34</sup>) (Figure S6A). We identified the locations of reactive astrocytes *in situ* using glial fibrillary acidic protein (GFAP) immunofluorescence. GFAP<sup>high</sup> astrocytes are enriched in the thalamus and the corpus callosum of 7- to 11-month-old and 18- to 19-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains compared with age-matched *Csf1r*<sup>WT/WT</sup> brains (Figures 3B and 3C). In the older group of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, abundant GFAP<sup>high</sup> astrocytes were present throughout all observable white matter tracts (including the fimbria and internal capsule), whereas in the thalamus they were largely restricted to its ventral portion (Figure 3C). Numbers of GFAP<sup>high</sup> astrocytes had also increased in the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> hippocampus and cortex by 18–19 months of age, but they did not differ in the hypothalamus at either age (Figure 3B).

Oligodendrocyte-lineage cells were also dysregulated in old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. For the dysregulated cluster of mature oligodendrocytes enriched in 16- to 18-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (<sup>O</sup>mOligo2), there were 1,741 upregulated and 1,072



**Figure 2. Increased glial cell disruption with aging in the absence of microglia**

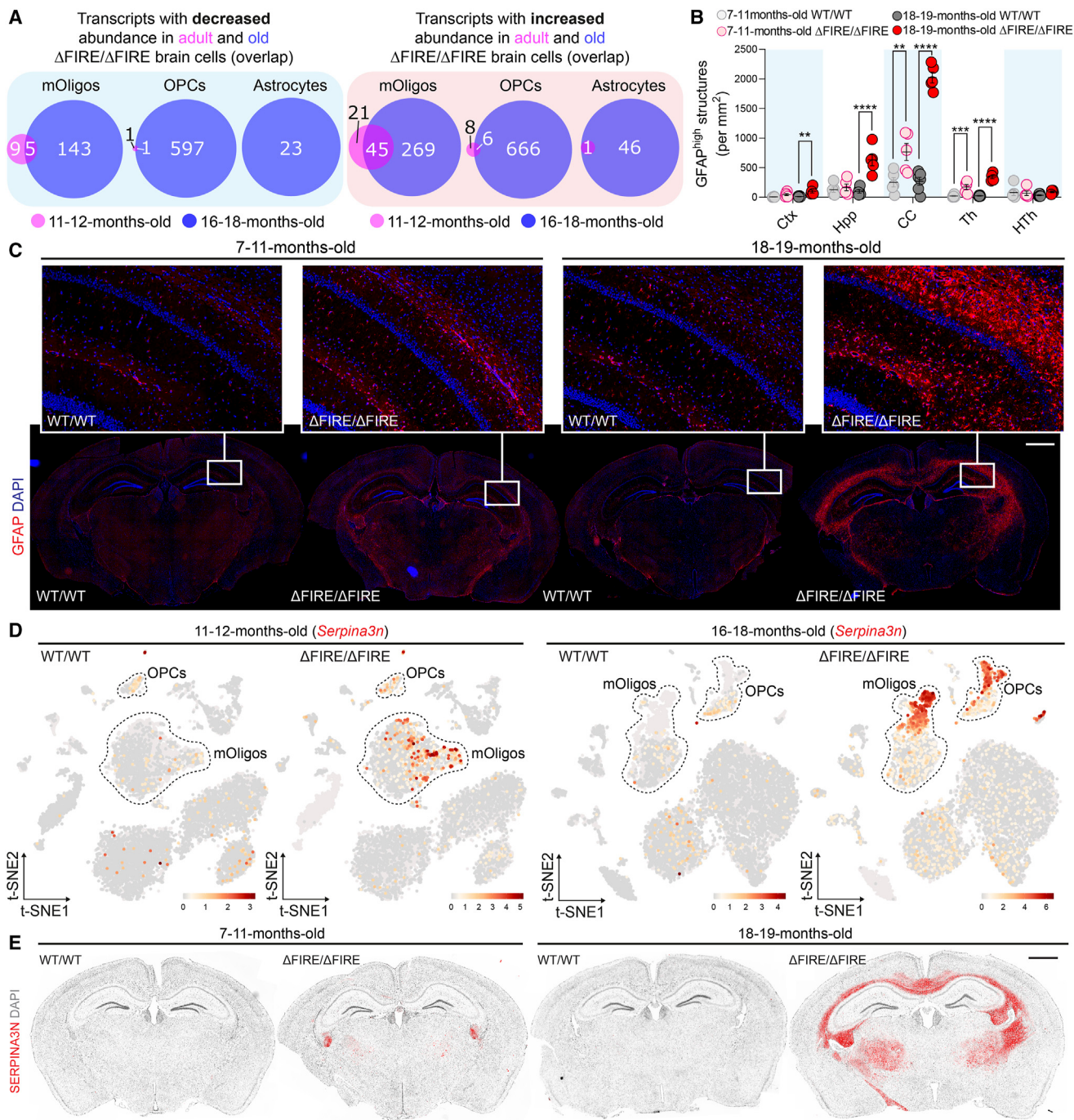
(A) Kaplan-Meier plot shows *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice are more likely to reach humane endpoints with aging ( $n = 20$  *Csf1*<sup>WT/WT</sup> mice [8 females, 12 males], 22 *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice [11 females, 11 males];  $p = 6 \times 10^{-4}$  using Mantel-Cox/log-rank test; chi-square = 11.65; df = 1). The ages used for single-cell RNA sequencing experiments are indicated.

(B–G) Single cells captured and sequenced from (B and C) 11- to 12-month-old and (E and F) 16- to 18-month-old *Csf1*<sup>WT/WT</sup> and *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice ( $n = 3$  *Csf1*<sup>WT/WT</sup> and 3 *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice per age group, all males). (D and G) Milo differential abundance plots showing enriched and de-enriched neighborhoods of cells in (D) 11- to 12-month-old and (G) 16- to 18-month-old *Csf1*<sup>WT/WT</sup> and *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice. Arrows in (F) indicate the *Csf1*<sup>ΔFIRE/ΔFIRE</sup> enriched clusters, <sup>o</sup>OPC2 and <sup>o</sup>mOligo2.

(H and I) Differential expression plots of main captured cell types in (H) 11- to 12-month-old and (I) 16- to 18-month-old *Csf1*<sup>WT/WT</sup> and *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice (gene colored only when significantly altered [red = upregulated; blue = downregulated], based on adjusted  $p < 0.05$ ).

downregulated transcripts compared with cluster <sup>o</sup>mOligo1 (based on adjusted  $p < 0.05$ ) (Figure S6B). Upregulated mRNAs include previously described DOL markers, such as *C4b* and

*Serpina3n*,<sup>30</sup> and transcripts induced in oligodendrocytes during demyelination, such as *Syt4* and *Gdf15*.<sup>35</sup> Based on the DEGs in the <sup>o</sup>mOligo2 cluster, Ingenuity pathway analysis (IPA) predicted



**Figure 3. Macrogial dysregulation in the white matter and thalamus with aging in the absence of microglia**

(A) Venn diagrams showing overlap of upregulated and downregulated transcripts in 11- to 12-months-old (ilac) and 16- to 18-months-old (blue)  $Csf1^{\Delta$ FIRE/ $\Delta$ FIRE astrocytes, OPCs, and mature oligodendrocytes (based on adjusted  $p < 0.05$ ). For illustrative purposes, Venn diagrams, which were created using DeepVenn,<sup>31</sup> are area proportional based only on the age group for a given cell type (not across cell types).

(B and C) Increased numbers of GFAP<sup>high</sup> astrocytes with aging in the absence of microglia. Corpus callosal (CC), thalamic (Th), hippocampal (Hpp), and cortical (Ctx) GFAP<sup>high</sup> astrocytes are increased in old brains. Hypothalamic (HTh) GFAP<sup>high</sup> astrocyte numbers are not significantly altered ( $n = 5-6$  mice per group [2-3 females and 2-3 males per group]); groups compared by two-way ANOVA with Tukey's multiple comparison post hoc testing; \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; mean  $\pm$  SEM shown for each group).

(legend continued on next page)

increased activation of several pathways, including those related to myelination ( $Z$  score = 2.33;  $p = 7.25 \times 10^{-7}$ ), interferon signaling ( $Z$  score = 3;  $p = 2.8 \times 10^{-6}$ ), and wound healing ( $Z$  score = 4;  $p = 8.33 \times 10^{-10}$ ). WNT/ $\beta$ -catenin signaling, on the other hand, was predicted to be inhibited ( $Z$  score =  $-2.236$ ;  $p = 3.56 \times 10^{-6}$ ).

For the dysregulated cluster of aged *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> OPCs (cluster <sup>OPC2</sup>), there were 2,673 upregulated and 2,320 downregulated mRNAs compared with cluster <sup>OPC1</sup> (with adjusted  $p < 0.05$ ) (Figure S6C). Based on the DEGs in the <sup>OPC2</sup> cluster, IPA predicted increased activation of eukaryotic initiation factor 2 (eIF2) signaling ( $Z$  score = 5.31;  $p = 7.83 \times 10^{-39}$ ) and inhibition of both neurovascular coupling signaling ( $Z$  score =  $-4.44$ ;  $p = 4.95 \times 10^{-15}$ ) and synaptogenesis signaling ( $Z$  score =  $-4.26$ ;  $p = 1.5 \times 10^{-11}$ ). Some of the upregulated genes in <sup>OPC2</sup> were also increased in the dysregulated cluster of mOligos (<sup>OPC2</sup>), such as *Serpina3n*, *C4b*, *Piezo2*, *Bmp4*, *Socs3*, and *Fos*; however, the dysregulation profiles of <sup>OPC2</sup>s and <sup>OPC1</sup>s were largely distinct from each other (Figures S6D and S6E).

As expression of *Serpina3n*, a serine protease inhibitor, was massively enriched in clusters <sup>OPC2</sup> and <sup>OPC1</sup> (Figure 3D), we investigated the location of these dysregulated cells *in situ* by performing immunohistochemistry for SERPINA3N and SOX10 (marking oligodendrocyte-lineage cell nuclei). SERPINA3N-immunoreactivity greatly increased with aging and was particularly high throughout the white matter and ventral thalamus of 16- to 18-month-old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains (Figure 3E). Notably, the distribution pattern of SERPINA3N changed with aging: in juvenile (6–7 weeks old) *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> white matter, SERPINA3N signal was predominantly perinuclear (mostly abutting oligodendrocyte-lineage nuclei; Figure S6F), whereas puncta of SERPINA3N immunoreactivity in old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains were more widely distributed throughout the tissue (Figure S6G).

Next, to examine whether the effects of genotype on gene expression differ across ages (i.e., the genotype  $\times$  age interaction), we integrated, re-clustered, and re-analyzed the 1- to 2-, 11- to 12-, and 16- to 18-month-old scRNA-seq datasets (Figures S7A and S7B). OPCs were by far the cell type with the most transcripts showing a significant genotype  $\times$  age interaction, reflecting their dysregulation only in 16- to 18-month-old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains (example genes include *Sox11*, *Gap43*, and *Tmem176a*; Figure S7C; Table S2). Several mature oligodendrocyte transcripts also exhibited a significant genotype  $\times$  age interaction, such as *Fxyd1* and *Cxcl14*, which are elevated with aging only in *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> oligodendrocytes (Figures S7D–S7F). Few significant genotype  $\times$  age interactions were found for transcripts in endothelia, mural cells, or astrocytes (for full results, see Table S2). Notably, re-analysis of all astrocytes after dataset integration shows that astrocyte subclusters 1 and 2, which transcriptomically resemble subcortical and gray matter astrocytes, respectively (e.g., *Agt* and *Nnat* enriched for subcortical, and *Igf2bp2*, *Nupr1*, and *Il33* enriched for gray

matter<sup>36</sup>), had more significantly altered transcripts than sub-cluster 3, which is enriched with white matter-associated transcripts (e.g., *Gfap*, *Tnc*, *Vim*, and *Thbs4* enriched<sup>36</sup>) (Figure S8; Table S3).

Taken together, these data reveal progressive dysregulation of macroglial cells with aging in the absence of microglia.

### Atrophy, neuron loss, MRI hypointensities, and white matter deterioration in the microglia-deficient brain with aging

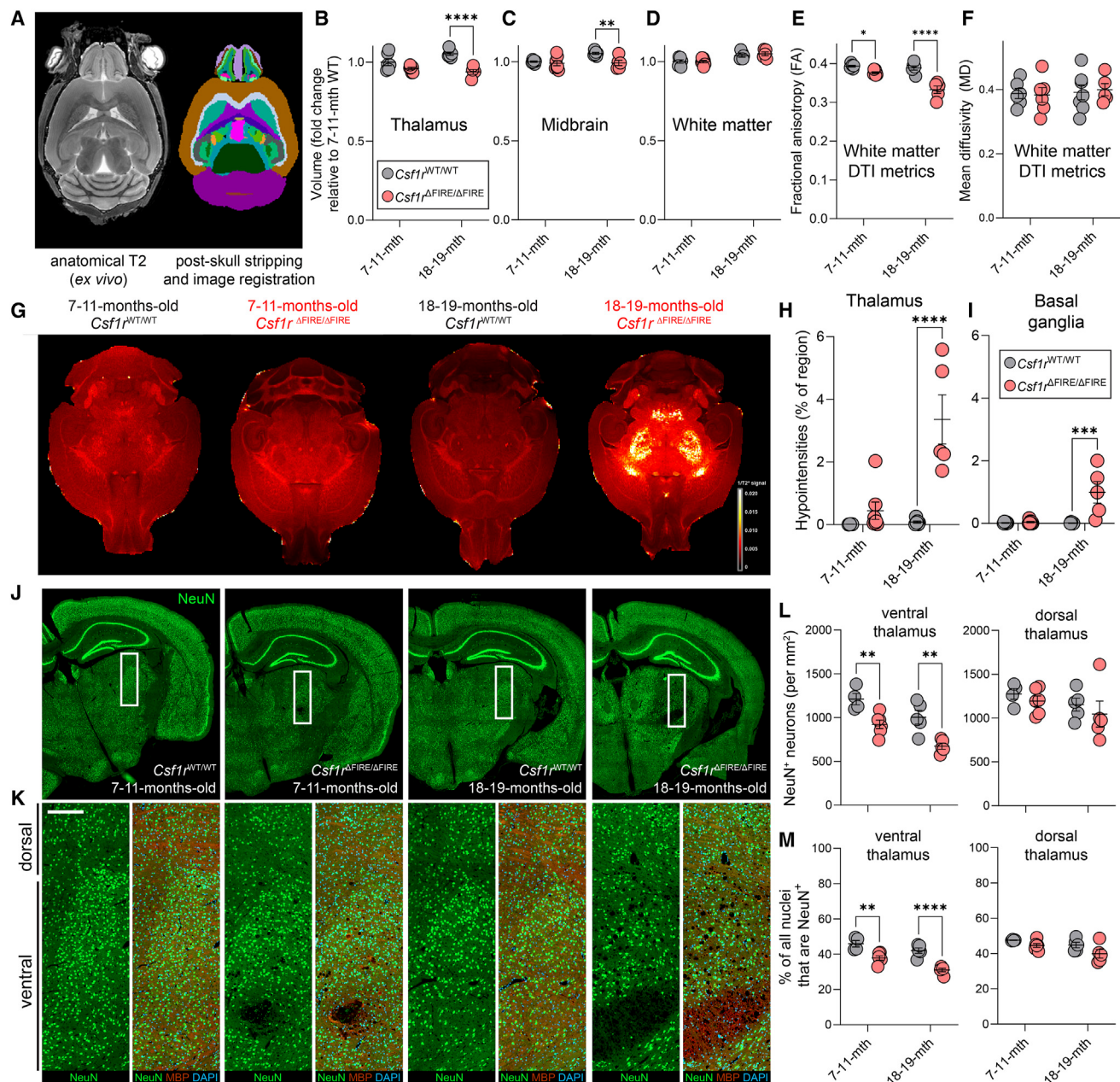
Based on our findings of localized cellular disruption with aging (particularly in the white matter and thalamus), we next sought to determine whether we could detect any corresponding regional abnormalities in the aging brain in the absence of microglia. To do so, we examined groups of adult (7–11 months old) and old (18–19 months old) *Csf1r*<sup>WT/WT</sup> and *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> mouse brains using various MRI scanning protocols. Brains were scanned *ex vivo* within the cranial vault (to preserve morphology) using a 9.4T MRI scanner after immersion in a gadolinium-based contrast agent (Figure S9A).

We first performed region-wise volumetric quantifications by analyzing structural T2 scans using an image analysis pipeline that involved skull-stripping and image registration with a brain atlas (Figure 4A; Video S1). The average volume of *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains was marginally increased at 7–11 months of age, but we found no differences in ventricular volume for either age group (relative to total brain volume; Figures S9B and S9C), despite ventriculomegaly being commonly reported in the literature in rodents and humans with *Csf1r*/*CSF1R* mutations that lead to a more global macrophage loss.<sup>18,19,37</sup> The relative volumes of the olfactory bulbs and hypothalami were decreased only in 7- to 11-month-old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains, albeit by a small amount (Figure S9C). The relative volumes of the thalamus, basal ganglia, and midbrain were subtly but significantly decreased only in 18- to 19-month-old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains (Figures 4B, 4C, and S9C).

White matter volume did not differ based on genotype (Figure 4D), but since we observed severe glial cell reactivity in white matter tracts (Figure 3), we next wanted to evaluate its structural integrity via diffusion-tensor magnetic resonance imaging (DTI). We reapplied the image registration used for the volumetric analysis to assess fractional anisotropy, mean diffusivity, radial diffusivity, and axial diffusivity across the volume of the white matter (by calculating a weighted mean score combining values from the corpus callosum, internal capsule, fimbria, and cerebellar white matter). These quantitative diffusivity metrics probe the relative diffusivity of water molecules along the different directions of fiber tracts and are commonly used to make inferences about white matter integrity. From this, we identified subtly but significantly reduced fractional anisotropy values (a measure of the directionality of diffusion anisotropy) for 7- to 11-month-old *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> white matter compared with *Csf1r*<sup>WT/WT</sup> (values lower by 4.3% on average) and more strongly reduced fractional

(D) t-SNE plots showing *Serpina3n* mRNA levels in the different cell types captured at 11–12 and 16–18 months-old ( $n = 3$  *Csf1r*<sup>WT/WT</sup> and 3 *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> mice per age group).

(E) Immunohistochemistry shows increased SERPINA3N immunoreactivity in the white matter and thalamus of aged *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> brains (images representative of 5–6 mice per group [2–3 females and 2–3 males per group]; colors inverted for illustrative purposes). Scale bars, 1 mm.



**Figure 4. Regional atrophy, neuron loss, MRI hypointensities, and white-matter deterioration in the aging brain without microglia**

(A) Example of a raw ex vivo MRI scan slice and a skull-stripped registered image used for analysis.

(B–D) Region-wise volumetric analysis of thalamus, midbrain, and white matter based on anatomical T2 scans ( $n = 5–7$  mice per group [3 females and 2–4 males per group]; groups compared by two-way ANOVA with Tukey's multiple comparison post hoc testing;  $^{*}p < 0.01$ ,  $^{****}p < 0.0001$ ).

(E and F) Diffusion-tensor magnetic resonance imaging (DTI) values for fractional anisotropy and mean diffusivity ( $n = 5–7$  mice per group; groups compared by two-way ANOVA with Tukey's multiple comparison post hoc testing;  $^{*}p < 0.05$ ,  $^{****}p < 0.0001$ ).

(G–I) Susceptibility-weighted imaging (SWI) MRIs show regional hypointensities in *Csf1<sup>ΔFIRE/ΔFIRE</sup>* mice (hypointensities pseudo-colored based on intensity;  $n = 5–7$  mice per group [3 females and 2–4 males per group]; groups compared by two-way ANOVA with Tukey's multiple comparison post hoc testing;  $^{***}p < 0.001$ ,  $^{****}p < 0.0001$ ).

(J and K) Immunohistochemistry for neuronal nuclear antigen (NeuN) and myelin basic protein (MBP) with DAPI labeling of *Csf1<sup>WT/WT</sup>* and *Csf1<sup>ΔFIRE/ΔFIRE</sup>* brain sections (region shown in K corresponds to the area within the rectangular box in J).

(L and M) Quantifications of neuron numbers (per mm<sup>2</sup>) and the percentage of cells that are neurons show fewer neurons in the *Csf1<sup>ΔFIRE/ΔFIRE</sup>* ventral thalamus at 7–11 and 18–19 months ( $n = 4–6$  mice per group [2–3 females and 2–3 males per group]; groups compared by two-way ANOVA with Tukey's multiple comparison post hoc testing  $^{**}p < 0.01$ ,  $^{****}p < 0.0001$ ). Mean  $\pm$  SEM shown for plotted data. Scale bars, 200  $\mu$ m.

anisotropy values for 18- to 19-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> white matter (values lower by 14.4% on average) (Figure 4E), suggesting worsening white matter condition with aging (two-way ANOVA interaction  $p = 0.001$ ; full two-way ANOVA tables can be found in Table S4). Other DTI metric values (i.e., axial, radial, and mean diffusivity) did not significantly differ between genotypes (Figures 4F, S9D, and S9E).

Next, our findings of mild regional atrophy led us to examine brains for further abnormalities using 3D high-spatial-resolution susceptibility-weighted imaging (SWI) MRI. SWI sequences are particularly sensitive to compounds that distort the magnetic field (appearing as dark, hypointense signals in scans), such as blood products, iron, and calcium, which can be difficult to identify using other MRI sequences.<sup>38</sup> Using SWI, we identified several brain regions with significantly more hypointensities in the 18- to 19-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> group (namely, the thalamus, basal ganglia, and pons; Figures 4G–4I and S9F; Video S2). The tissue volume covered by hypointensities in other brain regions, such as the white matter and hippocampus, did not differ based on genotype (Figures S9G and S9H).

Based on the atrophy, MRI hypointensities, and glial cell reactivity observed in the aging *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus, we next examined whether neuronal phenotypes were also altered in this region. The fixed brains used for MRIs were dissected from their cranial vaults, and coronal sections were cut to the appropriate depth for processing. Anti-NeuN immunohistochemistry demonstrated that the number of neurons per area, as well as the proportion of cells that were neuronal, were reduced in the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus (Figures 4J, S9I, and S9J). Notably, the ventral portion of the aging *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus had a particularly low number (per area) and proportion of neurons (Figures 4K–4M). The number of NeuN<sup>+</sup> neurons per area and the proportion of neurons did not significantly differ in other brain regions examined, except in the cortex of 7- to 11-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice (compared with age-matched *Csf1r*<sup>WT/WT</sup> controls; Figures S9I and S9J).

Together, these data show highly selective regional atrophy, MRI hypointensities, neuron loss, and white matter deterioration with aging in the absence of microglia. These anatomical and cellular defects spatiotemporally correspond to the brain regions with the most striking macroglial reactivity (i.e., the white matter and thalamus; Figure 3).

### Thalamic calcification is massively accelerated and intensified with aging in the absence of microglia

To identify the source(s) of the hypointensities observed in Figure 4G, we stained coronal sections with various histostains at the depth of the thalamus, where hypointensities appeared most severe. We show that thalamic regions in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> groups show marked calcium deposition, as evidenced by staining for alizarin red and von Kossa (Figures 5A, 5B, and S10A). We did not see positive staining for iron (Prussian blue) or amyloid fibrils (Congo red) on sections examined containing calcified thalamic regions (Figures S10B and S10C). Thalamic calcifications often centered on ventral posteromedial and ventral posterolateral nuclei, while dorsal lateral regions of the thalamus were relatively preserved (Figure S11). Calcifications were already present in the 7- to 11-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> group but progressively worsened

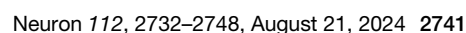
with aging (two-way ANOVA interaction  $p < 0.0001$ ; Figures 5A and 5B). Thalamic calcifications also immunostained for the bone-matrix protein, osteopontin (SPP1), and we found that GFAP<sup>high</sup> astrocytes encircled these SPP1<sup>+</sup> structures in 18- to 19-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figure 5C).

The thalamus is particularly prone to ectopic calcifications in the mouse brain, as shown in normal aging<sup>39–42</sup> and in several genetically modified mouse lines.<sup>43–46</sup> A suggested mechanism by which brain calcifications may form is due to phenotypic changes of resident brain cells (such as astrocytes, endothelial cells, and pericytes) toward an osteoblast-like/pro-mineralization state.<sup>47</sup> To assess whether any resident brain cells change phenotype in a manner that may promote calcification deposition in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, we explored thalamic cell profiles using scRNA-seq. Cell clusters from this dataset are labeled with a bold superscript “TH” for thalamus (e.g., <sup>TH</sup>Astro1) (Figure 5D).

Microglia were completely absent from 17- to 19-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalami, whereas some cellular neighborhoods of OPCs and mature oligodendrocytes were over-represented compared with control mice (Figure 5E). Transcriptomic profiles of captured thalamic *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> endothelia and mural cells were largely unchanged (Figure 5F). By contrast, thalamic *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> astrocytes, OPCs, and mature oligodendrocytes were enriched for previously described CNS injury/disease-associated markers (such as *Gfap*, *Stat3*, and *Egr1* for astrocytes; *Slc14a1* and *Mylk* for OPCs; and *Serpina3n*, *C4b*, and *Tnfrsf1a* for mature oligodendrocytes)<sup>29,30,32,48,49</sup> (Figure 5F). Within the thalamus, immunohistochemical staining demonstrated that GFAP<sup>high</sup> astrocytes and SERPINA3N puncta were particularly abundant in calcified regions (Figures 5C and S12A).

We did not observe differential expression of mRNAs previously linked to calcification formation/osteogenic states (such as *Bglap*, *Ibsp*, *Sp7*, *Alpl*, *Spp1*, and *Runx2*) in any cell type. Rather, IPA predicted pathway activation related to osteoclasts (bone-clearing cells) in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> OPCs and mature oligodendrocytes (Figures S12B and S12C). Interferon signaling was also predicted to be activated in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> oligodendrocytes, whereas cell cycle pathways were predicted to be activated in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> OPCs (Figures S12D and S12E). Indeed, there were consistently more marker of proliferation Kiel 67 (*mki67*)<sup>+</sup> OPCs in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalami compared with in *Csf1r*<sup>WT/WT</sup> thalami (Figure S12F). Few pathways were predicted to be activated or inactivated in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> astrocytes, perhaps owing to the small number of DEGs identified for this cell type.

As the thalamus is prone to calcification formation with aging,<sup>39,42</sup> we reasoned that we may also be able to observe these structures forming in the thalamus of aging wild-type mice. Using immunohistochemistry, we detected a few sporadic SPP1<sup>+</sup> structures, which were enwrapped by GFAP<sup>+</sup> astrocytes, in the ventral thalamus of 18- to 19-month-old *Csf1r*<sup>WT/WT</sup> mice (Figure S13A). Thalamic microglia were also intimately associated with these SPP1<sup>+</sup> structures in *Csf1r*<sup>WT/WT</sup> brains (Figures 5G and 5H). We next mined our thalamic scRNA-seq dataset to identify cellular sources of *Spp1* in *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. Most *Spp1*-expressing cells in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalami were in clusters of non-microglial myeloid cells and in a small cluster of perivascular fibroblasts<sup>50</sup>



(Figure S13B). The same cellular sources of *Spp1* were present in the *Csf1r*<sup>WT/WT</sup> thalamus, with the addition of microglia as an *Spp1* source in these brains (Figure S13B).

These data show that ectopic calcification with aging is dramatically accelerated in the absence of microglia. Non-microglial thalamic *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> glial cells display reactive phenotypes, but they appear not to shift toward an osteoblast-like/pro-mineralizing state.

### Vascular alterations in severely calcified regions of the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus

The thalamus is a densely vascularized brain region that is vulnerable to vascular damage with aging, and vascular dysfunction is a suggested route by which thalamic calcifications may form.<sup>47,51,52</sup> We therefore analyzed the thalamic vasculature in 6- to 11-month-old *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice. At this age, *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains show incipient thalamic calcification, which has not yet expanded extensively (Figure 5A). To assess the microanatomical localization of calcifications within *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalami, we co-immunostained free-floating sections for SPP1 and an astrocyte end-feet marker, β-dystroglycan. We confirmed that SPP1<sup>+</sup> demarcates the boundaries of calcified structures using two fluorescently labeled bisphosphonates, zoledronate and risedronate, which bind hydroxyapatite crystals<sup>53</sup> (Figures 6A and 6B). Volume rendering of confocal z-stacks demonstrated that some SPP1<sup>+</sup> structures were in contact with the vasculature (45% ± 3.8% SEM), while others were parenchymal (Figures 6C and 6D). Vessel-associated SPP1<sup>+</sup> structures varied greatly in size and were occasionally almost entirely intravascular (Figures S14A and S14B). We also show that thalamic calcifications label positively for amyloid precursor protein (APP) and/or osteocalcin, proteins detected in calcifications in other neurodegenerative diseases<sup>47</sup> (Figure S14C). These data indicate that thalamic calcifications are heterogeneous in terms of both their microenvironments and features in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

As many calcifications associate with the vasculature in thalami of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, we next assessed whether blood flow and/or vascular status are altered in this brain region of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice. To quantify thalamic blood flow *in vivo* in 8- to 11-month-old mice, we performed arterial spin labeling (ASL) MRIs after identifying the correct scanning depth using *in vivo* coronal T2-scans to localize calcifications (Figure 6E). Notably, the thalamus was the most highly perfused brain region examined in both *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, but we did not identify genotype-based blood flow differences in any region (Figure 6F).

Next, to examine the status of the thalamic vasculature, we co-immunolabeled free-floating sections with anti-CD31 (which labels endothelia), anti-ERG (which labels endothelial nuclei), and anti-CD13 (which labels pericytes), followed by spinning disk confocal imaging (Figure 6G). We identified no differences in vascular density in the dorsal or ventral thalamus of 8- to 11-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice compared with *Csf1r*<sup>WT/WT</sup> mice (Figure 6H). However, pericyte coverage of the vasculature was subtly decreased throughout the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus across a range of vessel diameters (Figures 6I and 6J). We also observed a small corresponding increase in average blood vessel diameter in the ventral thalamus (Figure 6K).

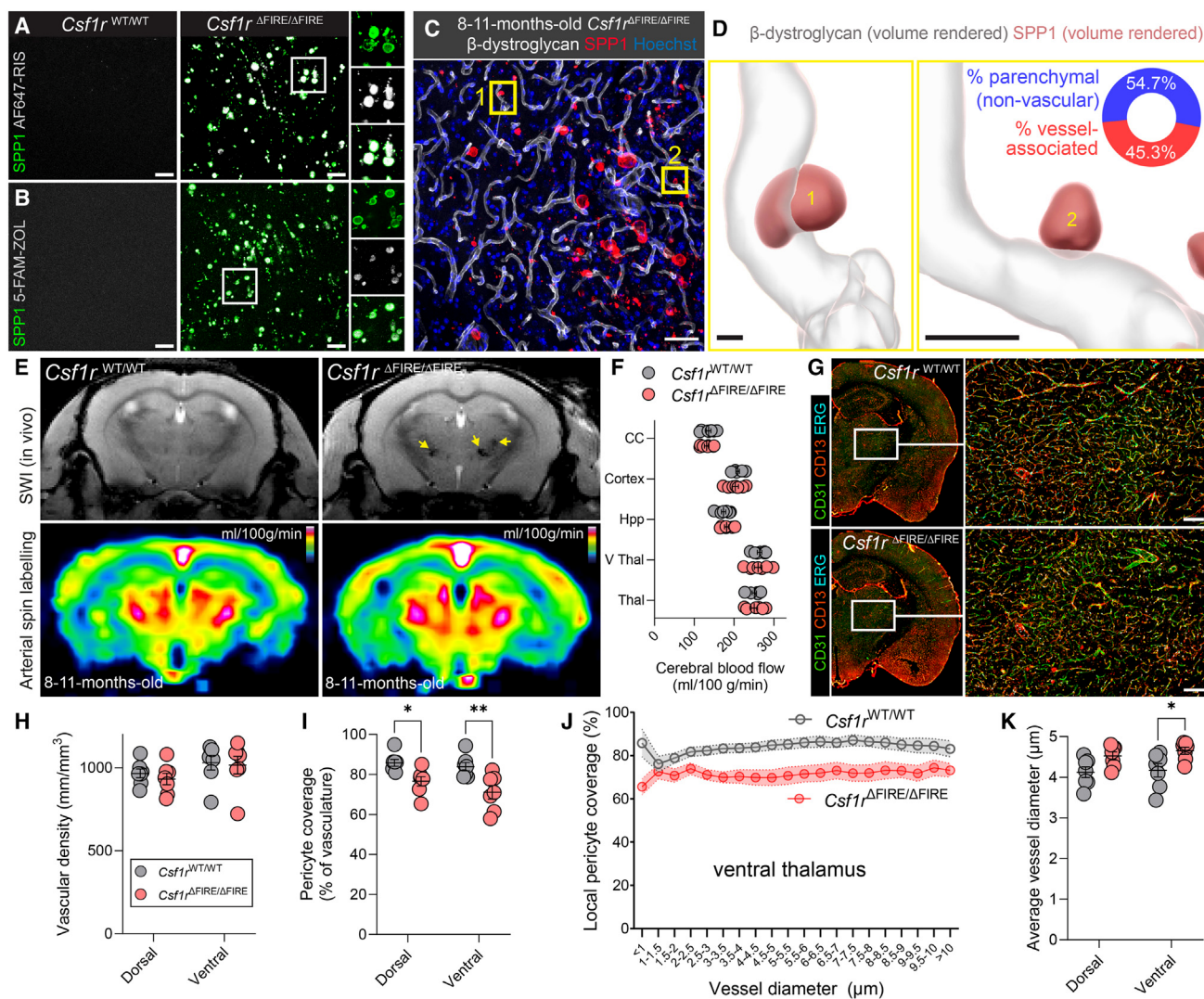
A recent study in a mouse model of dominantly inherited primary familial brain calcification (PFBC) suggested that astrocytes in the vicinity of vascular calcifications exhibit increased reactivity and reduced end-feet coverage of the cerebral vasculature.<sup>54</sup> Consistent with this report, we observed increased GFAP levels (Figures 3B and 5C) and a mis-localization of the astrocytic end-feet markers, aquaporin 4 and β-dystroglycan, from some blood vessels in heavily calcified regions of the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> ventral thalamus (Figures S14D and S14E). Reduced end-feet coverage and reduced pericyte coverage of vessels often coincides with blood-brain barrier (BBB) breaches, but we did not find evidence for increased fibrinogen deposition, a marker indicating a leaky BBB, in the thalamus of *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice or elsewhere in the brain (Figure S14F). Nor did we identify differences in endothelial, mural cell, or astrocytic expression of transcripts linked to BBB maintenance (Figures S14G–S14I).

### Populating *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains with wild-type microglia prevents pathology development

To verify that microglia provide resilience against brain calcification, we populated the empty parenchymal niche of postnatal day (P)1 *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains with donor wild-type microglia, using a previously validated approach.<sup>55</sup> A total of 50,000 P2 wild-type C57BL/6 primary mouse microglia were injected into the brain of each pup. Groups of microglia-injected and PBS-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice were then aged to 9 months of age, a time point at which thalamic calcifications have started forming in the absence of microglia. Microglial transplantations resulted in brain-wide engraftment and the population of the empty microglial niche in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figures 7A and 7B). Through these microglial injections, we prevented thalamic calcification formation in 9-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, as shown via staining with both alizarin red (Figures 7C and 7D) and risedronate-647 (Figure S15). We also show reduced astrocyte reactivity and increased neuronal numbers in the thalami of microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains compared with PBS-injected controls (Figures 7E and 7F).

Besides the thalamic pathologies, oligodendrocytes with “disease-associated” profiles<sup>29,30</sup> reside within white matter tracts of microglia-deficient *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, and axonal spheroids are also present.<sup>1,14</sup> We next assessed whether microglia-transplanted *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains are also protected from these phenotypes. For DOLs, we immunolabeled sections for anti-SERPINA3N, showing its levels are strongly reduced in the fimbriae of microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains compared with age-matched PBS-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, whereas the numbers of OLIG2<sup>+</sup> oligodendrocytes do not differ (Figures 7G and 7H). To examine axonal spheroids, characteristic features of adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) cases,<sup>56</sup> we stained sections for anti-SMI312, which labels phosphorylated axonal epitopes of neurofilaments.<sup>57</sup> As expected, many SMI312<sup>+</sup> axonal spheroids were present in control *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, whereas very few were present in age-matched microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains (Figure 7I).

These data show that populating the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain with donor wild-type microglia from early in postnatal life protects against the subsequent development of various age-associated brain pathologies.



**Figure 6. Mild vascular disruption in heavily calcified thalamic regions**

(A and B) SPP1<sup>+</sup> demarcates the boundaries of calcified structures, as shown by co-labeling with two fluorescently modified bisphosphonates, zoledronate (5-FAM-ZOL) and risedronate (AF647-RIS).

(C and D) Immunohistochemistry for SPP1 and β-dystroglycan shows that capillary calcifications are common in the thalami of 8- to 11-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice (45% ± 3.8% SEM of thalamic SPP1<sup>+</sup> structures are in contact with blood vessels; *n* = 7 male *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice). Images in (D) volume rendered from the yellow boxed regions in (B).

(E and F) Arterial spin labeling (ASL) MRIs indicate no differences in cerebral blood flow *in vivo* in 8- to 11-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains compared with age-matched *Csf1r*<sup>WT/WT</sup> brains (*n* = 7 mice per group [all males]; groups compared by two-way ANOVA). ASL images in (E) were smoothed for illustrative purposes using median filtering in ImageJ. CC, corpus callosum; Hpp, hippocampus; V Thal, ventral thalamus. SWI scans in (E) confirm calcifications had already started forming in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalami at the ASL scanning depth.

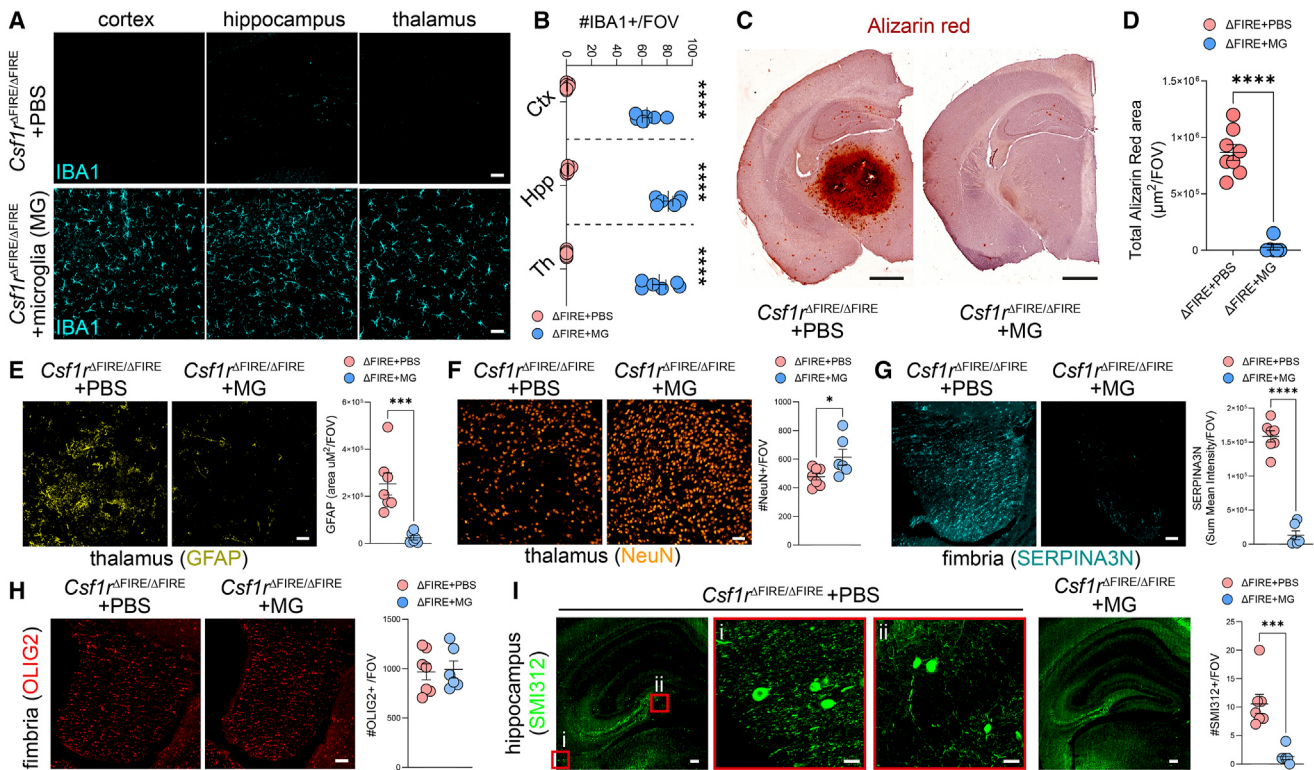
(G) Immunohistochemistry for CD31, CD13, and ETS-related gene (ERG; marking endothelial nuclei) in thick free-floating brain sections from 8- to 11-month-old *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice (images representative of *n* = 7 mice per group [all males]).

(H–K) Quantification of (H) vascular density, (I and J) pericyte coverage, and (K) vessel diameters in the dorsal and ventral thalamus (through analysis of stitched spinning disk confocal microscopy images shown in G; *n* = 7 mice per group [all males]; groups compared by two-way ANOVA with Šidák's multiple comparison post hoc testing; \**p* < 0.05, \*\**p* < 0.01). Mean ± SEM shown for plotted data. Scale bars: 50 μm for (C); 5 μm for (D) (left image) and 10 μm for (D) (right image); 100 μm for (G).

## DISCUSSION

Many non-immune functions have been assigned to microglia in development and adulthood.<sup>2</sup> Here, we found no compelling evidence that microglia are required for the maturation of other brain cell types. Rather, using diverse tools,

our data suggest that microglia provide resilience against detrimental brain changes with aging. Our findings thus highlight important new functions of microglia with important implications for the pathogenesis of neurodegenerative diseases and the development of CSF1R-related leukoencephalopathies.



**Figure 7. Populating *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains with wild-type microglia protects against the development of diverse pathologies**

(A and B) Transplantation of wild-type donor microglia at P1 results in brain-wide microglial engraftment, as shown in 9 months of age *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. CTX, cortex; Hpp, hippocampus; Th, thalamus; MG, microglia.

(C and D) Microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains are protected against calcification development at 9 months of age compared with PBS-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

(E) Reduced thalamic GFAP immunoreactivity in microglia-transplanted *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

(F) Increased thalamic NeuN<sup>+</sup> neurons per field of view (FOV) in microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

(G) Reduced SERPINA3N levels in the fimbria of microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

(H) Numbers of OLIG2<sup>+</sup> oligodendrocytes do not differ per FOV in microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains.

(I) Numbers of SMI312<sup>+</sup> axonal spheroids per FOV are reduced in microglia-injected *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. *n* = 6–8 mice per group (3–4 males and females per group); groups compared by unpaired t tests; \*\*\*\**p* < 0.0001; \*\*\**p* < 0.001; \**p* < 0.05. Mean ± SEM shown for plotted data. Scale bars: 50 μm for (A) and (E)–(H); 1 mm for (C); 100 μm for (I) (20 μm for images in Ii and Iii).

The most striking abnormality observed with aging in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains was the dramatic acceleration and intensification of thalamic calcification, which was prevented by reconstituting *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice with wild-type microglia. Around 30% of humans over 60 years of age have brain calcifications detectable by computerized tomography scanning, while calcification rates in those under 40 years of age are near zero.<sup>58</sup> Individuals with mutations causing either reduced microglial numbers or dysfunctional microglia (due to *CSF1R* and negative regulator of reactive oxygen species [*NRROS*] mutations, respectively) develop brain calcifications at young ages,<sup>18,19,59</sup> illustrating the relevance of our findings to microglial functions in humans. Additionally, recent findings demonstrate that microglia control vascular calcification in mouse models of PFBC,<sup>46</sup> a neurodegenerative disease characterized by small vessel calcification. These data suggest that microglia may hold promise as therapeutic targets in a wider range of neurodegenerative diseases.

The mechanisms underlying calcium phosphate deposition in the microglia-deficient thalamus and in the thalamus in normal

aging require further investigation but could be due to several factors.<sup>47</sup> The thalamus may be a region of high calcium phosphate deposition due to normal cellular processes,<sup>60,61</sup> and the clearance of these deposits may be hindered in the absence of microglia. Alternatively, calcification formation can be initiated when non-osteogenic cells aberrantly adopt osteogenic traits.<sup>62</sup> We did not find evidence supporting this mechanism in our scRNA-seq dataset of the microglia-deficient thalamus. The expression of genes encoding calcification-associated proteins, such as osteopontin (*Spp1*) and osteocalcin (*Bglap*), was unaltered in the *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamus despite their considerable accumulation around calcifications. These findings raise questions regarding the source(s) of osteopontin, osteocalcin, and other bone-matrix proteins, as well as their precise roles in brain calcification formation in the absence/presence of microglia.

A limitation of this study is that relatively few neurons were captured in our scRNA-seq datasets. Evidence from recent reports indicates that neurons are altered in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains. For example, a prior study presented single-nuclei RNA

sequencing data from 5- to 6-month-old *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brains, revealing transcriptional changes in various neuronal subtypes.<sup>55</sup> Moreover, we recently reported deficits in excitatory neurotransmission in *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> hippocampi, whereas we found no differences in synapse numbers, spine density, or neuronal morphology.<sup>15</sup> For the non-neuronal cell types we examined, further studies are necessary to establish links between the described RNA changes and alterations in their cellular functions. A further consideration is that we examined *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice on a mixed C57BL/6JCrI and CBA/Ca genetic background, and it is possible that phenotype severity and age of onset due to microglial deficiencies may vary depending on the background of the mice.<sup>63</sup> Despite these points, we believe our profiling of various brain cell types and regions across the lifespan in the microglia-deficient brain will be a valuable resource for the field.

Taken together, our observations reveal a previously unappreciated requirement for microglia in safeguarding the brain parenchyma with aging. These findings also lend conceptual support to strategies aimed at replacing dysfunctional endogenous microglia, a concept further explored in the accompanying study by Chadarevian and colleagues.

## STAR★METHODS

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.neuron.2024.05.018>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, D.A.D.M. and J.P.; methodology, D.A.D.M., N.B.-C., A. Chagnot, A. Corsinotti, A.W., R.J.L., and M.A.J.; software, N.B.-C. and A. Chagnot; validation, D.A.D.M., N.B.-C., and A. Corsinotti; formal analysis, D.A.D.M., N.B.-C., C.M., A. Chagnot, S.K.S., and J.P.C.; investigation, D.A.D.M., C.M., S.K.S., J.P.C., S.S., K.M., U.M., M.M., and B.B.; resources, J.P., C.P., M.B.-J., and A.M.; data curation, D.A.D.M., N.B.-C., R.J.L., and M.A.J.; writing – original draft, D.A.D.M.; writing – review & editing, D.A.D.M., N.B.-C., J.P.C., N.M., A.K., M.B.-J., and J.P.; visualization, D.A.D.M.; supervision, N.M., A.K., B.W.M., M.B.-J., A.M., A.W., and J.P.; funding acquisition, J.P.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE                                  | SOURCE                                  | IDENTIFIER                        |
|------------------------------------------------------|-----------------------------------------|-----------------------------------|
| <b>Antibodies</b>                                    |                                         |                                   |
| Rat PE anti-CD11b                                    | BioLegend                               | Cat# 101207, RRID:AB_312790       |
| Rat PE/Cyanine7 anti-CD45                            | BioLegend                               | Cat# 103114, RRID:AB_312979       |
| Rat APC anti-CD115                                   | BioLegend                               | Cat# 135510, RRID:AB_2085221      |
| Rat anti-GFAP                                        | Thermo Fisher                           | Cat# 13-0300, RRID:AB_2532994     |
| Goat anti-SERPINA3N                                  | R and D systems                         | Cat# AF4709, RRID:AB_2270116      |
| Rabbit anti-NeuN                                     | Abcam                                   | Cat# ab177487, RRID:AB_2532109    |
| Goat anti-osteopontin                                | R and D systems                         | Cat# AF808, RRID:AB_2194992       |
| Rabbit anti-IBA1                                     | FUJIFILM Wako Pure Chemical Corporation | Cat# 019-19741, RRID:AB_839504    |
| Rabbit anti-OLIG2                                    | Millipore                               | Cat# AB9610, RRID:AB_570666       |
| Rabbit anti-SOX10                                    | Abcam                                   | Cat# ab155279, RRID:AB_2650603    |
| Rabbit anti-Fibrinogen                               | Agilent                                 | Cat# A008002, RRID:AB_578481      |
| Rat anti-CD31                                        | BD Biosciences                          | Cat# 553370, RRID:AB_394816       |
| Goat anti-CD13                                       | R and D systems                         | Cat# AF2335, RRID:AB_2227288      |
| Mouse anti- $\beta$ -dystroglycan                    | Santa Cruz Biotechnology                | Cat# sc-33702, RRID:AB_627292     |
| Rabbit anti-ERG                                      | Abcam                                   | Cat# ab92513, RRID:AB_2630401     |
| Goat anti-IBA1                                       | Abcam                                   | Cat# ab5076, RRID:AB_2224402      |
| Rabbit anti-aquaporin 4                              | Novus                                   | Cat# NBP1-87679, RRID:AB_11006038 |
| Rabbit anti-APP                                      | Thermo Fisher Scientific                | Cat# PA5-19923, RRID:AB_11152072  |
| Goat anti-osteocalcin                                | Thermo Scientific Alfa Aesar            | Cat# J65216                       |
| Goat anti-osteopontin                                | R and D systems                         | Cat# AF808, RRID:AB_2194992       |
| Rat anti-CD31                                        | Dianova                                 | Cat# DIA-310, RRID:AB_2631039     |
| Goat anti-CD206                                      | R and D systems                         | Cat# AF2535; RRID:AB_2063012      |
| Rabbit anti-IBA1                                     | Abcam                                   | Cat# ab178846; RRID:AB_2636859    |
| Mouse anti-Neurofilament (SMI312)                    | Biolegend                               | Cat# 837904; RRID:AB_2566782      |
| Rabbit anti-NeuN                                     | Abcam                                   | Cat# abn78; RRID:AB_10807945      |
| Chicken anti-GFAP                                    | Abcam                                   | Cat# ab4674; RRID:AB_304558       |
| Goat anti-OLIG2                                      | R and D systems                         | Cat# AF2418; RRID:AB_2157554      |
| <b>Biological samples</b>                            |                                         |                                   |
| C57BL/6 primary mouse microglia                      | ScienCell                               | Cat# M1900-57                     |
| <b>Chemicals, peptides, and recombinant proteins</b> |                                         |                                   |
| Actinomycin D                                        | Sigma                                   | Cat# A1410                        |
| Trehalose dihydrate                                  | Sigma                                   | Cat# T0167                        |
| MultiHance® (gadobenate dimeglumine)                 | Bracco                                  | Cat# 0270-5164                    |
| AF647-RIS                                            | Biovinc                                 | Cat# BV500101                     |
| 5-FAM-Zol                                            | Biovinc                                 | Cat# BV111001                     |
| Alizarin Red S                                       | Millipore-Sigma                         | Cat# TMS-008                      |
| Perl's Prussian Blue                                 | TCS Biosciences                         | Cat# HS652                        |
| Microglial media                                     | ScienCell                               | Cat# 1901                         |
| DPX mounting media                                   | CellPath                                | Cat# SEA-1304-00A                 |
| ProLong™ Gold Antifade Mountant                      | Invitrogen                              | Cat# P36930                       |
| <b>Critical commercial assays</b>                    |                                         |                                   |
| Adult brain dissociation kit                         | Miltenyi Biotec                         | Cat# 130-107-677                  |

(Continued on next page)

**Continued**

| REAGENT or RESOURCE                                                                                                                 | SOURCE                                     | IDENTIFIER                                                                                                                                                                                               |
|-------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Chromium Single Cell 3' Reagent Kit v.3.1                                                                                           | 10x Genomics                               | Cat# PN-1000121                                                                                                                                                                                          |
| Chromium Next GEM Chip G kit                                                                                                        | 10x Genomics                               | Cat# PN-1000120                                                                                                                                                                                          |
| <b>Deposited data</b>                                                                                                               |                                            |                                                                                                                                                                                                          |
| Raw and analyzed data                                                                                                               | This paper                                 | GEO: GSE267545                                                                                                                                                                                           |
| Analyzed scRNA-seq data<br>(from 6- to 7-week-old <i>Csf1r</i> <sup>WT/WT</sup><br>and <i>Csf1r</i> <sup>ΔFIRE/ΔFIRE</sup> samples) | McNamara et al. <sup>14</sup>              | GEO: GSE215440                                                                                                                                                                                           |
| <b>Experimental models: Organisms/strains</b>                                                                                       |                                            |                                                                                                                                                                                                          |
| <i>Csf1r</i> <sup>ΔFIRE/ΔFIRE</sup> mice                                                                                            | Rojo et al. <sup>13</sup>                  | N/A                                                                                                                                                                                                      |
| <b>Software and algorithms</b>                                                                                                      |                                            |                                                                                                                                                                                                          |
| Imaris, version 9.9                                                                                                                 | Oxford Instruments                         | <a href="https://imaris.oxinst.com/">https://imaris.oxinst.com/</a>                                                                                                                                      |
| Fiji (Image J), version 2.0.0 – 2.3.0                                                                                               | NIH                                        | <a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>                                                                                                                                      |
| QuPath, version 0.4.3                                                                                                               | Bankhead et al. <sup>64</sup>              | <a href="https://qupath.github.io/">https://qupath.github.io/</a>                                                                                                                                        |
| Ingenuity Pathway Analysis                                                                                                          | Qiagen                                     | <a href="https://digitalinsights.qiagen.com/">https://digitalinsights.qiagen.com/</a>                                                                                                                    |
| Prism, version 10                                                                                                                   | GraphPad                                   | <a href="https://www.graphpad.com/">https://www.graphpad.com/</a>                                                                                                                                        |
| FCS express 7                                                                                                                       | De Novo software                           | <a href="https://denovosoftware.com/">https://denovosoftware.com/</a>                                                                                                                                    |
| CatWalk XT gait analysis                                                                                                            | Noldus                                     | <a href="https://www.noldus.com/catwalk-xt">https://www.noldus.com/catwalk-xt</a>                                                                                                                        |
| Any-Maze video tracking software (v.6.3)                                                                                            | Stoelting Europe                           | <a href="https://www.any-maze.com/">https://www.any-maze.com/</a>                                                                                                                                        |
| Cell Ranger (v5.0.0-v7.0.0)                                                                                                         | 10x Genomics                               | <a href="https://www.10xgenomics.com/support/software/cell-ranger/latest">https://www.10xgenomics.com/<br/>support/software/cell-ranger/latest</a>                                                       |
| MATLAB                                                                                                                              | The MathWorks, Inc                         | <a href="https://www.mathworks.com/products/matlab.html">https://www.mathworks.com/products/matlab.html</a>                                                                                              |
| Bruker's Paravision 360                                                                                                             | Bruker                                     | <a href="https://www.bruker.com/en.html">https://www.bruker.com/en.html</a>                                                                                                                              |
| R v.4.1.1-v.4.2.1-v.4.3.1                                                                                                           | CRAN                                       | <a href="https://cran.r-project.org/">https://cran.r-project.org/</a>                                                                                                                                    |
| Scater                                                                                                                              | McCarthy et al. <sup>65</sup>              | <a href="https://bioconductor.org/packages/release/bioc/html/scater.html">https://bioconductor.org/packages/<br/>release/bioc/html/scater.html</a>                                                       |
| Scran                                                                                                                               | Lun et al. <sup>66</sup>                   | <a href="https://bioconductor.org/packages/release/bioc/html/scran.html">https://bioconductor.org/packages/<br/>release/bioc/html/scran.html</a>                                                         |
| Seurat                                                                                                                              | Hao et al. <sup>67</sup>                   | <a href="https://satijalab.org/seurat/">https://satijalab.org/seurat/</a>                                                                                                                                |
| scDblFinder                                                                                                                         | Germain et al. <sup>68</sup>               | <a href="https://bioconductor.org/packages/release/bioc/html/scDblFinder.html">https://bioconductor.org/packages/<br/>release/bioc/html/scDblFinder.html</a>                                             |
| clustree                                                                                                                            | Zappia and Oshlack <sup>69</sup>           | <a href="https://github.com/lazappi/clustree">https://github.com/lazappi/clustree</a>                                                                                                                    |
| Milo                                                                                                                                | Dann et al. <sup>21</sup>                  | <a href="https://github.com/MarioniLab/miloR">https://github.com/MarioniLab/miloR</a>                                                                                                                    |
| DropletUtils                                                                                                                        | Lun et al. <sup>70</sup>                   | <a href="https://bioconductor.org/packages/release/bioc/html/DropletUtils.html">https://bioconductor.org/packages/<br/>release/bioc/html/DropletUtils.html</a>                                           |
| VasOMatic v1.0                                                                                                                      | Audrey Chagnot                             | <a href="https://doi.org/10.5281/zenodo.11198372">https://doi.org/10.5281/zenodo.11198372</a>                                                                                                            |
| Slingshot (v 2.0.0)                                                                                                                 | Street et al. <sup>71</sup>                | <a href="https://bioconductor.org/packages/release/bioc/html/slinsshot.html">https://bioconductor.org/packages/<br/>release/bioc/html/slinsshot.html</a>                                                 |
| ShinyCell                                                                                                                           | Ouyang et al. <sup>72</sup>                | <a href="https://github.com/SGDDNB/ShinyCell">https://github.com/SGDDNB/ShinyCell</a>                                                                                                                    |
| batchelor                                                                                                                           | Haghverdi et al. <sup>73</sup>             | <a href="https://bioconductor.org/packages/release/bioc/html/batchelor.html">https://bioconductor.org/packages/<br/>release/bioc/html/batchelor.html</a>                                                 |
| harmony                                                                                                                             | Korsunsky et al. <sup>74</sup>             | <a href="https://portals.broadinstitute.org/harmony/">https://portals.broadinstitute.org/harmony/</a>                                                                                                    |
| Fluoview FV31S-DT software                                                                                                          | Olympus                                    | <a href="https://www.olympus-lifescience.com/en/downloads/detail-iframe/?0[downloads][id]=847252002">https://www.olympus-lifescience.com/en/<br/>downloads/detail-iframe/?0[downloads][id]=847252002</a> |
| Custom code                                                                                                                         | This paper                                 | <a href="https://doi.org/10.5281/zenodo.11198851">https://doi.org/10.5281/zenodo.11198851</a>                                                                                                            |
| <b>Other</b>                                                                                                                        |                                            |                                                                                                                                                                                                          |
| Interactive databases for mining of sequencing data                                                                                 | This paper                                 | <a href="https://annawilliams.shinyapps.io/shinyApp_jpriller/">https://annawilliams.shinyapps.io/shinyApp_jpriller/<br/>https://annawilliams.shinyapps.io/shinyApp_jpriller/</a>                         |
| Computer cluster - Eddie                                                                                                            | Edinburgh Compute and Data Facility (ECDF) | <a href="http://www.ecdf.ed.ac.uk/">http://www.ecdf.ed.ac.uk/</a>                                                                                                                                        |

## RESOURCE AVAILABILITY

### Lead contact

Requests for further information, resources, and/or reagents should be directed to the lead contact, Josef Priller ([josef.priller@ed.ac.uk](mailto:josef.priller@ed.ac.uk)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- Single-cell RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#). The cellranger matrices, cell information, and sample metadata are available at GEO: GSE267545 and at <https://doi.org/10.5281/zenodo.11198851>. We also provide an online resource to visualise the single-cell RNA-sequencing data generated for this manuscript ([https://annawilliams.shinyapps.io/shinyApp\\_jpriller/](https://annawilliams.shinyapps.io/shinyApp_jpriller/)). Microscopy and MRI data reported in this paper will be shared by the [lead contact](#) upon request.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the [key resources table](#).
- Any additional information required to reanalyse the data reported in this paper is available from the [lead contact](#) upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Animals (mice)

Mice were bred and maintained under specific pathogen-free conditions in temperature- and humidity-controlled rooms in accordance with institutional guidelines. Mice were bred and maintained in 12 h light and dark cycles with chow and water provided ad libitum. *Csf1*<sup>ΔFIRE</sup> mice used in this study were on a mixed C57BL/6JCrI and CBA/Ca genetic background, as originally described in Rojo et al.<sup>13</sup> Group sizes, ages, and numbers of males/females used for each experiment are provided in respective figure legends. In cases where mice of both sexes were used in an experiment, we were underpowered to confidently report on any influence (or association) of sex on the findings. Mice were culled by qualified staff of a UK home office-licensed animal house and experiments were carried out under the authority of a UK Home Office Project License following guidelines set under the Animal (Scientific Procedures) Act 1986.

For the microglial transplantation experiments, all animal procedures were conducted in accordance with the guidelines set forth by the National Institutes of Health (NIH) and the University of California, Irvine Institutional Animal Care and Use Committee. Equivalent numbers of male and female mice were used, and all groups were age matched and group housed on a 12h/12h light/dark cycle with food and water ad libitum. *Csf1*<sup>ΔFIRE</sup> mice for this experiment were originally generated on a B6CBAF1/J background. Founders were then crossed with C57BL/6J mice for two generations and their offspring interbred.

## METHOD DETAILS

### Single-cell isolations and library preparation

Brain tissue harvests and dissociations were performed starting at approximately the same time of day for all mice (09:00–10:00 am). Processing of mice with different genotypes was mixed in a manner designed to reduce batch effects. Mice were culled via cervical dislocation and immediately processed without perfusion to reduce processing times. For 6–7-week-old “juvenile” samples, hippocampi from both hemispheres and the remainder of the left hemisphere were dissected (after removal of olfactory bulbs and cerebellum) and separately placed in 50 ml conical tubes containing 2 ml ice-cold HBSS (w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>, with 5% trehalose, 30 μM actinomycin D; A1410, Sigma) and finely minced using a disposable 22A scalpel to ensure rapid cooling. Hippocampi were processed separately to examine whether impacts of microglia deficiencies were particularly strong in this region compared to the rest of the brain. The same steps were carried out for all other single-cell RNA sequencing datasets: for the 11–12-months-old “adult” samples, the left hemisphere was processed (after removal of olfactory bulbs and cerebellum); for the 16–18-months-old “old” samples, the whole brain was processed (after removal of olfactory bulbs and cerebellum); for the 17–19-months-old “thalamus” samples, thalami were dissected from both hemispheres and processed. Although care was taken during hippocampal and thalamic dissections, it is possible that small numbers of cells from adjacent tissues may have unintentionally been retained.

Following tissue mincing, the dissociation protocol was performed as previously described<sup>14</sup> to generate brain single-cell suspensions. Brains were digested using the Adult Brain Dissociation Kit (130–107–677, Miltenyi Biotec) with the following modifications: (1) tissues were dissociated as described in the “manual dissociation” section of the Neural Tissue Dissociation Kit protocol (130–092–628, Miltenyi Biotec); (2) enzymatic digestions were performed at 35°C; (3) half the concentration of enzyme P was used; (4) actinomycin D was used to limit dissociation-induced transcriptional changes; (5) 5% trehalose was added in all buffers to increase cellular viability; (6) cell clusters were removed by filtration through pre-moistened 70 μm (352350, Falcon) and 40 μm (352340; Falcon) cell strainers; (7) erythrocyte and myelin debris removal steps were omitted; and (8) all centrifugations were performed at 200 g at 4°C.

After generating a single cell suspension, cells were collected in PBS with 0.2% BSA before being sorted on a Sony SH800 cell sorter or a BD FACSAria II cell sorter. Gates were chosen based on forward and side scatter to include live cells (based on trial experiments; Figure S1A), while excluding doublets, erythrocytes, and debris using FSC and SSC parameters (Figure S1B). Non-viable cells were excluded based on DRAQ7 and/or DAPI staining. After confirming the post-sorting viability of cells, using trypan blue and a haemocytometer, single cells were processed through the Chromium Single Cell Platform using a Chromium Next GEM Single Cell 3' GEM Library and Gel Bead kit (v.3.1 chemistry, PN-1000121, 10x genomics) and a Chromium Next GEM Chip G kit (PN-1000120) and processed following the manufacturer's instructions. Libraries were sequenced using a NovaSeq 6000 sequencing system (PE150 (Hi-Seq), Illumina).

### Flow cytometry

To characterise CD115 expression levels, an enzyme-free dissociation protocol was used to isolate and characterise brain myeloid cells from 10–11-week-old female mice. Mice were transcardially perfused with ice-cold PBS. Brains were then dissected and minced with a 22A scalpel in HBSS (w/o  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; 14175-053, Gibco) with 25 mM HEPES (10041703, Fisher Scientific). Samples were then homogenised with a Dounce homogeniser (D9938, Kimble) in HBSS (w/o  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) with 25 mM HEPES. Brain homogenates were separated using a 35% Percoll gradient, with centrifugation at 800 g for 20 min at 4 °C (with a slow acceleration/deceleration). Cell pellets were collected and washed in PBS (w/o  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; 14190-094, Gibco) with 0.1% low endotoxin BSA (A8806, Sigma Aldrich). Fc receptors were blocked (1:100; 101302, BioLegend) for 15 min at 4 °C on a shaker. Cells were stained with primary antibodies directed against CD11b (PE; 1:200; 101207; BioLegend, clone M1/70), CD45 (PECy7; 1:200; 103114; BioLegend, clone 30-F11) and CD115 (APC; 1:200; 135510; BioLegend, clone AFS98) for 30 min at 4 °C on a shaker. Samples were then washed and resuspended in PBS (w/o  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) with 0.1% low endotoxin BSA. DAPI was used for viability gating. Single-fluorochrome stained beads, unstained samples, and fluorescence-minus-one samples were used as controls. Data were acquired using a BD LSRFortessa flow cytometer. FCS express 7 was used for data analysis. Data from the *Csf1r*<sup>WT/WT</sup> brain myeloid cells in this experiment were previously compared to *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain myeloid cells.<sup>14</sup>

### Behavioral tests

Juvenile behavioural studies were performed on mice of both sexes, with females and males being tested on different days and analysed separately. Mice were between 4–8 weeks of age when tested. Mice were housed under standard conditions with 12-hour light/dark cycles in temperature and humidity-controlled rooms. Experimenters were blinded to genotypes and remained blinded during data analysis. Handling was carried out 3–4 days prior to testing to minimise stress during testing. Open-field tests were carried out first, followed by the elevated plus maze. Equipment was cleaned with 70% ethanol between tests. No outliers were removed (based on lack of significance using Grubb's outlier testing). For open field testing, mice were placed in a 47 x 47 cm field to freely explore for 10 min. Total ambulatory distance travelled (in metres) and time spent in the edges (9 cm from the wall) and centre (29 x 29 cm) were quantified. For the elevated plus maze, the maze had four arms: two open, and two enclosed by walls. Mice were left to explore the maze for 5 min. Mice that fell from the maze during the test run were excluded from analyses. A camera was set up directly above the open field and elevated plus maze, and Any-Maze video tracking software (Stoelting Europe, v.6.3) was used to quantify exploration in the different regions of the field/maze.

For behavioural testing of aged mice, open field testing was performed first, followed by the CatWalk XT gait analysis. The open field test was performed as described above, with the exception that old males and females were not tested or analysed separately. The CatWalk XT gait analysis was performed on a glass plate walkway equipped with light-emitting diodes, which were used to identify paw contact points on the plate. A camera positioned underneath the glass plate captured images for analysis. Animals walked along the walkway 3 times on the day before evaluation to habituate them. On the testing day, recordings were repeated until three satisfactory walks were obtained. Trial statistics for each mouse were based on averaged values from 3 satisfactory runs.

### Ex vivo MRI scanning

Mice were anaesthetised with isoflurane before being transcardially perfused with PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free) with 0.05% heparin. Mice were then immediately perfusion fixed with 4% PFA in PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free), which was pre-warmed to 37 °C. Samples were prepared by dissecting surrounding hair, skin, and muscles from heads, while leaving the brain within the cranial vault. Samples were then immersion fixed in 4% PFA in PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free) overnight, before being immersed in PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free) with 0.01% sodium azide and placed on a rocker at 4 °C for several days to leach out any remaining PFA (with the PBS solution being changed several times). Samples were then transferred to a 2.5 mM solution of the gadolinium-based contrast agent, gadobenate dimeglumine (MultiHance, Bracco Diagnostics Inc., Milan, Italy), with 0.01% sodium azide in PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free) and placed on a rocker at 4 °C for a further 24–48 hr prior to imaging.

MRI was performed using a 9.4T horizontal bore Biospec AVANCE neo preclinical imaging system equipped with a 116 mm bore gradient insert (Bruker BioSpin GmbH, Germany, maximum gradient strength 660 mT/m). For scanning, samples were immersed in Fomblin in 15 ml tubes and secured in place using plastic wedges. An 86 mm quadrature volume coil was used for transmission with signal reception by a four-channel phased-array mouse brain coil (Bruker BioSpin GmbH, Germany). Scout images were taken to confirm correct positioning, and the magnetic field was optimised using automated 3D field mapping routine.

For anatomical imaging, a 3D Gradient Echo T2\*-weighted sequence covering the entire brain and surrounding tissues was acquired. Slices were acquired using the following parameters: image/matrix size 300 x 210 x 180, field of view = 20 x 14 x 12 mm (for an isotropic resolution of 0.067 mm), repetition time = 35 ms, echo time 6.8 ms, number of signal averages 8. For high resolution T2-weighted RARE scans, the same field of view and matrix size as for T2\* and an effective echo time of 43.29 ms, repetition time of 850 ms, RARE factor of 16, and signal averaging of 10. For 3D diffusion tensor imaging (DTI) MRIs, 8 T2-weighted (A0) images and sets of diffusion-weighted ( $b = 1800$  s/mm<sup>2</sup>) echo planar imaging (EPI) volumes were acquired with diffusion gradients applied in 60 non-collinear directions, producing a total of 68 volumes. The acquisition matrix was 180 x 140 x 144, acquired in 4 segments. The repetition time and echo time for each EPI volume were 400 and 20.6 ms, respectively. The field of view was 18 x 14 x 14.4 mm (field of view saturation slabs were used to null signal derived from outside of the sample field).

### In vivo MRI scanning

Mice were initially anaesthetised with 3% isoflurane and secured in an MRI compatible cradle (Rapid Biomedical GmbH, Rimpar, Germany). Anaesthesia was maintained during scanning using 1.5%–2% isoflurane in oxygen/air (50/50, 1 L/min). The respiration rate and rectal temperature were monitored throughout, with body temperature maintained at 37°C (SA Instruments Inc., Stony Brook, NY, USA). Arterial spin labelling (ASL) MRIs were performed using the same MRI scanner as described above for ex vivo scans. Scout images were taken to confirm correct positioning and the magnetic field was optimised using an automated 3D field mapping routine. A 2-dimensional 33-slice T2-weighted (RARE) anatomical scan was performed to identify the scanning depth required for the calcified thalamic regions (0.5 mm slice thickness, image size/data matrix = 260 x 260 pixels, field of view = 18 x 18 mm, repetition time = 3600 ms, echo time = 44 ms, signal averages = 3). For ASL, a flow-sensitive alternating inversion recovery (FAIR) spin-echo EPI sequence was used. A single 1 mm thick slice at the region of interest was selected based on the T2 structural scans (image size = 96 x 96, field of view = 18 x 18 mm, echo time = 10.92 ms). Finally, we acquired a susceptibility-weighted gradient echo scan (SWI, flow compensated FLASH sequence; slices = 33, image size = 180 x 180 pixels, field of view = 18 x 18 mm, echo time = 8 ms, repetition time = 662 ms, averages = 4, flip angle = 30°).

### Immunolabeling

For paraffin-embedded sections, mice were perfused with PBS and then perfusion fixed with 4% PFA. Brains and/or peripheral organs were extracted and then immersion fixed in 4% PFA overnight at 4°C. Samples were paraffin embedded before being cut into 5 µm sections. For immunostaining, samples were first dewaxed in xylene and then rehydrated, using standard procedures. Different antigen retrieval methods (using citrate-based [H-3300-250] or tris-based [H-3301-250] antigen unmasking solution) were performed depending on antibody requirements, using a water bath (80–97.5°C for 30 min) to heat sections. Samples were blocked using 10% normal serum (derived from the species of the secondary antibodies) with 0.1% triton x-100 for 1 h. Primary antibodies were applied overnight in 5% normal serum in PBS at 4°C. Primary antibodies included anti-GFAP (2.2B10; 1:500), anti-SERPINA3N (AF4709; 1:100), anti-NeuN (ab177487; 1:400), anti-osteopontin (SPP1, AF808, 1:200), anti-IBA1 (019-19741; 1:2,000), anti-IBA1 (ab178846; 1:500), anti-OLIG2 (AB9610; 1:500), anti-SOX10 (ab155279; 1:1,000), anti-CD206 (AF2535; 1:200) and anti-fibrinogen (A008002-2; 1:500). Samples were repeatedly washed in PBS and then fluorescently conjugated secondary antibodies were applied for 1–2 hr at room temperature (RT) in the dark (1:500, Life Technologies–Molecular Probes). Samples were repeatedly washed in PBS and then counterstained with 1 µm DAPI for 3 min at RT. Sections were repeatedly washed in PBS, then allowed to dry before being mounted with anti-fade mounting medium. Finally, slides were coverslipped and left to cure and protected from light prior to imaging.

For free-floating sections, *Csf1*<sup>ΔFIRE/ΔFIRE</sup> mice and littermate controls were injected intraperitoneally with 100 µl of pentobarbital and then transcardially perfused with cold PBS with EDTA (1:1000), followed by perfusion fixation with 10% formalin. Brains were then dissected and post-fixed in 10% formalin for 24 hours. Brains were washed with PBS 3 times and cryopreserved in a 20% sucrose solution for 48 hours. After this, brains were frozen using isopentane and stored at -80°C until sectioning. Mouse brains were sectioned coronally using a cryostat. Each brain slice was 40 µm thick and stored at -20°C in an antifreeze solution (250 mM sucrose, 0.1 M PBS solution [Na-PBS, pH 7.4], 7 mM MgCl<sub>2</sub>, and made to 1 L with glycerol). For immunostaining, brain slices were transferred into well plates and washed 3 times with PBS. Slices were left for 2 hours in blocking solution (10% donkey serum diluted in 2% PBS-Triton). Blocking solution was then removed, and slices were incubated at 4°C overnight in primary antibodies diluted in blocking solution. Primary antibodies included anti-CD13 (AF2335, 1:100), anti-CD31 (553370, 1:500), anti-osteopontin (AF808, 1:200), anti-β-dystroglycan (sc-33702, 1:1000), and anti-ERG (ab92513; 1:1000). After overnight incubation, the primary antibodies were discarded, and the slices washed 3 times with 2% PBS-Triton-X. Brain slices were then incubated for 2 hours, protected from light, in fluorophore-conjugated secondary antibodies (Alexa Fluor 488 [A-21208, 1:1000], 568 [A-11057, 1:1000], and 647 [A-31571, 1:1000]) diluted in blocking solution. Slices were then washed once with 2% PBS-Triton. Nuclei were stained for 5 minutes with Hoechst (1:1000). Slices were washed 3 times with PBS and mounted onto slides using prolong Glass mounting media, stored at 4°C and protected from light until imaged.

For co-staining with bisphosphonates (and for the data shown in Figures S14C and S14D), brains were sectioned with a vibratome (Leica VT1000S) into 60 µm thick slices and stored in 24 well plates in 0.01% NaN<sub>3</sub> in PBS until further processing. Brain slices were blocked overnight in 1% BSA, 0.1% Triton™ X-100 in PBS at 4°C. Slices were then incubated for 3 days at 4°C in primary antibodies in 0.5% BSA, 0.05% Triton™ X-100 in PBS. Brain slices were washed 4–5 times with 0.5% BSA, 0.05% Triton™ X-100 in PBS at RT. Slices were incubated overnight at 4°C with secondary antibodies diluted in 0.5% BSA, 0.05% Triton™ X-100 in PBS. Brain slices were then incubated with AF647-RIS (Biovinc, stock concentration 0.02 nmol/µl in 0.9% NaCl) or 5-FAM-Zol (Biovinc, stock

concentration 0.02 nmol/ $\mu$ l in 0.9% NaCl) prepared at 1:200 dilution in 0.5% BSA, 0.05% Triton™ X-100 in 1X PBS for 2 hours at RT. Brain slices were washed 4–5 times with 0.5% BSA, 0.05% Triton™ X-100 in PBS at RT. Slices were then mounted in ProLong® Gold Antifade (Invitrogen).

### Histological staining

Mice were deeply anaesthetized and transcardially perfused with ice-cold PBS followed by perfusion fixation with ice-cold 4% paraformaldehyde (PFA) in PBS. Tissue was post-fixed in 4% PFA and then processed for paraffin embedding. Brains were sectioned into 5  $\mu$ m thick slices using a Leica microtome. For H&E staining, sections were deparaffinized and rehydrated using alcohol, followed by staining with haematoxylin for nuclei and subsequent counterstaining with eosin for cytoplasmic structures (using standard histological procedures). For Alizarin red staining, samples were incubated in an Alizarin Red S solution for 5 minutes after being dewaxed and rehydrated. Samples were then rinsed, counterstained with 0.05% Fast Green in 0.2% acetic acid for 1 min, and then rinsed again before mounting. For von Kossa staining, samples were incubated in 2% silver nitrate solution and exposed to strong light (after being dewaxed and rehydrated). Samples were then washed in distilled water before being placed in a 5% sodium thiosulphate solution. Samples were washed again and then counterstained with “red” or “green” solution. Samples were washed in distilled water a final time, then dehydrated, cleared in xylene, and mounted. Perl’s Prussian Blue Reaction (PBR) staining was performed using a kit from TCS Biosciences (HS652), as per the manufacturer’s instructions. For Congo Red staining, slides were stained in Congo Red solution for 5 min, washed in potassium hydroxide for 1–3 min, washed in tap water for 1 min, counterstained with Mayer’s haemalum for 1 min, washed in running water for 10 s, blued in Scott’s tap water substitute for 30 s, and washed once more under running water for 10 s. Slides were then dehydrated, cleared in xylene, and mounted. Mounting was performed using a DPX mountant (CellPath; SEA-1304-00A).

### Microglial transplantations

We previously validated a commercial source of C57BL/6 primary mouse microglia (ScienCell, Catalog #M1900-57) that express high levels of the homeostatic microglia markers P2RY12 and TMEM119 and can be readily transplanted into the brains of haplotype-matched *Csf1* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> recipient mice.<sup>55</sup> Donor microglia were thawed into commercially available microglial media (ScienCell, Catalog #1901), allowed to recover for 1 h in a 37°C cell culture incubator, and then resuspended in sterile 1  $\times$  PBS at a concentration of 6,250 cells/ $\mu$ l and placed on ice immediately prior to transplantation.

Microglial transplantations of neonatal mouse pups were performed as previously described.<sup>75</sup> Briefly, P1 *Csf1* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> pups were placed in a clean cage over a heating pad with a nestlet from the home cage. Pups were then placed on ice for 2–3 min to induce hypothermic anaesthesia. Free-hand transplantation was performed using a 30-gauge needle (7803-07; Hamilton) affixed to a 10  $\mu$ l Hamilton syringe (7653-01; Hamilton), mice received 1  $\mu$ l of primary mouse microglia suspended in sterile 1  $\times$  PBS at 6,250 cells/ $\mu$ l at each injection site (eight sites) totalling 50,000 cells/pup. Bilateral injections were performed at 2/5th of the distance from the lambda suture to each eye, injecting into the lateral ventricles at 3 mm and into the overlying superior cortex at 1 mm, and into the posterior cortex in line with the forebrain injection sites, and perpendicular to lambda at a 45° angle. Transplanted pups were then returned to their home cages and weaned at P21.

At 9-months-of-age, PBS- and microglia-injected *Csf1* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> mice were perfused with ice-cold 1xPBS and isolated brains were drop fixed in 4% (wt/vol) PFA for 48 h then cryoprotected in a 30% (wt/vol) sucrose at 4°C. Brains were sectioned coronally into 30- $\mu$ m-thick slices on a freezing microtome (Leica, SM 2010R) cooled to -70°C and stored in a solution of 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (S2002; Sigma-Aldrich) in 1  $\times$  PBS (P44017-100TAB; Sigma-Aldrich) as free-floating slices. Alizarin Red S (TMS-008, Millipore-Sigma) staining was performed following manufacturer’s protocol and imaged using Keyence BZ-X810 Widefield Microscope/Maestro Edge. ImageJ software was used to analyse stained tissue. Antibodies used for immunofluorescence staining on these sections were goat anti-IBA1 (1:300; ab5076; Abcam), mouse anti-SMI312 (1:1000; 837904; biolegend), rabbit anti-NeuN (1:1000; abn78; Abcam), chicken anti-GFAP (1:2000; ab4674; Abcam), goat anti-Olig2 (1:500; AF2418; R&D biosystems), goat anti-SerpinA3N (1:150; AF4709; R&D biosystems), and Ris-647 (1:1000; BV500101; Biovinc). Microglia engraftment and resdronate-positive calcifications were captured using an Olympus FX3000 confocal microscope, and hemibrain stitch assembled using Fluoview FV31S-DT software.

### Hematology

To examine the cellular composition of blood from aged *Csf1*<sup>WT/WT</sup> and *Csf1* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> mice, blood was collected in EDTA-coated “microvettes” (Sarstedt, cat# 20.1341.100) and immediately inverted multiple times to prevent clotting. Routine hematology was carried out using an automated analyzer (ADVIA 2120i, Simens). Blood smears were prepared manually, stained with modified Wright-Giemsa, and reviewed by light microscopy by a veterinary clinical pathologist (Easter Bush Pathology, Edinburgh University).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Single-cell RNA sequencing analysis

Alignment to the reference genome, feature counting, and cell calling were performed following the 10x Genomics Cell Ranger pipeline (v5.0.0), using the default mm10 genome supplied by 10x Genomics (<https://cf.10xgenomics.com/supp/cell-exp/refdata-gex-mm10-2020-A.tar.gz>). From the output, the filtered matrices were used for downstream analyses. Pre-processing was performed

on the University of Edinburgh's compute cluster Eddie. The analysis was performed with R v.4.1.1.(v.4.2.1 for thalamus). Full details to replicate the analysis pipelines described below, including the code and package versions used, can be found in code scripts available on the GitHub repositories linked in the code availability section.

In brief, all the datasets were processed as follows. Cells were first filtered using dataset-specific parameters based on genes and unique molecular identifiers (UMIs) per cell, the ratio between these two parameters and the percentage of mitochondrial gene reads per cell. Thresholds were computed with the `isOutlier` function from `scater`,<sup>65</sup> and modified if necessary, to keep them relaxed at this first stage (for details, see [Table S1](#)). The gene quality control was also permissive, keeping all genes that were detected in at least two cells. Using `scran`,<sup>66</sup> the data were normalized by deconvolution, and the top 15% highly variable genes were selected. Following principal component analysis (PCA), between 20 and 30 principal components (PCs) were kept for downstream analysis (cut-off selected by examination of an Elbowplot). Two nonlinear dimensional representation were computed, the t-distributed stochastic neighbour embedding (t-SNE)<sup>76</sup> and the uniform manifold approximation and projection (UMAP).<sup>77</sup> A graph-based clustering approach was used to cluster the cells using the `clusterCells` function from `scran` and/or the `FindClusters` function from `Seurat`.<sup>67</sup> The cell type identity of each cluster was identified using a combination of canonical marker genes, top expressed genes, and differentially expressed genes between clusters computed with `findMarkers` from `scran`. As in Ximerakis et al.<sup>78</sup> we performed a cluster QC, to exclude clusters formed by doublets or low quality cells. We excluded clusters where most cells had low UMI counts or high mitochondrial RNA; protecting clusters that were mostly formed by *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> brain cells, as the difference could be due to their condition; dataset specific thresholds were chosen for these parameters, and they can be found in the GitHub code. The potential doublets were computed with `findDoubletClusters` from `scDblFinder`, and the top hits were manually evaluated and selectively excluded. Following this preliminary analysis, a second round of analysis was performed. Cell and gene quality control were further adjusted, this time using not only dataset specific thresholds, but also cell type specific thresholds (for details, see [Table S1](#)). The normalisation, feature selection, and dimensional reduction were repeated with the clean datasets following the same procedure described above. Choosing the clustering resolution and performing the cluster annotation were done hand-to-hand, using `clustree`<sup>69</sup> as a reference to understand the differences between cluster resolutions, and ensuring there was no over-clustering or under-clustering of the different cell types, merging clusters if necessary.

In order to study the differences between *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> mice, a differential abundance analysis was performed with `Milo`<sup>21</sup> and differentially expressed genes (DEGs) per cell type and per cluster were computed by pseudo-bulk with `edgeR`,<sup>79,80</sup> adding the batch as a covariate. As background microglia genes were appearing as differentially expressed between the two conditions we decided to perform an ambient RNA removal from the DEGs lists, using `DropletUtils`.<sup>70</sup> The first ambient profile was computed with `ambientProfileEmpty`, and it was then further refined using known microglia genes that should not be expressed in other cell types, using `ambientContribNegative` in all non-immune cell types. The minimum ambient contribution was calculated by taking the minimum from the two ambient contribution estimates, which was then used for filtering any gene with an estimated contamination proportion greater than 0.25. To investigate the pseudotime trajectories of the oligodendroglia we used `Slingshot` (v 2.0.0). To focus the analysis, we subsetting for the clusters labelled as mOligo1-4, iOligo, pOPC and OPC. Within this subset, we repeated feature selection and batch correction. For input into `Slingshot`, we utilised 25 dimensions from the corrected PCA resulting from the fastMNN batch correction method. It is important to note we selected this embedding as the UMAP yield spatially separated clusters, which were not suitable for computing a continuous trajectory. Finally, an interactive application was produced with `ShinyCell`<sup>72</sup> (link provided in the “[data and code availability](#)” section).

The following modifications were performed on the juvenile and thalamus datasets: In the juvenile dataset, one of the batches was of lower quality; therefore, it was disregarded when deciding the QC thresholds by using the “subset” argument in the `isOutlier` function. In addition, there was a batch effect in this dataset, revealed in the first dimensional reduction and by the gene expression variance explained, computed with `scater`. Batch correction was performed using mutual nearest neighbours with fastMNN from `batchelor`.<sup>73</sup> The thalamus fastq reads were aligned to the reference genome with `CellRanger` v.7.0.0 that also includes intron reads. A sex effect was observed in this dataset and two integration methods were tested to correct for the effect, fastMNN and harmony.<sup>74</sup> Finally, harmony was kept as it yielded a better integration. Due to an unbalanced number of female and male mice between *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup> thalamic samples, differences between these two groups are reduced in the dataset corrected for sex. This affects the dimensional reduction representations and the clustering, which are therefore underestimating the differences between *Csf1r*<sup>WT/WT</sup> and *Csf1r*<sup>ΔFIRE/ΔFIRE</sup>. The DEGs are not affected by this correction, as they were computed back into the raw values, adding the sex as an additional covariate.

The integration between the three ages was performed with fastMNN. The corrected PCs were used to recompute the dimensional reduction and to perform a re-clustering, with the `quickClusters` function from `scran`. The cluster annotation was performed using the previously described annotations obtained when analysing the datasets separately. The interaction between the changes in genotype and the changes with aging was assessed for the major cell types with `edgeR`, by adding an interaction factor to the linear model design.

## MRI analysis

Dicom files were exported to NIFTI using FIJI prior to image processing. Brains were computationally extracted from surrounding tissue using a custom MATLAB script. Briefly, the 3D volumes were binarized with a threshold equal to 6 times the signal of surrounding Fomblin. Signal in the skull bone is low and hence included in the negative mask. A spherical element of 5 pixels radius was used to

dilate the negative mask, which separated the brain tissue signal from other tissues. All unconnected regions were suppressed, then the brain mask was dilated using a spherical element of 6 pixels radius to close holes. Finally, the mask was eroded using a spherical element of 2 pixels radius to remove the subarachnoid space. Images were all visually checked, and parameters refined as needed. We re-used the mouse brain MRI atlas from Dorr et al.,<sup>81</sup> which includes 115 lateralized labels. Skull-stripped scans were exported at the same resolution, then we used custom scripts in ANTs to apply the N4 algorithm and perform scan registration to template. Jacobian matrices were then used to warp the labelled atlas into each scan's space.

Volumetry was computed with custom MATLAB scripts using the warped labels. Region volumes were computed as the sum of the voxels of a certain label multiplied by individual voxel size. DTI metrics were recovered from the values computed by Bruker's Paravision 360 software. Briefly, we extracted fractional anisotropy (FA), axial diffusivity (AD) and radial diffusivity (RD). Mean diffusivity (MD) was defined as the sum of AD and RD. Hypointensities were automatically detected independently per region. Voxels whose signal was below the regional median of 3 median absolute deviations (MAD) were considered as hypointensities. Hypointensities closer than 210  $\mu\text{m}$  to the mask border were excluded as border effects and minor field distortions created false positives.

For the arterial spin labelling (ASL) analysis, cerebral blood flow maps were generated using the Bruker software (Paravision 360) using equation [1] as discussed by Belle et al.<sup>82</sup> Measurements were taken from cerebral blood flow (CBF) maps in ImageJ after manually drawing regions of interest based on the in vivo structural T2 scans. CBF measurements were taken using the original/harsh voxels of the CBF maps. For representative ASL images, CBS maps were subsequently smoothed for illustrative purposes using median filtering in ImageJ.

### Microscopy and image analysis

For confocal microscopy, Zeiss LSM 710 and Leica SP8 confocal microscopes were used with images taken using x5-x60 objectives. For full brain section scans, a Zeiss AxioScan was used with images taken using a 20x objective lens. For the free-floating sections, a Leica SP8 confocal microscope with 5 detectors and an Opera Phoenix High Content Screening System was used to image slides.

For the GFAP quantification in Figure 3B, QuPath (version 0.4.3) was used for image analysis. Areas of interest were manually drawn. Total numbers of GFAP<sup>high</sup> structures (defined based on the use of a consistent intensity threshold) were quantified per area using the Analyze > Cell detection > Cell detection command. For the NeuN quantification, QuPath (version 0.4.3) was used for image analysis. Areas of interest were manually drawn. Total numbers of DAPI<sup>+</sup> nuclei and numbers of NeuN<sup>+</sup>DAPI<sup>+</sup> nuclei were quantified per area using the Analyze > Cell detection > Positive cell detection command. Numbers of NeuN<sup>+</sup>DAPI<sup>+</sup> nuclei per area were calculated, as well as the percentage of all nuclei that were neuronal (i.e., % of DAPI<sup>+</sup> nuclei that were also NeuN<sup>+</sup>). For the alizarin red quantification (in Figure 5B), ImageJ was used to manually draw the thalamic region (using the polygon selection tool). The Threshold > Default > Over/Under > Apply command was then used to define thresholds, and measurements were taken for both the area and the % of area covered by thresholded pixels.

For the quantifications in Figures 5C and 6D, IMARIS (version 9.9) was used for volume rendering of individual channels for the quantification of the numbers of SPP1<sup>+</sup> structures that were in contact/not in contact with GFAP<sup>high</sup> astrocytes (Figure 5C) or the vasculature (as labelled by  $\beta$ -dystroglycan; Figure 6D). For the vascular analyses in Figures 6H–6K, the thalamus was subdivided into 4 regions for each animal (dorsal-left and right and ventral-left and right) and a region of 1000x700  $\mu\text{m}$  was analysed, with 3 replicates (slices) per animal (7 *Csf1r*<sup>WT/WT</sup> and 7x *Csf1r* <sup>$\Delta$ FIRE/ $\Delta$ FIRE</sup> mice for a total of 168 images). The images were all analysed using Vas-O-Matic, a homemade ImageJ-based tool used to perform automated analyses of 2D fluorescence microscopy datasets. Vas-O-Matic, through thresholding and particle filtering, segments the vasculature and correlates pericyte coverage to vessel diameter. Vas-O-Matic was used to quantify pericyte coverage (%), vessel diameter ( $\mu\text{m}$ ), and vascular density ( $\text{mm}^3$ ) as independent results.

### Statistical analyses

Sample sizes and statistical tests used for each experiment are provided in respective figure legends. Significance was defined as P (or adjusted P) < 0.05. Experimenters were blinded where possible. Statistical methods were not used to pre-determine study group sizes. Statistical analyses were performed using GraphPad Prism (version 10), unless otherwise stated in respective methods sections. Data are expressed as biologically independent samples with mean  $\pm$  S.E.M. unless indicated otherwise. Full two-way ANOVA test statistics tables can be found in Table S4.

### ADDITIONAL RESOURCES

#### Interactive database

For an interactive database to mine single-cell RNA sequencing datasets produced for this manuscript, please use the following link: [https://annawilliams.shinyapps.io/shinyApp\\_jpriller/](https://annawilliams.shinyapps.io/shinyApp_jpriller/)