Epigenetic regulation of brain region-specific microglia clearance activity

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The rapid elimination of dying neurons and nonfunctional synapses in the brain is carried out by microglia, the resident myeloid cells of the brain. Here we show that microglia clearance activity in the adult brain is regionally regulated and depends on the rate of neuronal attrition. Cerebellar, but not striatal or cortical, microglia exhibited high levels of basal clearance activity, which correlated with an elevated degree of cerebellar neuronal attrition. Exposing forebrain microglia to apoptotic cells activated gene-expression programs supporting clearance activity. We provide evidence that the polycomb repressive complex 2 (PRC2) epigenetically restricts the expression of genes that support clearance activity in striatal and cortical microglia. Loss of PRC2 leads to aberrant activation of a microglia clearance phenotype, which triggers changes in neuronal morphology and behavior. Our data highlight a key role of epigenetic mechanisms in preventing microglia-induced neuronal alterations that are frequently associated with neurodegenerative and psychiatric diseases.

Development and aging of the brain are associated with the attrition and loss of neurons. Despite the overall low rate of neuronal death during adulthood, functionally distinct brain areas display significant differences in neuronal loss. One of the brain regions displaying the most pronounced age-dependent neuronal loss is the cerebellum, where neuronal numbers start declining at the end of adolescence. The loss of neurons in the cerebellum contrasts with that of other brain structures, such as the striatum, cortex, and hippocampus, which stably maintain their size and neuronal numbers throughout adulthood.

The clearance of dying cells and debris in the brain is carried out by microglia, the brain-resident macrophages. Brain-region-specific differences in neuronal attrition suggest the possibility that microglia may fine-tune their clearance activity to the load of cell debris. Accordingly, the clearance activity of microglia must be suppressed in areas with low rates of neuronal death in the adult brain. Exacerbated activation of microglia has been shown to promote microglia-mediated neuronal attrition. These findings underscore the essential role of mechanisms that modulate microglia activation in the maintenance of brain integrity.

Here we show regional differences in microglia clearance activity in the adult brain and demonstrate the importance of the tight regulation of such clearance activity for normal brain function. We found that cerebellar microglia display a distinct clearance phenotype characterized by the expression of numerous genes supporting the engulfment and catabolism of cells and cellular debris. The cerebellar microglia phenotype is reminiscent of microglia during early brain development as well as microglia associated with neurodegenerative disease. In contrast, microglia in the striatum—which, like the cerebellum, is characterized by a rather homogenous neuron population—display a homeostatic surveillance phenotype. We found that the suppression of clearance activity in striatal microglia has an epigenetic underpinning. The suppression of clearance genes in striatal microglia is governed by PRC2, which catalyzes the repressive chromatin modification histone H3 lysine 27 trimethylation. Accordingly, microglia-specific ablation of PRC2 leads to the aberrant acquisition of a clearance phenotype in striatal and cortical microglia even in the absence of dying neurons. This aberrant clearance activation has a negative impact on neuronal morphology and associated neuron-mediated behaviors, leading to impaired motor responses, decreased learning and memory, and the development of anxiety and seizures in mice.

Results

Cerebellar microglia display cellular phenotypes and gene-expression patterns associated with cell clearance. We found that cerebellar microglia (cbMg), unlike microglia in the striatum (stMg), display morphological features and gene expression patterns that support clearance functions (Fig. 1 and Supplementary Fig. 1). cbMg in the adult mouse brain show a primed or phagocytic microglia phenotype characterized by a less-ramified morphology, reduced branching sites (Supplementary Fig. 1a), decreased cell volume (Supplementary Fig. 1b,c), and an increase in CD68+ lysosome content (Fig. 1a and Supplementary Fig. 1b,c). A significant fraction of microglia in the cerebellum contain lysosomes carrying cell nuclei fragments as determined by the presence of DAPI nuclear material (Fig. 1b and Supplementary Fig. 1d), which is indicative of apoptotic cell clearance. The relatively high CD68+ lysosome content of cbMg is reminiscent of microglia in the dentate gyrus and olfactory bulb (Supplementary Fig. 1b,c), where ongoing neuronal turnover is associated with high rates of cell death in the adult brain.
To study gene expression in microglia in a minimally invasive fashion in vivo, we established a microglia-specific translating ribosome affinity purification (TRAP) approach (Fig. 1c). To generate microglia-specific TRAP mice, we bred mice that carry a loxP-flanked STOP cassette (LSL) upstream of the gFP-L10a coding sequence under the control of ubiquitously expressed eukaryotic translation elongation factor 1 alpha 1, Eeffa1 (Eeffa1LSL<sup>+</sup>gFP<sup>+</sup>L10a<sup>+</sup>), with mice that express Cre recombinase under the control of a microglia-specific gene promoter. The Cx3cr1<sup>CreERT2<sup>+</sup>Litt</sup> strain was chosen based on the inducibility, selectivity, and highest efficiency of Cre expression in microglia in the adult brain (Supplementary Fig. 2). Notably, we show that the TRAP approach not only allows for region-specific analysis of microglia-enriched mRNA expression (Supplementary Fig. 3 and Supplementary Table 1) but also precludes nonspecific microglia activation and concurrent upregulation of immediate early and pro-inflammatory genes that occur during commonly used microglia isolation approaches (Supplementary Fig. 4 and Supplementary Table 2).

We found that stMg display a substantial enrichment in mRNAs associated with cell clearance functions (Fig. 1d,e, Supplementary Fig. 5a,b, and Supplementary Table 3). In contrast, stMg are enriched in mRNAs encoding mature microglia-specific homeostatic...
surveillance proteins\(^{14,15}\), including those that mediate G-protein-coupled chemosensing, chemotaxis, GTP signaling, and actin polymerization\(^{20}\) (Fig. 1d,e, Supplementary Fig. 5a,b, and Supplementary Table 3). A large fraction of the cbMg-enriched genes, including Apoe, Axl, Colec12, Cd74, Librb4, Mrc1, and Ms4a7 (Fig. 1d,e, Supplementary Fig. 5a,b, and Supplementary Table 3), encode proteins involved in apoptotic cell detection, cell engulfment, clearance of cell debris, and lipid and protein catabolism and have been shown to support clearance activity in phagocytic macrophages in peripheral tissues\(^{16}\). Furthermore, cbMg show an increase in the expression of genes characteristic of immature microglia\(^{14,15}\), as well as genes that are expressed in neurodegenerative disease-associated microglia (such as primed microglia, microglia with neurodegenerative phenotype, and disease-associated microglia)\(^{11–13}\) (Supplementary Fig. 5c,d and Supplementary Table 3). Notably, in contrast to neurodegenerative disease-associated microglia, cbMg are not enriched in the expression of pro-inflammatory genes such as Cxcl10, Il1b, Il6, and Tnf (Supplementary Fig. 5b).

The differential cbMg clearance–gene versus stMg surveillance–gene expression patterns were confirmed by microglia single-nuclei RNA-sequencing analysis (Fig. 2a,b, Supplementary Fig. 6, and Supplementary Tables 5 and 6) and RNA in situ hybridization...
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Fig. 3 | cbMg clearance phenotype is associated with exposure to dying cells. a,b. Representative immunofluorescence images are shown (Neun+ neurons, red; cCASP3+, green; DAPI, blue). Dotted circles, cCASP3+Neun+ cell. Scale bar, 10 μm. (a) Quantification of cCASP3+ cells per cm² from 4-month-old control (striatum: mean = 0, s.e.m. = 0; cerebellum: mean = 2,500, s.e.m. = 1.443) or PLX-treated mice (striatum: mean = 2,500, s.e.m. = 1.443; cerebellum: mean = 119.2, s.e.m. = 20.02; P < 0.0001; F = 67.40; 14 cerebellum or striatum sections from n = 4 mice per group). (b) Quantification of cCASP3+ cells per cm² from 4- to 5-month-old control (striatum: mean = 0, s.e.m. = 0; cerebellum: mean = 2,500, s.e.m. = 1.443) or Axl−/−Merk−/− mice (striatum: mean = 0, s.e.m. = 0; cerebellum: mean = 33.17, s.e.m. = 8.355; P = 0.0003; F = 14.62; 12 cerebellum or striatum sections from n = 4 mice per group; one-way ANOVA with Tukey’s multiple comparison test). Bar graphs with individual data points show mean ± s.e.m. c. Horizontal bar graph shows relative expression (quantitative PCR) of selected cbMg-enriched (orange) and stMg-enriched (purple) genes in microglia after 12 h of exposure to vehicle or early apoptotic cells (Pparg: P = 0.002, t4 = 7.144; Jdp2: P < 0.0001, t4 = 15.16; Rarg: P = 0.001, t4 = 10.16; Tfel: P < 0.0001, t4 = 11.47; Ahr: P = 0.004, t4 = 6.033; En2: P = 0.025, t4 = 3.493; Tead4: P = 0.002, t4 = 7.013; Anxa2: P < 0.0001, t4 = 36.96; Colec12: P = 0.002, t4 = 7.498; Libr6: P < 0.0001, t4 = 14.04; Apoe: P = 0.002, t4 = 6.964; Cd74: P = 0.001, t4 = 8.070; Pchtl: P = 0.037, t4 = 3.066; Clec7a: P = 0.015, t4 = 4.064; Msr1: P = 0.011, t4 = 4.448; Lyz2: P = 0.024, t4 = 3.529; Ptp1ld2: P = 0.009, t4 = 4.809; Kdm6b: P < 0.0001, t4 = 15.33; Kdm6c: P = 0.004, t4 = 5.87; Hhex: P = 0.025, t4 = 3.501; Esr7: P = 0.018, t4 = 3.885; Irf8: P = 0.001, t4 = 9.580; Sall3: P = 0.139, t4 = 1.842; Sall3: P = 0.449, t4 = 0.838; Slc2a6: P < 0.0001, t4 = 15.30; Asb2: P < 0.0001, t4 = 25.35; Tmem119: P = 0.003, t4 = 6.721; Fcrl: P < 0.0001, t4 = 18.93; P2ry12: P = 0.733, t4 = 0.3659; and Forc1: P = 0.001, t4 = 10.31; two-tailed unpaired t test, n = 3 wells of primary microglia cultures obtained from four 3-month-old mice). Bar graphs show mean ± s.e.m. Experiment was independently reproduced 4 times. ***P ≤ 0.001.

(Cerebellar microglia are involved in the clearance of dying cells. The cerebellar microglia clearance phenotype, which is stably maintained during adulthood (Fig. 1d), could reflect either an inherent region-specific feature of the cbMg and/or the activation of clearance function by exposure to dying cells. While the age-dependent loss of cerebellar mass and neuronal numbers implicates ongoing cell death, we could not detect dying cells in the cerebellum of adult wild-type mice. We speculated that dying cells in the cerebellum are rapidly removed by microglia. In such a scenario, one would anticipate an increase in the number of apoptotic cells in the absence of microglia. Microglia can be efficiently ablated in mice by chronic treatment with PLX5622 (PLX), a pharmacological inhibitor of the colony stimulating factor 1 receptor, CSF1R, which is required for microglia survival. Treatment of 2-month-old wild-type mice for >3 weeks with PLX (Supplementary Fig. 8a) resulted in the near complete ablation of microglia (~99%) in the adult brain, including in the cerebellum and striatum (Supplementary Fig. 8b). The chronic depletion of microglia led to the accumulation of a significant number of apoptotic cells positive for cleaved-Caspase 3 (cCASP3) and TUNEL in the cerebellum but not in the striatum (Fig. 3a and Supplementary Fig. 8c–e). Co-labeling with different cell-type-specific markers revealed that the majority of the apoptotic cell-type expression in cbMg as compared to in stMg (Fig. 2d and Supplementary Fig. 7f). Cerebellar microglia are involved in the clearance of dying cells. The cerebellar microglia clearance phenotype, which is stably maintained during adulthood (Fig. 1d), could reflect either an inherent region-specific feature of the cbMg and/or the activation of clearance function by exposure to dying cells. While the age-dependent loss of cerebellar mass and neuronal numbers implicates ongoing cell death, we could not detect dying cells in the cerebellum of adult wild-type mice. We speculated that dying cells in the cerebellum are rapidly removed by microglia. In such a scenario, one would anticipate an increase in the number of apoptotic cells in the absence of microglia. Microglia can be efficiently ablated in mice by chronic treatment with PLX5622 (PLX), a pharmacological inhibitor of the colony stimulating factor 1 receptor, CSF1R, which is required for microglia survival. Treatment of 2-month-old wild-type mice for >3 weeks with PLX (Supplementary Fig. 8a) resulted in the near complete ablation of microglia (~99%) in the adult brain, including in the cerebellum and striatum (Supplementary Fig. 8b). The chronic depletion of microglia led to the accumulation of a significant number of apoptotic cells positive for cleaved-Caspase 3 (cCASP3) and TUNEL in the cerebellum but not in the striatum (Fig. 3a and Supplementary Fig. 8c–e). Co-labeling with different cell-type-specific markers revealed that the majority of the apoptotic cells were of neuronal origin and were distributed within all layers of the cerebellum (Supplementary Fig. 9a,b). The clearance...
of dying cells by microglia was further supported by a substantial accumulation of cCASP3+ cells in the dentate gyrus and the olfactory bulb (Supplementary Fig. 9c,d), two brain regions displaying continuous neuronal turnover in the adult brain. At the same time, the lack of microglia did not lead to the appearance of cCASP3+ cells in the striatum or cortex (Supplementary Fig. 9c,d). In a complementary approach, we assessed the presence of apoptotic cells in the cerebellum of mice that lack the receptor tyrosine kinases Axl, and Merkt receptors, which regulate apoptotic cell detection and clearance by microglia21. We found that the combined deficiency of Axl and Merkt resulted in the accumulation of apoptotic cCASP3+ cells in the cerebellum of adult mice (Fig. 3b), suggesting that the presence of dying cells was not caused by the loss of microglia but reflected the attenuation of microglia clearance function. These data suggest that microglia are responsible for the continuous clearance of dying cells in the cerebellum and that the presence of dying cells may instruct the activation of cbMg clearance function.

Exposure to dying cells triggers the expression of clearance genes in microglia in vitro. We found that exposure of primary forebrain microglia to apoptotic cells (Supplementary Fig. 10a) led to the progressive induction of numerous prophagocytic genes that were also enriched in cbMg (Fig. 3c and Supplementary Fig. 10b). This exposure to dying cells was associated with the time-dependent induction of cbMg-enriched transcription factors (Fig. 3c and Supplementary Fig. 10c) and with the downregulation of stMg-enriched homeostatic surveillance genes (Fig. 3c and Supplementary Fig. 10d,e) in the absence of pro-inflammatory gene activation (Supplementary Fig. 10f). Among the genes that were rapidly induced in microglia upon exposure to apoptotic cells were the cbMg-enriched Kdm6a and Kdm6b, which encode histone demethylases26 (Fig. 3c and Supplementary Fig. 10g). Kdm6A and Kdm6B mediate the repair of the suppressive chromatin modification H3K27me3, which is associated with silenced genes26. Upregulation of Kdm6a/b and the associated induction of clearance genes in response to apoptotic cells are reminiscent of Kdm6a/b induction and H3K27me3 demethylation at inflammatory gene loci during macrophage activation17,21. These findings point to a possible role for H3K27me3 in modulating brain-region-specific microglia clearance functions in vivo.

PRC2 controls a clearance-related gene-expression program in vivo. H3K27me3 is mediated by PRC2, which has a well-established role in the regulation of cell differentiation during development18. Similarly to neurons and other cell types, microglia express all of the core components of PRC2: EZH1, EZH2, EED, and SUZ12 (data not shown). Enrichment of mRNAs encoding the H3K27me3-specific demethylases, KDM6A and KDM6B, in cbMg (Supplementary Fig. 5b and Supplementary Table 3), as well as the upregulation of Kdm6a/b in microglia upon exposure to dying cells in vitro (Fig. 3c and Supplementary Fig. 10g), point to the possible role of PRC2...
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Supplementary Table 7). The deficiency in were direct PRC2-target genes (Fig. 5e, Supplementary Fig. 11e, and Supplementary Tables 8 and 9) or genes characteristic of microglial nuclei, as well as on the cbMg-enriched transcription factors Rarg, Ahr, and Tead4 in stMg (Fig. 4c,d and Supplementary Table 7).

To address the role of PRC2-mediated H3K27me3 in the suppression of clearance genes, we inactivated PRC2 specifically in microglia. PRC2 inactivation was achieved by genetic ablation of EED, a core subunit of PRC2, which is essential for complex stability and catalytic activity. To ablate EED specifically in adult microglia, we generated and bred EedloxP/loxP mice, which carry a loxP-flanked Eed allele (Supplementary Fig. 11d), to Cx3cr1CreErt2+ mice to generate Cx3cr1CreErt2+;EedloxP/loxP mice (Fig. 5a). Microglia-specific Eed ablation was induced by tamoxifen treatment from 4 to 6 weeks of age, to exclude any impact of PRC2 inactivation on microglia development (Fig. 5a).

Inactivation of Eed resulted in the complete and selective loss of H3K27me3 in microglia (Fig. 5b-d). We found that deficiency in Eed and the ensuing loss of H3K27me3 had no immediate impact on microglia activation but led to rather moderate and progressive changes in stMg gene expression between 3 and 9 months of age (Fig. 5e,f). About 50% of the genes that become progressively upregulated in Eed-deficient microglia were H3K27me3+ and hence were direct PRC2-target genes (Fig. 5e, Supplementary Fig. 11e, and Supplementary Table 7). The deficiency in Eed and the ensuing loss of H3K27me3 led to the upregulation of several known PRC2 targets, such as Tbx15 and Hoxd8 (Supplementary Fig. 11f, g). However, it did not affect the expression of genes that define microglia lineage identity (Supplementary Fig. 11f and Supplementary Tables 8 and 9), and the vast majority of stMg cells (313 of 643) implicated in the regulation of complex behaviors that include control of motor activity, mood, and reward. While Cx3cr1CreErt2+/;EedloxP/loxP mice displayed normal motor activity at baseline (Supplementary Fig. 12a), we found a significant reduction in stance numbers in mice with Eed-deficient microglia as compared to controls (Fig. 7a). Notably, changes in MSN spine numbers were not caused by the overall negative effect of Eed-deficient microglia on MSN survival. Deficiency in Eed had no effect on the overall size and morphology of the striatum (data not shown), MSN density (Supplementary Fig. 12a), or survival as defined by the presence of cCASP3+ cells (Supplementary Fig. 12b).

The changes in spine density of MSNs were associated with alterations in specific MSN-controlled behaviors. MSNs are involved in the regulation of complex behaviors that include control of motor activity, mood, and reward. While Cx3cr1CreErt2+/;EedloxP/loxP mice displayed normal motor activity at baseline (Supplementary Fig. 12a), we found a significant reduction in stance numbers in mice with Eed-deficient microglia as compared to controls (Fig. 7a). Notably, changes in MSN spine numbers were not caused by the overall negative effect of Eed-deficient microglia on MSN survival. Deficiency in Eed had no effect on the overall size and morphology of the striatum (data not shown), MSN density (Supplementary Fig. 12a), or survival as defined by the presence of cCASP3+ cells (Supplementary Fig. 12b).

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In contrast to stMg, H3K27me3 was not present at clearance-related gene loci in cbMg (Fig. 4c,d). Instead, H3K27me3 in cbMg was enriched on genes that are transcriptionally active in stMg. Accordingly, loss of PRC2 in cbMg, while following a similar temporal pattern of progressive PRC2 target gene induction (Fig. 5f and Supplementary Fig. 11e), was associated with the upregulation of selected stMg-enriched genes and the downregulation of cbMg-enriched genes (Fig. 6d,e). The changes in PRC2-deficient cbMg gene expression patterns were associated with enhanced cbMg-ramification, a reduction in cbMg lysosomal content (Fig. 6f), and an increased number of cCASP3+ cells in the cerebellum (Fig. 6g). Collectively, our data point to a largely selective role of PRC2 in the maintenance of regional microglia specification in the adult brain, where it controls the cell-intrinsic suppression of an aberrant prophagocytic and clearance-like phenotype in stMg in vivo.

Aberrant expression of clearance-specific genes in striatal microglia has a negative impact on striatal neuron morphology and function. We found that the switch in PRC2-deficient stMg from a surveillance phenotype to a more clearance-promoting phenotype had a profound impact on striatal medium spiny neuron (MSN) morphology and associated behaviors. Quantitative spine analysis of MSNs revealed a significant reduction in spine numbers in mice with Eed-deficient microglia as compared to controls (Fig. 7a). Notably, changes in MSN spine numbers were not caused by the overall negative effect of Eed-deficient microglia on MSN survival. We found no significant reduction in stance numbers in MSN-mediated locomotor sensitization in response to increased dopamine levels upon chronic cocaine treatment (Fig. 7b and Supplementary Fig. 12d). The induction of behavioral locomotor sensitization in response to cocaine depends on the structural plasticity of MSNs and is associated with

Fig. 5 | Selective effect of Eed inactivation on stMg and cbMg gene expression. a, Schematic showing microglia-specific Eed deletion in adult mice. b, H3K27me3 levels quantified by western blot analysis of isolated microglial nuclei (50,000 nuclei per genotype) relative to total H3 (two independent experiments). Left: representative blot (cropped to show the specific band); right: quantification. Ratio of intensities (ImageJ) from control (Cx3cr1CreErt2+/;EedLoxP/loxP;Eedflox/flox;EedCre+); mean = 0.9725, s.e.m. = 0.04116) and mutant mice (Cx3cr1CreErt2+/;EedLoxP/loxP;Eedflox/flox;EedCre+); mean = 0.01006, s.e.m. = 0.00556; P < 0.0001, F = 54.90, t = 22.02; two-tailed paired t test). *P < 0.001. c, Quantification of the number of H3K27me3+ cells from control (microglia: mean = 96.50; nonmicroglia: mean = 89.70) and mutant mice (microglia: mean = 3.500; nonmicroglia: mean = 90.33; > 50 cells from n = 2 mice per genotype). Bar graphs with individual data points show mean ± s.e.m. d, H3K27me3 (red) in YFP or GFP microglia (green) using immunofluorescence of brain sections (DAPI, blue). Scale bars, 10 μm. Representative image from two independent experiments. Dotted circles, microglial nuclei. e, Bar graph shows number of genes up- or downregulated in Eed-deficient stMg at 3, 6, and 9 months by TRAP (DESeq2, n = 2 mice per region per genotype). Number of H3K27me3+ genes (red) is shown. f, Principle component analysis (PCA) of stMg- or cbMg-TRAP-seq of 3-, 6-, and 9-month-old Cx3cr1CreErt2+/;EedLoxP/loxP;EedCre+ and control (Cx3cr1CreErt2+/;EedLoxP/loxP;EedCre+); mice (n = 2 mice per genotype per age). g, h, MA plots (representing log-ratio (M) on the y axis and mean average (A) on the x axis) show gene expression changes (red, upregulated; blue, downregulated) caused by deletion of Eed in stMg (g) and cbMg (h) of 9-month-old mice (DESeq2, n = 2 mice per genotype). Genes in green are equally expressed. Pie charts show the GO-based categories of upregulated genes with selected genes named.
last changes in MSN spine density. PRC2 deficiency in cbMg, on the other hand, had no significant impact on cerebellar Purkinje cell spine numbers (P = 0.1905; Supplementary Fig. 12c) or on cerebellum-dependent behaviors (Supplementary Fig. 12f).

As in the striatum, we found that loss of PRC2 activity in cortical microglia (cxMg) was associated with a similar switch toward a microglia clearance phenotype (Supplementary Fig. 12g), including an increase in cxMg lysosomal content (Supplementary Fig. 12h).

The changes in cxMg were associated with a reduction in cortical pyramidal neuronal spine density (Supplementary Fig. 12i) in the absence of changes to neuronal survival (Supplementary Fig. 12j).

Disorders accompanied by alterations in spine numbers, including neurodegenerative and neuropsychiatric disorders, are frequently associated with the development of anxiety and seizures and cognitive decline. Accordingly, we found that loss of PRC2 activity in microglia led to enhanced anxiety behavior in the open field.
cell sorting (FACS) led to the expression of numerous genes that were indicative of cell activation and that were barely detectable when measured by TRAP. In addition to the noninvasive nature of the TRAP approach, we applied a stringent bioinformatic step during data analysis that excluded genes not enriched in microglia as compared to the input brain tissue12 (Methods). Notably, this approach, while being well-suited to generate signatures of mRNAs enriched in microglia subpopulations in different brain areas, bears certain shortcomings, as it may omit genes that are shared between microglia, neurons, astrocytes, or other cell types in the specific region. These differences should be considered when comparing data generated by TRAP to those generated from purified microglia (Supplementary Fig. 14a).

Here we expanded the depth of the population-based microglia TRAP approach by using single-microglia RNA-sequencing analysis, which allowed further assessment of inter- and intrapopulational microglia diversity. We continued to adhere to less invasive approaches and used rapid nuclei purification from specific mouse brain regions followed by FACS-based isolation of the GFP+ microglial nuclei. We found that microglia nuclei isolation, in contrast to whole-microglia cell purification, diminished aberrant microglia activation. The cross-comparison between the microglia-region-specific TRAP and single-nuclei-based RNA-expression patterns confirmed the distinct clearance-supporting phenotype in cbMg. Moreover, single-microglia nuclei-based analysis allowed us to exclude perivascular macrophages (Cx3cr1+, Tmem119+, and P2ry12loxp) as potential factors driving the cbMg clearance-promoting gene expression signature.

In particular, a large fraction of the cbMg-expressed genes have been implicated in the detection, engulfment, and catabolism of debris both in peripheral macrophages carrying out cell clearance1, as well as in microglia associated with aging and neurodegeneration13–16. Recent evidence suggests that microglia can sense and clear apoptotic cells at the earliest stages of apoptosis17. This process, often referred to as efferocytosis, is a multistep process: (i) the recognition of ‘eat me’ signals presented by apoptotic cells,
(ii) engulfment of cells or debris, (iii) degradation of engulfed material, and (iv) excretion of metabolic products. The numerous efferocytosis and clearance-associated genes expressed in cbMg comprise a set that encodes transcription factors implicated in the regulation of macrophage polarization, clearance, and differentiation, including AHR, TFEC, and RARG. These transcription factors are also induced upon exposure to apoptotic cells, as well as in the Eed-deficient stMg, which acquired an aberrant clearance phenotype (Supplementary Fig. 14b).

Our findings suggest that the cerebellum displays relatively higher levels of cell loss as compared to the striatum or cortex. While dying cells in the adult cerebellum cannot be readily detected in the presence of microglia, we found that the ablation of microglia leads to an increase in the numbers of cCASP3+ apoptotic cells in...
the cerebellum. This scenario is compatible with rapid microglia-mediated removal of dying neurons in the subventricular zone\(^9\) as well as removal of apoptotic thymocytes during T cell development\(^8\). While most newly generated neurons and T cells undergo apoptotic cell death, the dying cells cannot be readily detected due to their rapid removal by resident macrophages/microglia\(^9,10\). Consistent with this model, microglia in the cerebellum are likely to contribute to the efficient removal of dying cells. Accordingly, we found that both deletion of microglia and attenuation of their cell-clearance capability were associated with the accumulation of dying cells in the cerebellum but not in the striatum or the cortex. The clearance activity of cbMg is further supported by recent live-imaging data from adult mice showing decreased parenchymal surveillance activity of cbMg as compared to forebrain microglia in vivo\(^11\). In parallel, we and others found that acutely isolated cbMg are much more efficient in the uptake of apoptotic cells or other biological materials\(^4\). Notably, while these data strongly imply the enhanced clearance activity of cbMg in the healthy brain, a comparative analysis that quantifies the levels of phagocytic activity in individual cbMg versus stMg in vivo is still needed.

We found that the cbMg-specific clearance gene expression pattern significantly overlapped with the signature of microglia associated with neurodegeneration \(P<0.0001\)^11–13 and early brain development \(P<0.0001\)^14–16, two stages of life characterized by enhanced neuronal attrition and heightened microglia clearance activity. In addition to the induction of cell clearance genes, the cbMg are further characterized by a clear reduction in homeostatic microglia genes\(^14,15\). This is another key characteristic of early microglia progenitors\(^9,10\) as well as of microglia exposed to dying neurons during different diseases\(^11–13\). Notably, one of the commonalities between the three previously discussed microglia phenotypes—microglia during early development, disease-associated microglia, and cbMg—is their likely active involvement in the clearance of apoptotic cells and cellular debris. Hence, we speculate that the similarities in their gene expression profiles may be driven by the activation of a microglia clearance function in response to dying cells.

While we consider the differential exposure to dying cells as a major contributor to regional microglia specification, we cannot fully exclude the potential impact of other cues, including region-specific differences in neuronal activity, neurotransmitter release, and other microglia-recognized ligands\(^15\). However, in strong support of a causal contribution of ongoing cerebellar cell death to the induction of the cbMg clearance phenotype, we found that the exposure of forebrain microglia to dying cells in vitro led to a progressive induction of some key microglia cbMg-enriched clearance genes.

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**Fig. 7 | Microglia-specific Eed deficiency alters MSN morphology and MSN-controlled behaviors in mice.** a, Representative images of Golgi-stained neuronal processes of MSNs from 15-month-old control \(\text{Eed}^{loxP/loxP}\) and \(\text{Cx3cr}^{Eert2/+/Eed}^{loxP/loxP}\) mice. Scale bar, 2 μm. Bar graph with individual data points shows total spine densities of MSNs of control (mean=1.205, s.e.m.=0.0487) and mutant mice (mean=1.009, s.e.m.=0.02326, \(P=0.0171, F=3.556, t_7=3.932; 30\) dendrites from \(n=3\) mice per genotype). b, Bar graph with individual data points shows the ratio of total distance traveled on day 7 vs. day 1 of daily cocaine administration (control: mean=3.497, s.e.m.=0.3380; mutant: mean=2.356, s.e.m.=0.3833; \(P=0.0361, F=1.286, t_{12}=2.232; n=12\) mice per genotype). c, Open field analysis (thigmotaxis) shows mutant mice spend less time in the center vs. periphery (mean=0.2714, s.e.m.=0.04387) and mutant mice (mean=1.009, s.e.m.=0.02326, \(P=0.0171, F=3.556, t_{7}=3.932; 30\) dendrites from \(n=3\) mice per genotype). d, Elevated plus maze analysis shows that mutant mice spend less time in the open arms (mean=60.09, s.e.m.=5.765) than control mice (mean=31.77, s.e.m.=11.23; \(P=0.0279, F=3.415, MWU=18.00, SWP=0.0210\)) and more time in closed arms (mutant: mean=163.0, s.e.m.=12.75; control: mean=207.5, s.e.m.=14.09; \(P=0.0314, F=1.100, t_{12}=2.346, n=10\) mice per genotype). Grubbs’ test was used to identify and exclude an outlier. e, Mutant mice develop seizures with age (Kaplan–Meier curve, \(n=12\) mutant, \(14\) control mice; \(P=0.0002; \chi^2=13.71\) log-rank Mantel–Cox test). Bar graphs with individual data points show mean±s.e.m. All t tests were unpaired and two-tailed.*\(P<0.05\), **\(P<0.001\).
including Colec12, Lirb4, and Apoe. Our findings are supported by data from Krasemann et al.13 showing an induction of selected cbMg-enriched clearance genes in cortical microglia upon injection of dead cells into the healthy mouse brain in vivo (Supplementary Fig. 14c). Overall, our data support the instructive nature of the cbMg phenotype, where continuous exposure to dying cells leads to the expression of genes that support cell clearance function. Our data also suggest that the exposure to and clearance of dying cells induces a distinct pattern of clearance-associated genes that is not intrinsically linked to the activation of pro-inflammatory genes. The lack of inflammatory gene activation in cbMg suggests that the microglia clearance activity may antagonize or suppress the activation of inflammatory genes in a fashion that has been observed in peripheral macrophages19–21. In such a scenario, active clearance of cell debris in cbMg may lead to tolerance of cbMg against self, and thereby preclude potential damaging effects of an aberrant attack by activated cbMg on healthy cerebellar neurons. Consequently, there could be a mechanistic dissociation between the induction of clearance-gene expression and microglia pro-inflammatory activation observed during neurodegenerative diseases11–13, which may be mediated by additional cues from the local environment.

Our data also show that microglia clearance function, while important for the removal of dying cells, must be tightly controlled in other brain regions to avoid microglia-mediated damage of healthy neurons. We found that transcriptional silencing of some clearance-related genes correlated directly with abundance of the major suppressive histone modification H3K27me3 in stMg. The possible causal role of H3K27me3 in suppressing clearance-promoting genes in microglia is supported at several levels. First, clearance-specific genes display negligible amounts of H3K27me3 in cbMg as compared to in stMg. Moreover, exposure of forebrain microglia to dying cells in vitro increases the expression of H3K27me3-specific demethylases, Kdm6a and Kdm6b, and is associated with progressive activation of cbMg-enriched clearance genes. While a potential demethylase-independent function of Kdm6b cannot be excluded, recent studies using a small molecule-based inhibitor specifically targeting Kdm6a/B demethylase activity revealed that H3K27 demethylation and specifically Kdm6a/B catalytic activity are critical determinants of pro-inflammatory gene activation in human primary macrophages26. Based on these data, it is plausible that clearance-gene expression in microglia is controlled in a Kdm6a/B–H3K27me3-dependent fashion and that the induction of clearance genes is associated with a targeted reduction in H3K27me3 levels at specific clearance-gene loci in vivo.

Our data show that PRC2 plays an important role in the maintenance of region-specific functional specification of microglia. The lack of H3K27me3 leads to slow but progressive transcriptional changes in all Eed-deficient microglia subpopulations analyzed. The delay between the inactivation of PRC2 and PRC2-target gene induction in stMg, cxMg, and cbMg mimics our previous findings in neurons and is likely based on the combination of slow histone H3 protein turnover, as well as the slow catalytic activity of the H3K27me3 demethylases, Kdm6a/B11. While following a similar temporal pattern, the nature of PRC2-controlled target genes differs among microglia subpopulations. In the striatum, deficiency in Eed leads to upregulation of specific clearance-associated genes and transcription factors. Only a small fraction of PRC2-controlled genes in stMg overlap with genes induced in PRC2-deficient cbMg or cxMg (Supplementary Fig. 14d).

Aberrant intrinsic activation of clearance phenotypes in PRC2-deficient microglia in the striatum and cortex is associated with alterations in neuronal spines. The observed microglia-mediated reduction in spine numbers resembles the patterns of spine alterations caused by pathological microglia activation in mouse models of Alzheimer’s disease14. While PRC2-deficient stMg and cxMg show no upregulation of complement or pro-inflammatory genes generally associated with microglia activation during neurodegeneration11–13,14, it is still plausible that their aberrant clearance activity may lead to the enhanced engulfment of C1q–C3-tagged spines, the presence of which increases with age41. Aberrant synaptic pruning might also be mediated in a complement-independent fashion by Tyro3, Axl, and Merk receptors, which can bind exposed phosphatidylycerine on synapses42. At the same time, it is possible that the reduction in spine density reflects the downregulation of stMg-enriched surveillance genes promoting spine formation and/or maintenance in the adult brain43.

The ability of aberrant intrinsically activated microglia to cause neuronal damage is further supported by recent findings showing that cell-autonomous activation of the RAF–MEK–ERK pathway in microglia leads to the loss of synapses and the development of a severe, late-onset neurodegenerative disorder17. Notably, the RAF–MEK–ERK signaling cascade has been intrinsically linked to the regulation of PRC2 activity at several levels. While the loss of PRC2 activity is associated with reduced MEK–ERK activation in embryonic stem cells38, the inhibition of MEK–ERK activity leads to attenuated PRC2 chromatin association on PRC2-target genes in these cells42. On the other hand, increased activation of the MEK–ERK pathway has been linked to Ezh2 overexpression45. Increased Ezh2 expression and activity is associated with a genome-wide redistribution of H3K27me3 that includes the loss of H3K27me3 and the transcriptional activation of numerous PRC2-suppressed genes in these cells42. Furthermore, aberrantly activated Ezh2 was recently found to synergistically cooperate with hyperactive BRAF in the regulation of tumorigenesis46. Therefore, it is possible that neuronal damage caused by the aberrant activation of the RAS–MEK–ERK pathway in microglia may reflect a role of this pathway in PRC2-mediated aberrant microglia clearance activity.

Our studies underscore the causal contribution of aberrant microglia clearance activation to neuronal damage and complex behavioral alterations associated with neurodegenerative and psychiatric disorders. The development of novel approaches to control microglia clearance activity via targeting specific epigenetic or signaling mechanisms may have therapeutic potential for the treatment of neurological diseases. Our studies may also have implications for understanding well-described brain-region-specific susceptibilities to different neurodegenerative disorders. For example, the cerebellum appears to be particularly resistant to Aβ accumulation and neurodegeneration in Alzheimer’s disease49. It is tempting to speculate that the enhanced cbMg clearance activity may contribute to more efficient clearance of Aβ aggregates and damaged cells in the absence of inflammation.
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Methods

Animals. Mice were housed two to five animals per cage with a 12 h light/dark cycle (lights on from 0700 to 1900 h) at constant temperature (23°C) with ad libitum access to food and water. All animal protocols were approved by IACUC at Ichinomata School of Medicine at Mount Sinai and were performed in accordance with NIH guidelines.

Cx3cr1CreErt2/ mice were crossed with Cx3cr1CdivD402/+; EedloxP/loxP, Cx3cr1CreErt2/ Tg(Prox-Cre)SJ359, Tg(Vav1-Cre), Tg(Csf1r-iCre), or Tg(Csf1r-Cre/Er81*) mice to obtain a microglia-specific TRAP mouse model. Tg(Vav1-Cre), Tg(Csf1r-iCre), and Tg(Csf1r-Cre/Er81*) lines were excluded due to nonspecific targeting. The Cx3cr1CreErt2/ line was selected for all subsequent experiments due to its highly efficient (>99% of microglia) and microglia-specific Cre expression pattern throughout the brain. This was visualized by the expression of YFP driven by an internal ribosome entry site (IRES) following the CreErt2 gene. The expression of IRES-YFP did not interfere with microglia TRAP efficiency. The Tg(Prox-Cre)SJ359 line showed highly efficient (99%) of microglia) and exclusive microglia targeting in a limited number of brain regions, including the striatum and cerebellum. This line was used as an alternative TRAP line to ensure that region-specific gene expression changes were not caused by Cx3cr1 haploinsufficiency in the Cx3cr1CreErt2/ mice. Tg(Addl1h11-eGFPL10a) mice were used as astrocyte tracer. R26R.SV-tdTom reporter mice were used as a reporter of Cre expression.

Methods

37°C for 30 min with trituration through a 5ml pipette every 10 min and was stopped by addition of EDTA to a final concentration of 1 mM. The homogenate was centrifuged at 2,000g for 7 min at 4°C, the pellet was resuspended in HBSS filtered through a 100-μm mesh filter. The centrifuge step was repeated once more, and the pellet was resuspended in 70% Percoll (17-0891-02, Amersham, Amersham, UK) in phosphate-buffered saline (PBS). A 50 mL Falcon tube was layered from bottom to top with the tissue in 70% Percoll, 57% Percoll, and PBS in 4:3:1 ratio. The Percoll gradient was centrifuged at 2,000g for 1 h at 4°C in an HS-4 Swinging Bucket Rotor (Life Technologies) in a Sorval RC 6 Plus Centrifuge (Life Technologies). The interface containing the microglia was collected and resuspended in PBS with 2% normal goat serum supplemented with 10 μl/mL RNasin, 10 μl/mL Superasein and LIVE/DEAD Fixable Dead Cell Stain (Life Technologies, CA). Microglia were sorted using a BD FACSAria cell sorter (BD Biosciences, San Jose, CA, USA) by gating for high GFP signal and low LIVE/DEAD Fixable dead cells (50,000) within 2,000g for 15 min at 4°C. Genomic DNA was extracted by RNase-Free DNeasy Set (Qiagen) following the manufacturer's instructions before RNA clean-up.

Acute isolation of adult microglia by CD11b expression. Adult microglia were isolated from 3- to 4-month-old wild-type (for protein isolation) or Cx3cr1CreErt2/ mice (for phagocytosis assay) using a protocol adapted from Botteret et al. Mice were killed by CO2 asphyxiation, and brain regions were immediately removed. Tissue was enzymatically and mechanically dissociated using the Miltenyi neural dissociation kit (130-092-628, Miltenyi, Auburn, CA) following the manufacturer's recommendations. Myelin removal was performed using myelin removal beads Plus (130-099-362, Miltenyi) with 15 minutes (130-091-001, Miltenyi) following the manufacturer's recommendations. After myelin removal, mice were selected by anti-CD11b-coated microbeads (130-093-636, Miltenyi) with the QuadroMACs separator following the manufacturer's recommendations. Cells were manually counted with a hemocytometer using trypan exclusion staining and were either plated at 56,000/cm² in a 48-well plate with DMEM supplemented with 10% FBS (Sigma F4135) and 1% penicillin–streptomycin. E7 was positively stained for 4, 12, and 24 h. For the phagocytosis assay, early apoptotic Jurkat cells were labeled with 10 μM fluo-3 and 0.5 mM EDTA for 5 h for phagocytosis assay or pelleted and immediately frozen on dry ice for protein isolation.

Primary neonatal microglia culture. Preparation of primary neonatal microglial cultures was adapted from Saur et al. Microglia were dissociated from mixed glial cultures by mild trypsinization. Briefly, 0.08% trypsin + 0.35 mM EDTA at 37°C for 5 min, and resuspended in 500 μl of DAPI and placed on ice. We recorded 10,000–20,000 events were gated on the SSC-A and FSC-A to exclude debris.

Treatment of adult or neonatal microglia cultures with early apoptotic Jurkat cells (EAJ). The Jurkat human T cell line was obtained from ATCC (TIB-152). Jurkat cells were regularly tested negative for mycoplasma using MycoAlert LT-07. According to the manufacturer's instructions (MycoAlert LT-07). Jurkat cells were cultured in RPMI medium (Gibco 11875) supplemented with 10% FBS, 10 mM HEPES, and 1% penicillin–streptomycin. On the day of microglia treatment, Jurkat cells were transferred to fresh growth medium at 1 million/mL and treated with 1 μM staurosporine for 3 h. After treatment, cells were collected and washed with 15 mL cold PBS by centrifugation at 400g for 10 min. Medium or PBS were discarded using pipettes. Cells were resuspended in PBS at 10 million/mL, and induction of apoptosis was verified using FITC-Annexin V/propidium iodide kit and LSR II flow cytometer (BD Biosciences). Staurosporine-treated Jurkat cells were ~70% positive for phosphatidylserine staining and ~95% negative for propidium iodide staining, identifying the major population as early apoptotic (Supplementary Fig. 7e).

For gene expression analysis, EAJ were added to primary neonatal cbMg/stMg cultures (from 3- to 4-month-old male and female mice) that were trypsinized 24 h earlier at a 1:1 ratio for 4, 12, and 24 h. For the phagocytosis assay, early apoptotic cells were labeled with 10 μg fluorescein isothiocyanate (Thermo Fisher Scientific, P36600) per 105 cells for 1 h at room temperature (RT, 22–26°C) in the dark. The cells were then washed with PBS and resuspended in microglia medium at 10 million/mL. Primary microglia from the cerebellum and striatum of adult mice that were cultured for 5 h were given an equal number of pHrodo-labeled EAJ, events were gated on the SSC-A and FSC-A to exclude debris.

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and then on GFP+ microglia to quantify the percent of GFP+H2O2+ cells. Data were analyzed; dot plots and histograms were generated using FCS Express 6 (De Novo Software). Per experiment, minima of 434 and 453 and maxima of 3,672 and 3,087 cells (medians: 1,539 and 1,545) were counted from stMg and cbMg, respectively. Histogram plots were smoothed by 30% and normalized to peak values based on the number of gated cells. Two-way ANOVA was used to compare between brain regions and dead cell exposure.

Isolation of microglial nuclei by EACS. Microglial nuclei from different brain regions of 4–6-month-old C3xCre1+/+;Eef1a1LSL.eGFPL10a/+ (ChIP sequencing, nuclear RNA sequencing, or single nuclei RNA sequencing), 2–3-month-old C3xCre1+/+;Eef1a1LSL.eGFPL10a/+ or C3xCre1+/+;Eef1a1+/+ (western blotting) mice were isolated based on the eGFP-L10a fluorescence of newly formed ribosomes in the microglial nuclei, as described in Kricunon et al. All mice were gavaged at 4–6 weeks of age with five doses of 100 mg/kg of tamafoxin with a separation of at least 48 hours between doses. Briefly, mice were killed by CO2 asphyxiation, and brain regions were quickly dissected and homogenized in 0.25 M sucrose, 150 mM KCl, 5 mM MgCl2, 20 mM tricine, pH 7.8, with a glass dounce homogenizer (1984-10002, Kimble Chase, Vineland, NJ). For RNA isolation, the homogenate was unixed and all buffers were supplemented with 10 μL/mL RNasin (Promega) and Superasin (Applied Biosystems). For chromatin immunoprecipitation, the homogenate was cross-linked with a final concentration of 1% formaldehyde for 8 min at room temperature (RT, 22–26 °C) and the reaction was quenched with 0.125 M glycine for 5 min at RT. All buffers were supplemented with 0.15 mM spermine, 0.5 mM spermidine, and EDTA-free proteinase inhibitor cocktail (1813617001, Roche). For protein and RNA extraction, the homogenate was spun through a 29-glass dialisol cushion. The resulting nuclear pellet was resuspended in 0.25 M sucrose, 150 mM KCl, 5 mM MgCl2, 20 mM tricine, pH 7.8, supplemented with 10 μM DyeCycle Ruby (V10504, Invitrogen) and 10% donkey serum (017-000-121, Jackson Immunoresearch, West Grove, PA). Microglial nuclei were homogenized in a BD FACsAria cell sorter by gating for the lowest DyeCycle Ruby, which serves as an indicator of nuclei singlets, and high GFP signal. For single-nuclei RNA sequencing, isolated nuclei were used immediately. For bulk nuclei sequencing (50,000), protein lysate preparation (50,000), and chromatin immunoprecipitation (50,000), nuclei were pelleted at 2,000g for 15 min at 4 °C. For bulk RNA analysis, genomic DNA was eliminated using RNA-Free DNase Set (Qiagen) following the manufacturer’s instructions before RNA clean-up.

## Quantitative PCR (qPCR)
Quantitative PCR (qPCR) was performed on tissue using SYBR green and a Stratagene Mx3000P real-time PCR system. RT-PCR was performed in triplicate using appropriate controls. Primers were used to amplify known microglial marker genes and housekeeping genes. The cycle numbers for each gene were calculated. Unpaired two-tailed t tests were used to compare biological replicates.

### Bulkle RNA sequencing
RNA clean-up from isolated microglial cells (50,000), TRAP samples, and 5% of the unreadable fractions from TRAP samples was performed using RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. RNA integrity was assayed using an RNA Pico chip on a Bioanalyzer 2100 (Agilent, Santa Clara, CA), and only samples with RIN > 9 were considered for subsequent analysis. Double-stranded cDNA was generated from RNA using TURBO DNA-free Kit (Life Technologies, CA) and Superscript III reverse transcriptase (Life Technologies, CA). Double-stranded cDNA was used for the amplification of all gene expression using the following gene-specific primers.

```
| Gene    | Forward Primer                  | Reverse Primer                  |
|---------|---------------------------------|---------------------------------|
| Fcrls   | ACTTCACTGAGATGCTGCTTTG         | CTCATTGCAATACTGGTGGTCC         |
| Tnfa    | CACTACCAACAGCGAATGAC           | GACCTTGGCCACAGATAACC          |
| Mx1     | CTGCTACGAGTGGTGGTGG            | TCAGTGCCTGGTGGTGCTA           |
| Ifnb1   | GAGCAGGGACATGATAGGGA           | GACAGGGGTGACATGGCATC          |
| Tmem119 | GTGAGGAGGAGGTGAGGAT            | ATGTTCTTGTTAGTTGTTG          |
| Asb2    | CCAGTGTTGTCAGCTTCTT            | CTCCACACACATCAGACAC           |
| Fcrls   | TCCTTTCTTCTTCTTCTTCTT          | TCATTGCAATACTGGTGGTCC         |
| Tnfa    | CACTACCAACAGCGAATGAC           | GACCTTGGCCACAGATAACC          |
| Mx1     | CTGCTACGAGTGGTGGTGG            | TCAGTGCCTGGTGGTGCTA           |
| Ifnb1   | GAGCAGGGACATGATAGGGA           | GACAGGGGTGACATGGCATC          |
| Tmem119 | GTGAGGAGGAGGTGAGGAT            | ATGTTCTTGTTAGTTGTTG          |
| Asb2    | CCAGTGTTGTCAGCTTCTT            | CTCCACACACATCAGACAC           |
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## Bioinformatic analysis of bulk sequencing
###reads were mapped to the mouse genome (mm9) using the TopHat2 package (v2.1.0). Reads were counted using HTSeq-count (v0.6.0) against the Ensembl v67 annotation. The read alignment, read count, and quality assessment using metrics, such as total mapping rate and mitochondrial and ribosomal mapping rates, were done in parallel using an in house workflow pipeline called SPEZtRA. The raw counts were processed through a variance stabilizing transformation (VST) procedure using the DESeq2 package to obtain transformed values that are more suitable than the raw read counts for certain data mining tasks. Principal component analysis (PCA) was performed on the top 500 most-variable genes across all samples, visualized on the VST data using the R package ggplot2. Additionally, hierarchical clustering was used to assess the outliers among the NCBI RefSeq transcripts using the VST data. Two-way ANOVA was performed on the count data of entire gene transcripts using the DESeq2 package (v1.2.3). For the comparison of microglia- to astrocyte-TRAP a cutoff of adjusted P value < 0.05, fold-change > 5, and mean expression > 30 (DESeq2; n = 2 mice per cell-type and per region; 3- to 6-month-old mice) was applied. To represent the most highly expressed cell-type-specific genes, a mean expression cutoff of >125 was used. To obtain the top 50 genes for these cell types, a fold-change cutoff of >20 was used. Genes with a log2 transformed mean expression are shown as bar graphs. For the comparison of microglia-TRAP to isolated microglia, a cutoff of adjusted P < 0.05, fold-change > 10, and mean expression > 30 was applied (DESeq2; n = 2 mice per method and brain region; 3- to 6-month-old males). Additionally, for the TRAP samples, an enrichment cutoff of P < 0.05 and fold-change > 2 over their respective unbound fraction was applied. For microglia brain-region-specific
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TRAP comparisons, first an enrichment cutoff of \( P < 0.05 \) and fold-change \( > 2 \) for each TRAP group over its respective unbound fraction was applied. Then a cutoff of \( P < 0.05 \), fold-change \( > 2 \), and mean expression \( > 0.5 \) between regions was applied (DESeq2 - median absolute deviation (MAD) and age/sex matched control samples). Log2 fold-change \( > 2 \) for each TRAP sample over its unbound fraction was applied. Comparisons for Cx3cr1CreErt2/+ and Eef1a1loxP/loxP mice were carried out with age- and sex-matched control Cx3cr1CreErt2/+ and Eef1a1loxP/loxP mice. We used 9-month-old control mice in heatmaps and box-and-whisker plots.

For all comparisons, non-protein-coding genes were ignored. All of the volcano, MA, and scatter plots and histograms for bulk sequencing were made using R (v3.1.1; https://www.R-project.org). For all heatmaps, expression of each gene in log2(RPKM) was normalized to the mean across all samples (BH correction) GO annotations for biological processes and are represented in a pie chart. Comparisons for Cx3cr1CreErt2/+ and Eef1a1loxP/loxP mice were carried out as follows. Raw bulk RNA-seq reads were aligned using STAR (2.4.0a) on GRCm38 (mm10) and quantitated on genic features using feature (counts for all TRAP, FACS, and nuclear RNA bulk RNA-seq samples). Differential expression analysis was performed using DESeq2 (v1.25.0). FDR-corrected \( P \) values for comparisons between single-cell and bulk and also significantly differentially expressed (FDR < 0.05), the Pearson correlation coefficients for the log-fold changes on the regional contrast were computed.

Chromatin immunoprecipitation (ChiP). ChiP was performed as previously published \(^1\) using LowCell ChiP kit (S海峡-Ramon, Diagene, Denville, NJ). For in vivo ChiP, we used 500,000 fixed nuclei (1% formaldehyde for 8 min at RT and quenched with 0.125 M glycine for 5 min at RT) isolated from the striatum and the cerebellum of 25 Cx3cr1CreErt2/+Eef1a1loxP/loxP (6- to 9-month-old males and females) or 500,000 fixed nuclei isolated from the cortex of 8 Cx3cr1CreErt2/+Eef1a1loxP/loxP (6- to 9-month-old male and female mice). Samples were sonicated for 13 cycles of 30 s on/30 s off at 4°C on high power to achieve fragments of sizes ranging from 200 to 500 bp with a Bioruptor (Diagene). After removal of an input sample equivalent to 1% of each ChiP reaction, sheared chromatin was incubated with 15 μg antibody against H3K27me3 (06999; Millipore) overnight at 4°C. After incubation, chromatin was immunoprecipitated by 50 μl Protein G magnetic beads (kch-818-220, Diagene) for 2 h at 4°C and immediately cleaned with the IPure kit (AL-100-0100, Diagene) according to the manufacturer’s instructions with the addition of Protease K treatment (03151828001, Roche) for 1 h at 55°C after the de-crosslinking step. Immunoprecipitated chromatin was further purified with two rounds of Dynabeads and are stored at −80°C in acetone precipitation. ChiP-sequencing libraries were prepared using the ChiP-Seq DNA sample preparation kit (IP-102-1001, Illumina) using an adaptor oligo dilution of 1:25 for all samples. Prepared samples were amplified onto flow cells using cBOT per manufacturer protocol and sequenced on the HiSeq platform for 50 cycles. Raw sequencing data was processed by using Illumina bcl2fastq2 Conversion Software v2.17.

Bioinformatic analysis of H3K27me3 ChiP sequencing. The ChiP-seq data was first checked for quality using the various metrics generated by FastQC (v0.11.2; http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The sequencing reads were then aligned to the mouse mm9 genome using the default settings of Bowtie (v2.2.0). Only uniquely mapped reads were retained, and the alignments were subsequently filtered using the SAMtools package (v0.1.19) to remove duplicate reads. Peak-calling was performed using MACS (v2.1.1) \(^2\) with default settings. Annotation of called peaks and differential regions to their genomic features (promoters, gene bodies, intergenic, etc.) was performed using region-analysis (v0.1.2) \(^3\), and read alignment profile plots and heatmaps were generated using ngsplot (v2.47) \(^4\) and Multiple Experiment Viewer 4.8.

To select for H3K27me3 genes in cbMg, stMg, and cxMg, the log(fold change) (log2fc) of ChiP signal over the corresponding input signal was calculated for each gene for each time point using the “mean of the mean” approach. The 1 kb for each gene. A cutoff of log2fc > 0 was used to define H3K27me3 genes for stMg, cbMg, and cxMg. To compare H3K27me3 targets between cbMg and stMg, a goodness-of-fit G-test was applied to the normalized read counts mapping within TSS ± 1 kb of each gene. A cutoff of \( P < 0.05 \) was used to define genes differentially marked with H3K27me3 in cbMg and stMg. Correlation coefficient between H3K27me3 enrichment and TRAP-seq enrichment was calculated by the Pearson method using R.

Protein preparation and western blot analysis. Pellets containing 50,000 microglia nuclei or 75,000 microglia (from a 2- to 3-month-old male or female mouse) were resuspended in 1% SDS (Ambion) supplemented with protease inhibitors (Complete, Roche) and 100 mM DTT. Cell lysis was carried out on ice for 30 min, followed by sonication in a Bioruptor on high power 30 s on/30 s off cycles for a total of 10 cycles at 4°C and boiled for 10 min in 1X LDS sample buffer (Invitrogen) supplemented with DTT to a final concentration of 200 mM (Sigma). Lysates were separated on 4–12% NuPAGE Bis-Tris or Bolt 4–12% Bis-Tris Plus precast denaturing gels (Invitrogen) and transferred onto PVDF membranes in Mini Gel Tank at 25 V for 2 h. Membranes were blocked with 5% milk–TBST for 1 h at RT. Membranes were then probed with primary antibodies diluted in 5% milk–TBST solution overnight at 4°C. Primary antibodies: anti-AXL (1:500, sc-1097, Santa Cruz), or 1:1000, ab227871, Abcam), anti-FSCN1 (1:5000, ab227872, Abcam), anti-MRCI (1:500, AF2353, R&D Systems), anti-LC3B (1:1000, 2775, Cell Signaling), anti-H3K27me3 (1:1000, 67-449, Millipore), anti-ACTB (1:2000, 4970, Cell Signaling), and anti-Histone H3 (12,000, ab1791, Abcam) and anti-Histone H4 (12,000, ab1791, Abcam) were then washed and probed with horseradish-peroxidase-conjugated anti-mouse (Life Technologies, 31438, 1:10000), anti-rabbit IgG secondary antibody (GE, NA934V, 1:10000), or anti-goat IgG antibody (Life Technologies, P31400, 1:1000) for 1 h at RT. Membranes were developed using enhanced chemiluminescence substrate (PerkinElmer, USA, 590949326) and exposed on film.
articles or imaging using ChemiDoc MP Imaging System and Image Lab software (BioRad). Exposed films were scanned, and protein bands were quantified using ImageJ Software (NIH). H3K27me3 quantities were normalized to total histone H3 for each lane. AXIN1, MIR1, LC3B, and FSCN1 were removed and dehydrated in 5%, 15%, and 30% (120 mg/kg) and xylazine (24 mg/kg) and perfused transcardially with 10 mL PBS microscope slides (Fisher Scientific), which were stored at AB947, Millipore)13, AXL (1:100, AF854, R&D Systems)25, cCASP3 (1:400, #9661, Teraxys, CA; NeuN (1:500, MB377, Millipore, Billerica, MA))25, GFRalpha2 (1:100, G8395, Sigma)25, OLG2 (1:250, MAB950, Wako Chemicals, Richmond, VA)97, P2RY12 (1:5,000, kind gift from O. Botsford, University of Washington, Seattle, WA)10, and CD11b (1:1,000, MCA711GT, Bio-Rad, Hercules, CA). Male and female 4-/6-month-old wild-type; P7 wild-type; 6–8 weeks; 2-/12-month-old C3H/HeN; and C57BL/6J (Jung);Eef1a1LSL.eGFPL10a/+ and C57BL/6J (Jung);EedloxP/loxP mice were anesthetized with ketamine (120 mg/kg) and xylazine (24 mg/kg) and perfused transcardially with 10 mL PBS followed by 40 mL 4% paraformaldehyde (Electron Microscopy Sciences) as previously described25. Fixed brains were removed and dehydrated in 5%, 15%, and 30% sucrose in PBS. Following dehydration, brains were frozen in Neg 50 (Life Technologies) on dry ice and stored at −80°C until further processing. Brains were cut using a cryostat, and 25–35 μm sections were mounted on Superfrost Plus microscope slides (Fisher Scientific), which were stored at −80°C until staining. Fixed-frozen brain tissue from Axl+/− Mertk−/− mice were generously provided by Dr. G. Lemke.

Slides were washed with PBS, permeabilized with PBS + 0.2% Triton X-100 (PBST) and incubated with Image-IT FX Signal Enhancer (Invitrogen) followed by blocking with 2% normal goat serum in PBST. Slides were incubated with primary antibodies in 2% normal goat serum in PBST overnight at 4°C. Primary antibodies: GFP (1:2,000, ab6556, Abcam, Cambridge, MA),25 IBA1 (1:500, 019-19741, Wako Chemicals, Richmond, VA)97, P2RY12 (1:5,000, kind gift from O. Botsford, University of Washington, Seattle, WA)10, and CD11b (1:1,000, MCA711GT, Bio-Rad, Hercules, CA). Male and female 4-/6-month-old wild-type; P7 wild-type; 6–8 weeks; 2-/12-month-old C3H/HeN; and C57BL/6J (Jung);Eef1a1LSL.eGFPL10a/+ and C57BL/6J (Jung);EedloxP/loxP mice were anesthetized with ketamine (120 mg/kg) and xylazine (24 mg/kg) and perfused transcardially with 10 mL PBS followed by 40 mL 4% paraformaldehyde (Electron Microscopy Sciences) as previously described25. Fixed brains were removed and dehydrated in 5%, 15%, and 30% sucrose in PBS. Following dehydration, brains were frozen in Neg 50 (Life Technologies) on dry ice and stored at −80°C until further processing. Brains were cut using a cryostat, and 25–35 μm sections were mounted on Superfrost Plus microscope slides (Fisher Scientific), which were stored at −80°C until staining. Fixed-frozen brain tissue from Axl+/− Mertk−/− mice were generously provided by Dr. G. Lemke.

Briefly, slides (prepared for immunofluorescence) from 4-month-old wild-type mice were sectioned at 5 μm apart originating from the soma, and intersections of each cell were spaced 10 μm apart. The percent area covered by a single microglia was calculated by dividing the percent area covered by microglia in an image by the total number of microglia in the same image. For each animal, 2–3 mice were used to generate at least 3 samples per condition. The percent area of CD68 (lysosomes), CD74, MHCI, C3, and YFP in brain sections from 4-/6-month-old wild-type and Cx3cr1GFP+/− or 12-month-old Cx3cr1GFP+/−;EedloxP/loxP mice was measured with ImageJ (National Institutes of Health) using the following image processing steps: (i) binary image was created from the raw single-channel images, and (ii) the ‘measure’ function was used to determine the percentage area coverage of CD68, ApoE, AXL, CD74, MHCI, C3, and YFP at 5–11 images from n = 3–6 mice. Percent area covered by a single microglia was calculated by dividing the percent area covered by microglia in an image by the total number of microglia in the same image. We analyzed 20-μm z-stacked confocal images taken with 40X/1.3 objective at 1X zoom.

Imaging quantification. The percent area of CD68 (lysosomes), CD74, MHCI, C3, and YFP in brain sections from 4-/6-month-old wild-type and Cx3cr1GFP+/− or 12-month-old Cx3cr1GFP+/−;EedloxP/loxP mice was measured with ImageJ (National Institutes of Health) using the following image processing steps: (i) binary image was created from the raw single-channel images, and (ii) the ‘measure’ function was used to determine the percentage area coverage of CD68, ApoE, AXL, CD74, MHCI, C3, and YFP at 5–11 images from n = 3–6 mice. Percent area covered by a single microglia was calculated by dividing the percent area covered by microglia in an image by the total number of microglia in the same image. We analyzed 20-μm z-stacked confocal images taken with 40X/1.3 objective at 1X zoom.

DAP1+ lysosomes in microglia from 4-/6-month-old wild-type mice were measured from 20-μm z-stacked confocal images taken with 40X/1.3 objective at 1X zoom with Imaris (Bitplane, UK) using the following image processing steps: (i) microglia and lysosomes were reconstructed in 3D; (ii) DAPI signal outside the PBS was excluded; (iii) DAPI channel was reconstructed in 3D; and (iv) microglia with overlapping lysosome and DAPI surface were manually counted to exclude extracellular nuclei (>200 cells per region from n = 3 mice). Microglia cell bodies from wild-type- and Cx3cr1GFP+/−;EedloxP/loxP mice, and control cells were counted manually from 3–20 20-μm z-stacked confocal images taken with 40X/1.3 objective at 0.6X zoom from n = 3 mice per condition. C3AP3, TUNEL+, and Kd67+ cells were counted manually from Oil Red B stain, striatum, somatosensory cortex, and cerebellum from 5 brain sections of n = 3 or 4 control, PLX-treated, and/or Axl−/−− Mertk−/− mice. Cells with somas filled by C3AP3 and with pyknotic nuclei were counted as C3AP3 cells. Cells with nuclei filled with Kd67 were counted as Kd67+ cells. Cx3cr1Gfp+/− cells were counted from 197–284 GFP and 50–55 GFP+ cells from n = 2 mice per genotype. Counting for R1A and R2A was done manually (50–73 pm). All images were acquired under identical conditions.

Using Imaris software, we reconstructed 20-μm z-stacked confocal images taken with 40X/1.3 objective at 1X zoom from n = 3–6 mice per condition, and DAPI+, CD68+, and 200 IBA1+ microglia counted manually from n = 3 wild-type mice.

The percentage of NeuN cells was determined using ImageJ (National Institutes of Health) using the following image processing steps: (i) DAPI-stained nuclei were segmented by automated thresholding and particle analysis; (ii) nuclear masses was then detected using the magic wand and transferred onto the NeuN-stained image by restore selection; (iii) fluorescence intensity for each cell was measured and tabulated, and the percentage of NeuN+ cells was calculated using a mean intensity threshold > 15 (5–6 images from n = 2–3 mice).

Morphological analysis of microglia. We obtained 20-μm z-stack images from 4-/6-month-old wild-type mice or 12-month-old Cx3cr1GFP+/−;EedloxP/loxP and Cx3cr1GFP+/−;EedloxP/loxP mice on a Zeiss LSM780 Confocal Microscope with 40X/1.3 objective at 1X zoom at 2 μm intervals and collapsed into 2D images. Neuronal-rich Explorers (MFB Bioscience, Williston, VT USA) was used to manually trace and generate analyses of microglia from the striatum and cerebellum. One microglial cell was selected using 0.045 μm2 of the cell body and soma were traced, and the thickness of each process was adjusted along its length. The number of primary processes and number of branch tips were measured. To account for cell complexity, Sholl analyses were performed for each cell. Concentric circles were spaced 10 μm apart originating from the soma, and intersections of each cell with concentric circles were measured. We used n = 5 control animals for brain region comparisons; n = 3 mice/genotype were used to compare 15-month-old control and Cx3cr1Gfp+/−;EedloxP/loxP mice.
Golgi analysis. Coronal brain sections of 15-month-old male Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls were stained using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Inc., Ellicott City, MD) following the manufacturer’s recommendations. We dissolved 200 mg/ml of EtoH in a vibrotome (Leica). All sections were visualized on a Zeiss Apotome 2 Microscope (Carl Zeiss, Thornwood, NY) and 30 dendrites from n = 3 mice for each genotype were analyzed using NeuroLucida360 (MBI Bioscience).

Statistical analysis of imaging data. Gender ratios and ages were matched within control and treatment groups. For the comparisons of brain regions, a paired t test was performed to compare two groups and one-way ANOVA was used for comparisons with more than two groups. For control vs. Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice comparisons, an unpaired t test was performed.

Behavioral analyses. All behavioral analyses were performed during the 0700–1900 light cycle as previously described<sup>106,108</sup>. All subjects correspond to data points within two s.d. from the sample mean, and no subjects were excluded from the behavioral analyses. For all behavioral experiments, a mixed population of males and females with sex- and age-matched corresponding littermate control animals were used. All mice were gavaged at 4–6 weeks of age with five doses of 100 mg/kg of tamofoxifen with a separation of at least 48 h between doses. A cohort of Cx3cr1<sup>fl/fl</sup> and their respective Cx3cr1<sup>+/+</sup> wild-type littermate controls were tested on selected behavior experiments to exclude potential behavioral alterations due to Cx3cr1 haploinsufficiency in Cx3cr1<sup>+/+</sup>; Eed<sup>loxP/loxP</sup> mice. Male and female mice were used in experiments in 70% males and 30% females to ensure uniform distribution of ages and sexes in each group. All procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC at Icahn School of Medicine at Mount Sinai.

Open field analysis. Locomotion and exploratory behavior for 3-month-old (n = 5/genotype) and 15-month-old (n = 12/genotype) Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their Cx3cr1<sup>+/+</sup>; Eed<sup>loxP/loxP</sup> littermate controls, and for 12-month-old PLX-treated or control mice (n = 8 × 2, 13, respectively), were analyzed by open field analysis as previously described<sup>20</sup>. For thigmotaxis, a 2 × 2 section in an 8 × 8 grid was designated as ‘center’ and the rest ‘periphery’. The ratio of time spent in center versus periphery in the first 10 min was analyzed.

Rotarod analysis. Balance and motor function of 3-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 5/genotype), and of 15-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 11 control and 7 mutant mice), were measured using the standard accelerated rotarod test (Med Associates, St. Albans, VT) with rotation speeds of 4–40 rpm within 5 min. Scoring using Rota-Rod Software and analysis was carried out as in Schafer et al.<sup>20</sup>

Cocaine induced locomotor sensitization. Eighteen-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> and their respective littermate controls (n = 12/genotype) were habituated in the locomotor activity apparatus (clear Plexiglas 40 × 40 × 30 cm open-field arena) for 30 min, then injected intraperitoneally with 10 mg/kg cocaine (Sigma, dissolved in 0.9% saline) and placed back into the apparatus for 60 min after injection. Activity in the open-field was quantified by a computer-operated Photobeam activity system (AccuScan Instruments, Columbus, OH). Mice were recorded for the total distance moved (cm), number of vertical episodes (rearing), and distance moved in the center of the arena (cm). Data were collected at 5- to 10-min intervals over 90-min test sessions. The protocol was repeated at 48-h intervals. The ratio of total distance traveled on day 7 of cocaine treatment over total distance on day 1 is used for representation.

Elevated plus maze. The elevated plus maze test was used to determine the unconditioned response to a potentially dangerous environment. Anxiety-related behavior was measured by the degree to which the rodent avoided the open arms of the maze. Eighteen-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 4 control, 5 mutant mice); 15-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 10 genotype); 15-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 8 control, 9 mutant mice); or 12-month-old chronic PLX-treated or control mice (n = 10 per group) were placed at the junction of the elevated four-arm maze, in which two arms are open and two are enclosed. The number of times the animal entered each of the arms and the time spent in each arm was recorded for 5 min by the EthoVision video-tracking system (Noldus Information Technology Inc., Leesburg, VA). Total arm entries, percentage of open arm entries, and percentage of time spent in the open arms were calculated.

Novel object recognition. Fifteen-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> and their respective littermate controls (n = 10/genotype); or 15-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> and their respective littermate controls (n = 9/genotype) were given two habituation sessions in which they were allowed to explore the apparatus (without objects) for 10 min. On the object trial, the mouse was placed into the box with two identical objects and allowed to explore for 10 min (training). The time spent by the animal exploring each object, and the time spent by the animal exploring both objects and the box, were measured by the EthoVision video-tracking system (Noldus Information Technology Inc., Leesburg, VA). The box was then removed to its home cage, and 1 h after the training it was placed in the box for the retention test and allowed to explore the objects for 10 min with one of the objects used for training and a novel object.

Fear conditioning. In a standard fear-conditioning experiment (Med Associates), the test chamber (neutral context) was made of clear Plexiglas. The bottom of the test chamber was a grid floor used to deliver a mild electric foot shock. The test chamber was placed inside a sound-attenuated chamber. One mouse was placed in the test chamber (house lights on) and allowed to explore freely for 200 s. A white noise (80 dB), which served as the conditioned stimulus, was then presented for 18 s, followed by a mild (2 × 0.5 mA) foot shock, which served as the unconditioned stimulus. The same sequence of auditory cue–shock pairing was repeated every 2 times with 2-min intervals. The mouse was removed from the chamber 30 s later and returned to its home cage. Freezing behavior was recorded every 10 s during the time spent in the test chamber. Chambers were cleaned with ethanol between experiments. Twenty-four hours later, the mouse was placed back into the test chamber for 3 min, and the presence of freezing behavior was recorded (context test). Two hours later, the mouse was tested for its freezing response to the auditory cue. Environmental and contextual cues were changed for the auditory cue test: a white Plexiglas circular insert was placed in the chamber to alter its shape, spatial cues and color; the grid floor was covered with white Plexiglas; and the chamber was wiped with isopropanol to alter the smell. The auditory cue test was divided into two phases. During the first phase, freezing behavior was recorded for 99 s in the absence of the auditory cue. In the second phase, the auditory cue was turned on, and freezing was recorded for another 60 s. The number of freezing intervals for each test was measured by Video Freeze Software (Med Associates) and converted to a percentage of freezing value per component time. Fifteen-month-old Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> and their respective littermate controls (n = 11 control and 10 mutant) were tested.

Seizure and survival curves. Frequency of seizure episodes were counted at magnitudes of stages 4–5 of the modified Racine’s seizure scoring system<sup>107</sup> in aged Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice, in Cx3cr1<sup>−/−</sup> littermate mice, and in PLX-treated mice in response to handling or exposure to novel environments. Dates of first observed seizure in the home cage for individual Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> mice and their respective littermate controls (n = 12 mutants and n = 14 controls), Cx3cr1<sup>−/−</sup> littermate mice and their respective littermate controls (n = 9 genotype), or PLX- and control diet-treated mice (n = 15 per group) were recorded and plotted as Kaplan–Meier curves. Dates of death in the home cage for Cx3cr1<sup>fl/fl</sup>; Eed<sup>loxP/loxP</sup> and their respective littermate controls (n = 28/genotype) were recorded and plotted as Kaplan–Meier survival curves.

Statistical analysis of behavior data. For two-group comparisons with equal variance as determined by the F-test, an unpaired Student’s t test was used. For the analysis of novel object recognition experiments, a two-tailed paired t test was used. For the analysis of accelerated rotarod analysis and locomotor sensitization, two-way ANOVA with repeated measures was used. For seizure and survival curves, a log-rank Mantel–Cox test was used. All behavioral data except rotarod, locomotor sensitization, and seizure/survival curves were represented as bar graphs with individual data points with mean ± standard error of the mean (s.e.m.). Rotarod and locomotor sensitization data were represented with mean ± s.e.m. Seizure and survival data were represented as Kaplan–Meier curves.

Statistical analysis. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications<sup>107,108</sup>. Data collection was randomized for all experiments. Experimenters were blinded to genotype during imaging and behavioral experiments. Gene expression and protein analyses were not performed blind to the conditions of the experiments. Statistical analyses were analyzed using GraphPad Prism V5.01, and significance was determined at P < 0.05. Normal distribution was assessed by the Shapiro–Wilk (SW) normality test. Grubbs’ test was used to identify outliers. Variance was determined by the F-test. All statistical analyses were two-tailed. Normally distributed data with unequal variance was analyzed with Welch’s correction. Non-normally distributed data was analyzed by Mann–Whitney U test. For two-group comparisons with equal variance, a paired or unpaired Student’s t test was used and clearly indicated in Methods and figure legends.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Accession Codes. Sequence data is available at NCBI GEO: GSE108356.

Code availability. All code used in this manuscript has been previously published, is described in the Methods section, and is also available from the corresponding author upon reasonable request.
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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a: Confirmed
- □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- □ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- □ The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- □ A description of all covariates tested
- □ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- □ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- □ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- □ Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- Data collection
  - RNA/DNA quality check: 2100 Expert Software
  - RNA/DNA sequencing: NextSeq System Suite, HiSeq Control Software, Illumina bcl2fastq2 Conversion Software v2.17
  - qPCR: StepOne Software
  - Single nuclei RNA sequencing: C1 System Software
  - Imaging: Zen 2011 software
  - Flow cytometry: FACSDiva v8.0.1
  - Mouse behavior: EthoVision, Home Cage photobeam, Video Freeze
  - Software and script usage is described in the method section.
Data analysis
Bulk RNA seq bioinformatics: TopHat2, HTSeq-count (v0.6.0), SPEctRA, DESeq2 package, R (v3.1.1), Enrichr, Ingenuity Pathway Analysis
Single cell sequencing bioinformatics: STAR (2.4.0a), edgeR (3.10.0), Monocle (1.2.0) toolkit
ChIP-seq bioinformatics: FastQC (v0.11.2), Bowtie v0.12.7, SAMtools package (v0.1.19), MACS (v2.1.1), region-analysis (v0.1.2), ngsplot (v2.47)
Published data analysis: GEO2R
Data representation: Multiple Experiment Viewer 4.8 (v.10.2), IGV Tools, GraphPad Prism v5.01
Imaging analysis: Imaris, Neurolucida Explorer, Neurolucida360, ImageJ, IncuCyte, Image Lab
Flow cytometry analysis: FCS Express 6
Software and script usage is described in the method section.

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Flow cytometry analysis: FCS Express 6
Software and script usage is described in the method section.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data
Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Accession Codes
Sequence data can be downloaded from the National Center for Biotechnology Information Gene Expression Omnibus Accession #GSE108356.

Field-specific reporting
Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences
☑ Behavioural & social sciences
☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

Sample size
We do not include a justification of sample size for this study. We used the minimum number of animals needed to reliably detect the expected effect size with an alpha rate set at .05 in a standardly powered experiment and based on extensive laboratory experience and literatures in the field.

Data exclusions
For all behavioral data Grubbs test was used to identify significant outliers and these outliers were excluded.

Replication
All attempts of replications were successful. Each experiment was reproduced with similar results. Reproducibility has been either indicated in the Figure Legends, or shown as a quantification.

Randomization
For all molecular, imaging and behavioral experiments, animals were randomly assigned to groups.

Blinding
Experimenter were blinded during imaging and behavioral experiments. Gene expression and protein analyses were not performed blind to the conditions of the experiments.

Reporting for specific materials, systems and methods
Involved in the study

- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Involved in the study

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Obtaining unique materials

All unique materials used are readily available from the authors.

**Antibodies**

**Antibodies used**

- Immunostaining: GFP (1:2000, ab6556 and 1:500 ab13970 Abcam, Cambridge, MA), IBA1 (1:500, 019-19741, Wako Chemicals, Richmond, VA), CD68 (1:250, MCA1957, Biorad, Hercules, CA), NeuN (1:500, MAB377, EMD Millipore, Billerica, MA), GFAP (1:500, G3893, Sigma), OLIG2 (1:250, MABN50, EMD Millipore), CD11B (1:1000, MCA711GT, Biorad, Hercules, CA), H3K27me3 (1:500, C36B11, Cell Signaling), CD74 (1:50, sc-5438, Santa Cruz), cCASP3 (1:400, #9661, Cell Signaling), P2RY12 (1:5000, kind gift from O. Butovsky), MHCII (1:200, ab23990, Abcam), ApoE (1:100, AB947, Millipore), AXL (1:100, AF854, R&D Systems), Ki67 (1:200, ab16667, Abcam), Alexa Fluor 488-labeled anti-mouse IgGs (H+L) (1:500, Life Technologies, A32723), Alexa Fluor 488-goat anti-rat IgGs (H+L) (1:500, Life Technologies, A-11066), Alexa Fluor 488-goat anti-chicken IgGs (H+L) (1:500, Life Technologies, A-11039), Alexa Fluor 488-goat anti-rabbit IgGs (H+L) (1:500, Life Technologies, A-11008), Alexa Fluor 568-labeled goat anti-mouse IgGs (H+L) (1:500, Life Technologies, A-11004), Alexa Fluor 568-goat anti-rat IgGs (H+L) (1:500, Life Technologies, A-11077), Alexa Fluor 568-goat anti-rabbit IgGs (H+L) (1:500, Life Technologies, A-11011), Alexa Fluor 568- donkey anti-goat IgGs (H+L) (1:500, Life Technologies, A-11057), and Alexa Fluor 647-goat anti-rat IgGs (H+L) (1:500, Life Technologies, A-21247)

- ChIP: H3K27me3 (1:100, 07-449, Millipore)

- Immunoblotting: AXL (1:500, sc-1097, Santa Cruz), AXL (1:1000, ab227871, Abcam), FSCN1 (1:5000, ab126772, Abcam), MRC1 (1:500, AF2535, R&D Systems), LC3B (1:1000, 2775, Cell Signaling), H3K27me3 (1:1000, 07-449, Millipore), ACTB (1:20,000, ab8227, Abcam), Histone H3 (1:2,000, ab1791, Abcam), horseradish-peroxidase-conjugated anti-mouse (Life Technologies, 31438, 1:10,000), horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody (GE, NA934V, 1:10,000), and horseradish-peroxidase-conjugated anti-goat IgG antibody (Life Technologies, P131400, 1:10,000)

- TRAP: mouse monoclonal anti-GFP (9F7 and I9C8, Antibody & Bioresource Core Facility Memorial Sloan Kettering Cancer Center)

**Validation**

- GFP (ab6556), GFAP (ab13970), IBA1 (019-19741), CD68 (MCA1957), NeuN (MAB377), CD11B (MCA711GT), H3K27me3 (C36B1), cCASP3 (#9661), MHCII (ab23990), and Ki67 (ab16667) are verified for immunostaining in mouse on the company websites, with the exception of GFP which is an exogenously-expressed protein but endogenous to A. victoria.

- GFAP (G3893) was verified for immunostaining in mouse by LeComte et al. 2015. OLIG2 (MABN50) was verified for immunostaining in mouse by Moyon et al. 2016. CD74 (sc-5438) was verified for immunostaining in mouse by Sun Hui et al. 2015. P2RY12 and ApoE (AB947) were verified for immunostaining in mouse by Krasemann et al. 2017. AXL (AF854) was verified for immunostaining in mouse by Fourgeaud et al. 2016. H3K27me3 (C36B1) for immunostaining in mouse was verified by von Schimmelmann et al. 2016 using Ezh1/Ezh2-deficient mouse neurons.

- H3K27me3 (07-449) was verified for ChIP in mouse on the company website and by von Schimmelmann et al. 2016 using Ezh1/Ezh2-deficient mouse neurons.

- AXL (ab227871), FSCN1 (ab126772), MRC1 (AF2535), LC3B (2775), H3K27me3 (07-449), ACTB (ab8227), and Histone H3 (ab1791) are verified for immunoblotting in mouse on the company websites.

- AXL (sc-1097) was verified for immunoblotting in mouse by Fourgeaud et al. 2016. H3K27me3 (07-449) and Histone H3 (ab1791) were verified for immunoblotting in mouse was verified by von Schimmelmann et al. 2016 using Ezh1/Ezh2-deficient mouse neurons.

- mouse monoclonal anti-GFP (9F7 and I9C8, Antibody & Bioresource Core Facility Memorial Sloan Kettering Cancer Center) were verified for TRAP in Heiman et al. and Doyle et al. 2008.
### Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**
- Jurkat human T cell line from ATCC (TIB-152)

**Authentication**
- None of the cell lines used have been authenticated.

**Mycoplasma contamination**
- All cell lines tested negative for mycoplasma contamination.

**Commonly misidentified lines**
- No commonly misidentified cell lines were used.

### Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

**Laboratory animals**
- Mice of C57Bl/6 background were used. For imaging and behavior experiments, young adult 2-4 mo or aged 17-20mo male and female mice were used. For gene expression analyses 3mo male, 6mo male, and 9 mo female mice are used. For TRAP proof of principle 3-6mo male mice were used. For phagocytosis assay and protein analysis, 2-4mo male and female mice were used. For ChIP sequencing assay adult 6-9mo male and female mice were used. For single nuclei sequencing adult 4-5mo female mice were used. Whenever male and female mice were used together, the ratio in control and treatment groups were equal.

**Wild animals**
- This study did not involve wild animals.

**Field-collected samples**
- This study did not involve samples collected from the field.

### ChIP-seq

#### Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

#### Data access links

- May remain private before publication.

#### Files in database submission

- CB_H3K27me3_ChIP
- CB_input
- ST_H3K27me3_ChIP
- ST_input
- H3K27_MGCX_Nolindex_L002_R1.fastq.gz
- Input_MGCX_Nolindex_L003_R1.fastq.gz
- 24_ST_ACAGTG_L007_R1_001.fastq.gz
- 22_ST_ACAGTG_L005_R1_001.fastq.gz
- 72_ST_GTGAAA_L005_R1_001.fastq.gz
- 77_ST_GTGAAA_L007_R1_001.fastq.gz
- 77_CB_GTGAAA_L006_R1_001.fastq.gz
- 72_CB_GTGAAA_L001_R1_001.fastq.gz
- 22_CB_ACAGTG_L001_R1_001.fastq.gz
- 24_CB_ACAGTG_L006_R1_001.fastq.gz
- 38-ST_S1_R1_001.fastq.gz
- 48-ST_S2_R1_001.fastq.gz
- 70-ST_S3_R1_001.fastq.gz
- 74-ST_S4_R1_001.fastq.gz
- 77-ST_S3_R1_001.fastq.gz
- 78-ST_S4_R1_001.fastq.gz
- 38-CB_S1_R1_001.fastq.gz
- 48-CB_S2_R1_001.fastq.gz
- 70-CB_S3_R1_001.fastq.gz
- 74-CB_S4_R1_001.fastq.gz
- 77-CB_S3_R1_001.fastq.gz
- 78-CB_S4_R1_001.fastq.gz
- 50-CB_S1_R1_001.fastq.gz
- 50-ST_S1_R1_001.fastq.gz
- 52-CB_S2_R1_001.fastq.gz
- 52-ST_S2_R1_001.fastq.gz
- CB_IN_GTCCGC_L003_R1_001.fastq.gz
- ST_IN_GTCCGC_L004_R1_001.fastq.gz
- ctrl_UB_CB_CAGATC_L008_R1_001.fastq.gz
Methodology

Replicates

Given the large number of mice (25) we had to utilize for a single ChIP experiment, we omitted doing replicates.
Sequencing depth

Total reads: CB H3K27 ChIP: 134508639, ST H3K27 ChIP: 128561066, ST H3K27 MG CX: 177410280, INPUT MG CX: 92850211 ; uniquely mapped reads: CB H3K27 ChIP: 91203884, ST H3K27 ChIP: 82153966, CB H3K27 IN: 87850086, ST H3K27 IN: 86077596, CB H3K27 MG CX: 121583596, INPUT MG CX: 60709894. Reads were 50 bp single-end reads.

Antibodies

H3K27me3 (07-449, Millipore, 1:100)

Peak calling parameters

Peak-calling was performed using MACS (v2.1.1) with default settings. Annotation of called peaks and differential regions to their genomic features (promoters, gene bodies, intergenic, etc) was performed using region-analysis (v0.1.2).

Data quality

The ChIP-seq data was first checked for quality using the various metrics generated by FastQC (v0.11.2). Raw sequencing reads were then aligned to the mouse mm9 genome using the default settings of Bowtie (v2.2.0). Only uniquely mapped reads were retained, and the alignments were subsequently filtered using the SAMtools package (v0.1.19) to remove duplicate reads.

To select for H3K27me3-positive genes in cbMg, stMg and cxMg, the log2 fold enrichment (log2FE) of ChIP signal over the corresponding input signal was calculated for a region spanning TSS ±1 kb for each gene. A cutoff of log2FE > 0 was used to define H3K27me3-positive genes for stMg, cbMg and cxMg. To compare H3K27me3 targets between cbMg and stMg, a goodness-of-fit G-test was applied to the normalized read counts mapping within TSS ± 1 kb of each gene. A cutoff of p-value<0.05 was used to define genes differentially marked with H3K27me3 in cbMg and stMg.

Software

Sequencing: HiSeq 2000 platform
Raw sequencing data processing: Illumina bcl2fastq2 Conversion Software v2.17
Quality control: FastQC (v0.11.2)
Alignment: Bowtie (v2.2.0)
Filtering: SAMtools package (v0.1.19)
Peak-calling: MACS (v2.1.1)
Annotation of called peaks: region-analysis (v0.1.2)
Profile plots and heatmaps: ngsplot (v2.47) and Multiple Experiment Viewer 4.8.

Flow Cytometry

Plots

Confirm that:
☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Nuclei isolation: Microglial nuclei from different brain regions of 4-/8-month-old Cx3cr1CreERT2/+(Litt);Eef1a1LSL.eGFPL10a/+ mice were isolated based on the eGFP-L10a fluorescence of newly formed ribosomes in the microglia nucleoli. All mice were gavaged at 4-6 weeks of age with five doses of 100 mg/kg of tamoxifen with a separation of at least 48 hours between doses. Briefly, mice were euthanized with CO2 and brain regions were quickly dissected and homogenized with a glass homogenizer. The homogenate was cross-linked with 1% formaldehyde for 8 min at room temperature and the reaction was quenched with glycine for 5 min at RT. The homogenate was then spun through a 29% iodixanol cushion. The resulting nuclear pellet was resuspended for FACS.

Microglia isolation: All mice were gavaged at 4-6 weeks of age with five doses of 100 mg/kg of tamoxifen with a separation of at least 48 hours between doses. Brain regions from adult mice were dissected, cut into small pieces and homogenized by manual compression. The tissue was incubated in digestion reaction at 37°C for 30 minutes with titration and was stopped by addition of EDTA. The homogenate was centrifuged, the pellet was resuspended and filtered. Sample was resuspended in 70% Percoll. A Percoll gradient (70%, 37%, and PBS in 4:3:1 ratio) was centrifuged to obtain the interphase containing the microglia. Interphase was collected and resuspended for FACS.

Early apoptotic Jurkat cells (EAJ): The Jurkat human T cells were transferred to fresh growth medium at 1 million/ml and treated with 1 μM Staurosporine for 3 hours. After treatment cells were collected and washed. Induction of apoptosis was verified using FITC-Annexin V / propidium iodide kit.

Phagocytosis assay: EAJ were incubated with 10 μg pHrodo dye per 106 cells for 1 hour at RT in the dark. The cells were then washed with PBS and resuspended in microglia medium at 10 million/ml. Primary microglia from the cerebellum and striatum of adult mice that were cultured for 5h were given an equal number of pHrodo-labeled EAJ. Cells pretreated with 2 μM Cytochalasin D for 30 min before and during the incubation of microglia with pHrodo-EAJ were used as a negative control. After 3 hours, the supernatant was discarded and microglia were trypsinized. Pooled wells were collected in one tube, centrifuged at 400 × g for 5 min, and resuspended in 500 μl 1% BSA in PBS with 1μg/ml DAPI and placed on ice.

More detail is provided in the Methods section.
| Instrument         | BD FACS Aria II and BD LSR II |
|--------------------|-------------------------------|
| Software           | BD FACSDiva v8.0.1 and FCS Express 6 |
| Cell population abundance | The purity of the samples were >95% as determined by re-analyzing the post-sort fractions. |
| Gating strategy    | Gates were made for live cells (FSC-A by SSC-A), then singlets (FSC-W by FSC-A) for all samples. Singlets of nuclei were additionally selected by lowest DyCycle Ruby signal. Microglial cells and nuclei were gated by high GFP/YFP signal from mice expressing GFP/YFP under Cx3cr1 promoter. For apoptotic Jurkat cells, high and low Annexin V and Propidium Iodide signals were quantified to measure percentage of early apoptotic cells. For phagocytosis assay, events were gated on the SSC-A and FSC-A to exclude debris, then on GFP+ microglia, and finally to quantify the percent of GFP+/pHrodo+ cells. pHrodo gate was determined by using the phagocytosis inhibitor as negative control. |

**Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.**