Microglial brain region–dependent diversity and selective regional sensitivities to aging

Kathleen Grabert, Tom Michoel, Michail H Karavolos, Sara Clohisey, J Kenneth Baillie, Mark P Stevens, Tom C Freeman, Kim M Summers & Barry W McColl

Microglia have critical roles in neural development, homeostasis and neuroinflammation and are increasingly implicated in age-related neurological dysfunction. Neurodegeneration often occurs in disease-specific, spatially restricted patterns, the origins of which are unknown. We performed to our knowledge the first genome-wide analysis of microglia from discrete brain regions across the adult lifespan of the mouse, and found that microglia have distinct region-dependent transcriptional identities and age in a regionally variable manner. In the young adult brain, differences in bioenergetic and immunoregulatory pathways were the major sources of heterogeneity and suggested that cerebellar and hippocampal microglia exist in a more immune-vigilant state. Immune function correlated with regional transcriptional patterns. Augmentation of the distinct cerebellar immunophenotype and a contrasting loss in distinction of the hippocampal phenotype among forebrain regions were key features during aging. Microglial diversity may enable regionally localized homeostatic functions but could also underlie region-specific sensitivities to microglial dysregulation and involvement in age-related neurodegeneration.

The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, UK. Correspondence should be addressed to B.W.M. (barry.mccoll@roslin.ed.ac.uk).

Received 24 September 2015; accepted 7 December 2015; published online 18 January 2016; doi:10.1038/nn.4222
and Supplementary Fig. 1a). We first validated the consistency of microglial extraction from all regions of interest (cerebellum, cerebral cortex, hippocampus and striatum). The CD11b antigen was ubiquitously expressed on microglia throughout all brain regions as shown by colocalization with GFP+ microglia in the Csf1r-EGFP 'MacGreen' reporter mouse (Supplementary Fig. 1b). The purified selected fraction consisted of a single population of CD11b+ and F4/80+ cells, and there was no detectable CD11b or F4/80 staining in the purified nonselected fraction, which confirmed CD11b as an efficient target for purification (Supplementary Fig. 1c). Microglia obtained from all regions showed a uniform CD11b+ F4/80+ CD45hi profile (Fig. 1a) characteristic of resident brain microglia and distinguishing them from CD45hi systemic macrophage populations. This indicated that we were extracting an equivalent microglial population from all brain regions. Expression of Itgam (encodes CD11b) and other established microglial/macrophage genes including Csf1r and Cx3cr1 was similarly enriched in purified microglia from each region in comparison to the respective mixed brain cell homogenates (Fig. 1b). Additional genes recently reported as microglial 'signature' genes (for example, Tmem119 and P2ry13)11,12,19 were also highly enriched in purified samples (Fig. 1c), whereas markers of neurons, astrocytes and oligodendrocytes were expressed at negligible levels in purified microglia (Fig. 1d). Genes highly expressed in blood leukocyte subsets including Cd3e (T lymphocytes), Cd19 (B lymphocytes) and Ly6g (granulocytes) were undetectable in purified microglia (Fig. 1e), and there was no expression of systemic macrophage-specific genes identified from a recent study (for example, Fabp4, Serpinb2)11 in microglial samples (Fig. 1f). Immunostaining of isolated cells in culture showed that all cells stained positively for the microglial/macrophage antigens IBA1 and F4/80 (Fig. 1g). Together these data verified the purity and consistency of microglial extraction across brain regions.

The microglial transcriptome is regionally heterogeneous

We initially determined whether the microglial transcriptome in the healthy young adult mouse brain (4 months of age) is regionally heterogeneous. Principal component analysis (PCA) showed clustering of samples in a region-dependent manner, and indicated a close relationship between microglial expression profiles of the cerebral cortex and striatum and relatively more distinct profiles in the cerebellum and hippocampus (Fig. 2a). We validated these relationships nonsubjectively using the network visualization and analysis tool BioLayout Express3D (http://www.biolayout.org/). Analysis of sample-to-sample correlation showed clustering according to brain region with similar interregional relationships as those identified by PCA (Fig. 2b). Thus, the global gene expression profile of adult microglia in the healthy brain was regionally heterogeneous.

Expression of 3,131 probe sets (~7% of total) representing 2,527 genes was differentially regulated in various brain regions (false discovery
rate (FDR) \( q < 0.05 \); Supplementary Table 1). Hierarchical clustering of samples based on differentially expressed probe sets demonstrated the marked contrast in expression profile between cortical/striatal and cerebellar microglia, and the intermediate profile in hippocampal microglia (Fig. 2c). Genes involved in multiple aspects of immune function were among the most differentially expressed by region (Supplementary Table 1), including those with established function and others previously unexplored in microglia. Analysis of Gene Ontology (GO) biological processes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) revealed 'immune response' and 'immune effector response' as significantly overrepresented (Supplementary Table 2). There was also a striking overrepresentation of multiple processes associated with energy metabolism (Supplementary Table 2). We used the Enrichment Map (http://baderlab.org/Software/EnrichmentMap/) network visualization tool to remove redundancy in GO enrichment annotation. The key feature of the network was the presence of two major clusters, each comprising functionally related and highly connected enriched gene sets with roles in immune function and energy metabolism (Fig. 2d). Full annotation of nodes within these clusters is presented in Supplementary Table 3. These data indicated immunoregulatory and bioenergetic/metabolic processes as the major contributors to regional diversity in microglial phenotype of the young adult brain.

Figure 2 The adult mouse microglial transcriptome is regionally heterogeneous. (a) PCA of microarray expression profiles for purified microglia from discrete brain regions. (b) Network graph showing sample-to-sample correlation of microarray data sets from the analysis performed in BioLayout Express3D (Pearson correlation threshold \( r \geq 0.96 \)). Nodes represent individual samples and edges the degree of correlation between them. (c) Heatmap showing the expression pattern of probe sets differentially expressed by brain region \((P < 0.05 \text{ with FDR correction})\). The scaled expression value (row \( Z \text{-score} \)) is displayed in a blue-red color scheme with red and blue indicating high and low expression, respectively. Str, striatum; Hpp, hippocampus; Ctx, cerebral cortex; Cbm, cerebellum. (d) Network graph showing enriched GO terms imported to Enrichment Map. Nodes represent individual GO terms (gene sets) and edges the relatedness between them. Two major clusters defined by immunoregulatory and metabolic function were identified.

Figure 3 Three major patterns of gene coexpression underpin regional microglial transcriptional heterogeneity. (a) A transcript-to-transcript correlation network graph of transcripts significantly differentially expressed by brain region was generated in BioLayout Express3D (Pearson correlation threshold \( r \geq 0.80 \)). Nodes represent transcripts (probe sets), and edges represent the degree of correlation in expression between them. The network graph was clustered using a Markov clustering algorithm, and transcripts were assigned a color according to cluster membership. (b) Mean expression profile of all transcripts within clusters 1, 2 and 3. (c) Heat maps showing the expression profile of all transcripts contained within clusters 1, 2 and 3. Each probe set is represented in a blue-red row \( Z \text{-score} \) scale with red indicating high expression and blue low expression. Str, striatum; Hpp, hippocampus; Ctx, cerebral cortex; Cbm, cerebellum.
Three major patterns of region-dependent microglial gene coexpression

We next sought to define the region-specific microglial phenotypes by assessing patterns of gene coexpression using BioLayout Express3D (ref. 20). The utility of BioLayout Express3D for the identification of spatiotemporal patterns of gene expression and the discovery of transcriptional networks underpinning common functional pathways has been described previously20. A network graph constructed from the 3,131 regionally differentially expressed probe sets was clustered using a Markov clustering algorithm to nonsubjectively subdivide the graph into discrete sets of coexpressed genes. Overall graph structure consisted of 14 clusters ranging in size from 10 to >1,000 nodes (Fig. 3a and Supplementary Fig. 2a). Three major clusters distributed across two distinct regions of the graph were evident (Fig. 3a and Supplementary Table 4). The mean expression profiles of these three clusters showed that cluster 1 contained genes whose expression was relatively greater in the cerebral cortex and lower in the cerebellum (Fig. 3b). In contrast, clusters 2 and 3, which were located together and were distant from cluster 1, both contained genes with relatively greater expression in the cerebellum (with greater hippocampal expression in cluster 2). The expression profile for individual genes within each cluster generally followed the cluster mean (Fig. 3c). Increasing the Pearson correlation threshold did not materially affect the overall graph or clustering structure (Supplementary Fig. 2).

Microglial immunophenotypic and bioenergetic heterogeneity

Genes that have highly correlated expression profiles across a range of experimental conditions (i.e., are coexpressed) are often distinct components of a common pathway or biological process21. A large number of cluster 3 genes (high expression in the cerebellum) were immune-related. GO analysis revealed ‘immune response’ and ‘defense response’ as the most overrepresented biological processes (Supplementary Table 5), and clustering using Enrichment Map underlined the array of enriched immune-related processes (Fig. 4a). To gain further insight to the molecular functions encoded in cluster 3, we manually annotated genes according to the following major categories: pathogen/self-recognition, cell adhesion and chemotaxis, signaling integration, antigen presentation and microbial killing/sequestration (Fig. 4b). Genes from multiple molecular classes involved in pathogen (or self) recognition were present in cluster 3.

Bacterial recognition genes included the C type lectins (Clec4e), bacterial recognition genes included the C type lectins (Clec4e), and some genes involved in pathogen recognition (for example, Ficolin and Fpr1 and Fpr2). Viral recognition was also evident in the high expression of the Zbp1 gene, which encodes cytoplasmic sensors of viral DNA22. Consistent with antiviral activity, there was a large number of interferon pathway genes (for example, Stat1, Stat4, Ifit2, Ifitm3, Ifr7, Oas1l and Pirc1l), and pathway analysis in Ingenuity identified an enriched interferon network and both interferon gamma and the type I interferon receptor (IFNAR) as top upstream regulators (Supplementary Fig. 3 and Supplementary Table 6). A striking feature of cluster 3 was the presence of multiple genes involved in antigen processing and presentation, including both MHC-I (H2-D1 and H2-K1) and MHC-II (H2-Aa, H2-Ab1, H2-Eb1 and Cd74) pathways. Upstream regulation was evident through the presence of Ciita encoding the master regulator of MHC-II expression and Nrloc5, the master regulator of MHC-I gene expression. Pathway analysis in KEGG identified antigen processing and presentation as a significantly overrepresented pathway (Supplementary Fig. 4 and Supplementary Table 7). Genes encoding several classes of immune effector molecules, many involved in antigen killing or sequestration, were present in this cluster. Camp and Ngp, genes encoding the antimicrobial peptide mCRAMP and neutrophilic granule protein, respectively, were of particular note because a recent study identified these genes as unexpectedly highly expressed in microglia compared to non-CNS macrophages11. We also assessed regional expression of selected genes from cluster 3 by quantitative PCR (Fig. 4c) and protein expression by flow cytometry, with both methods demonstrating comparable profiles to the microarray data (Fig. 4d,e). We also noted immunoregulatory molecules in cluster 1 (relatively high expression in the cortex and low in the cerebellum) indicating that some immunoregulatory pathways may be more active in regions other than the cerebellum (see below also). Those were largely immune signaling genes (for example, Cd47 and Cd300a) encoding molecules that limit the strength of myeloid cell responses to external stimuli.

The bioenergetic profile of myeloid cells is tightly linked to their immunophenotype and the environmental conditions to which they are exposed (for example, normoxia/hypoxia)23. We were therefore interested that cluster 2 (high expression in the cerebellum and the hippocampus) contained a large number of genes associated with key components of energy production systems and their regulation (Fig. 4f–h). We validated this by GO analysis, which revealed generation of precursor metabolites and energy, ‘electron transport chain’ and ‘oxidative phosphorylation’ as among the most highly overrepresented processes (Supplementary Table 8), and visualization in Enrichment Map showed multiple clusters associated with glycolysis, the electron transport chain, ATP synthesis and redox metabolic activity (Fig. 4f). Genes encoding most enzymes in the glycolytic pathway, the tricarboxylic acid (TCA) cycle, multiple subunit constituents of each of the proton pump complexes in the electron transport chain (I, III and IV) and the ATP synthase complex were present in cluster 2 (Fig. 4g). Key regulators of energy metabolism were also present, notably peroxisome proliferator-activated receptor gamma (Pparg) and the associated coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Ppargc1a), which cooperate to control transcription of an array of genes involved in mitochondrial function and energy metabolism. Antioxidant responses, important for counteracting oxygen radicals produced during oxidative phosphorylation, were also represented, including the superoxide dismutase (Sod1 and Sod2), catalase (Cat), peroxiredoxin (Prdx2 and Prdx5) and glutathione peroxidase (Gpx4 and Gpx8) families (Fig. 4g). Individual gene expression profiles of representative examples for each of the above classes are shown in Figure 4h.

Collectively, the above data support the suggestion that cerebellar and hippocampal microglia maintain a more immune-alert state than microglia in the striatum and cortex, and this is accompanied by relatively greater expression of an extensive set of co-regulated genes involved in energy metabolism.

Microglial steady-state heterogeneity in immune alertness

Immune cells maintain a balance between activating and inhibitory signals to fine-tune the strength of their responses in part through cell-surface activating receptors that associate with immunoreceptor tyrosine-based activation motifs (ITAMs) and counteracting inhibitory receptors containing immunoreceptor tyrosine-based inhibition motifs (ITIMs)24. Given the more immune-alert state of cerebellar and hippocampal microglia suggested above, we explored whether there were regional differences in microglial expression of genes encoding ITAM-associating and ITIM-containing immunoreceptors. We focused on the genes encoding triggering receptor expressed on myeloid cell (Trem), sialic acid–binding immunoglobulin–type lectins (Siglec), Cd200r, Cd300 and signal regulatory peptide (Siris) families, each of which contains activating and inhibitory members (Fig. 5a).
Figure 4 Regional transcriptional heterogeneity in microglial immunophenotype and bioenergetics. (a) Network graph of enriched GO biological processes among cluster 3 transcripts (analyzed using DAVID; \( P < 0.05 \) with Benjamini correction). Nodes represent individual GO terms (gene sets), and edges represent the relatedness between them. (b) Examples of individual genes in cluster 3 manually annotated to functional categories of immunoregulatory function. (c) mRNA expression intensity of selected genes in purified microglia measured by quantitative PCR. Data show mean \( \pm \) s.d., \( n = 4 \) independent samples, each from tissue pooled from eight mice. \( * P < 0.05, ** P < 0.01, *** P < 0.001 \), one-way ANOVA with Bonferroni correction. (d,e) Flow cytometry analysis of MHC-II protein expression on freshly isolated adult microglia identified by CD11b\(^{+}\)CD45\(^{lo}\) profile in mixed brain cell suspensions from discrete brain regions. CD11b\(^{+}\)CD45\(^{lo}\) microglia positive for MHC-II (d) and mean fluorescence intensity (MFI) of MHC-II expression on CD11b\(^{+}\)CD45\(^{lo}\) cells (e). Data show mean \( \pm \) s.d., \( n = 3 \) independent cell preparations. \( * P < 0.001 \), one-way ANOVA with Bonferroni correction. (f) Network graph of enriched GO terms generated in Enrichment Map for cluster 2 transcripts (\( P < 0.05 \) with Benjamini correction). (g,h) Examples of individual genes in cluster 2 manually annotated to functional categories of bioenergetic function. Data show mean \( \pm \) s.d., \( n = 4 \) independent samples, each from tissue pooled from eight mice. \( * P < 0.05, ** P < 0.01, *** P < 0.001 \), one-way ANOVA with Bonferroni correction. \( P \) values for all statistical comparisons are listed in Supplementary Table 13.
In all families, we found interregional differences in expression of genes encoding both ITAM- and ITIM-signaling members, and an opposing pattern of expression for those activating and inhibitory receptors (Fig. 5b,c). Activating ITAM-associating members were more highly expressed in cerebellar microglia, whereas ITIM-containing inhibitory members showed the reverse pattern. For example, in the CD300 family, CD300a is the only member with a cytoplasmic tail containing an ITIM, and CD300a was expressed at lower levels in cerebellum and hippocampus. In contrast, other CD300 members have consensus sequences enabling association with ITAM-containing adapters such as DAP12, and expression of all genes showed the opposite pattern of expression to CD300a. One caveat relates to TREM2: TREM2 associates with the ITAM-containing DAP12 adaptor molecule and dampens microglial proinflammatory reactions, and thus the expression profile of its encoding gene was consistent with those for other inhibitory immunoreceptor genes. Expression of the genes encoding the ITAM-containing adaptor proteins DAP12 and DAP10 was consistent across brain regions, perhaps reflecting their common use for signaling by several receptors (Fig. 5a).

We next determined whether the regional immunophenotypes of microglia extended to differences resembling overtly polarized states of microglia activation. We mined published microglial microarray data sets25 to establish a set of non-overlapping genes induced by classical (lipopolysaccharide; LPS) or alternative (interleukin-4; IL-4) activation. This identified 216 LPS-induced genes and 132 IL-4-induced genes (Fig. 5d). 17% of these LPS-induced genes and 18% of IL-4-induced genes were differentially expressed according to brain region. The LPS-inducible subset showed greater expression of the majority of genes in the cerebellum and the hippocampus (Fig. 5e). In contrast, greater expression of the differentially expressed subset of IL-4-inducible genes was not restricted to any particular brain region (Fig. 5f). The majority of microglial genes associated with classical or alternative activation was expressed at almost undetectable levels in all brain regions, including the archetypal marker genes Nos2 and...
Figure 6 Regional microglial heterogeneity is comparable to intertissue macrophage diversity. (a) PCA of the present regional microglial expression data sets and systemic macrophage data sets. PC, principal component; (b) Number of highly enriched genes (≥10-fold, P < 0.05 with FDR correction) in microglia compared to peritoneal macrophages for microglia from each brain region. (c) Regional overlap of the genes highly enriched in microglia versus peritoneal macrophages. (d) Fold change (microglia versus peritoneal macrophages) in expression of selected genes recently identified as signature genes distinguishing microglia from systemic macrophages. (h) Hippocampus; Cx, cerebral cortex; Cbm, cerebellum.

Arg1 (Fig. 5g). Thus it appears the more ‘alert’ phenotype of microglia in the cerebellum and the hippocampus is distinct from conventional states of activation or polarization.

We reasoned that steady-state differences in immune alertness could predispose microglia to region-dependent variations in responsiveness to immune stimulation. To assess this directly, it was important to use an assay with freshly isolated microglia (prepared as for microarray) exposed to an equivalent challenge and over a short timeframe to avoid prolonged culturing that could result in de-differentiation from in vivo regional phenotypes. We achieved this using a bacterial phagocytosis and replication assay. We recovered fewer bacteria from cortical microglia than from cerebellar microglia 1 h after treatment with gentamicin (Fig. 5h), which may reflect distinct phagocytic or killing capacity, or both. Relative to the population of bacteria present inside microglia at 1 h after treatment with gentamicin, there was a significant increase in intracellular net replication of bacteria in cortical but not cerebellar microglia by 4 h (Fig. 5h). This suggested that cerebellar microglia were better able to control the net replication of internalized bacteria and support distinct functional responses of microglia to challenge that correlate with their region-specific immune alertness transcriptional profiles.

Transcriptional regulators of region-dependent coexpression networks

As described above, clusters 2 and 3 contained genes encoding known transcriptional regulators (for example, Pparg and Nlrc5) of many of the respective cluster genes. To gain further insight to transcriptional control mechanisms that may drive microglial diversity, we searched the annotated promoter regions of genes within these clusters for overrepresentation of transcription factor binding motifs from the JASPAR collection using Clover. Motifs recognized by the specificity protein (Sp), nuclear hormone receptor 4A (NR4A), estrogen related receptor (ERR) and RAR-related orphan receptor (ROR) were significantly overrepresented in cluster 2 (Supplementary Table 9). Each of these families has established roles in regulating cellular energy metabolism, which is consistent with the prominence of bioenergetic genes in cluster 2. The NR4A, ERR and ROR transcription factors are all members of the nuclear receptor family that act as both metabolic sensors and transcriptional regulators, perhaps highlighting how the metabolic environment of microglia could direct region-dependent regulation of gene expression. Transcription factor binding motifs overrepresented in cluster 3 included those bound by early B-cell factor-1 (EBF1), forkhead box L1 (FOXL1), activator protein 1 (API) and c-Rel (REL) (Supplementary Table 9), all of which are known regulators of immune and inflammatory gene expression, and these data are therefore consistent with the immunoregulatory gene profile of cluster 3.

Brain region disproportionately affects cell-surface protein–encoding gene expression

A subset of genes encoding microglial cell surface proteins has been described recently and termed the microglial ‘sensome’ with reference to their involvement in sensing the environment. We hypothesized that brain region would have a substantial impact on the expression of the sensome genes given that regional heterogeneity of microglial phenotype may in part arise from exposure to varying local environmental demands. GO terms associated with the cell surface were highly enriched in the set of regional differentially expressed genes (Supplementary Fig. 5a) and 34 of the 100 sensome genes were differentially expressed according to brain region. The majority were expressed at greater levels in the striatum and the cortex (Supplementary Fig. 5b), and were related to immune signaling (Supplementary Table 10). Moreover, of the differentially regulated sensome genes involved in immune signaling, many encoded proteins involved in restricting overactivation of microglia, including Cx3cr1, Trem2, Cd33, Siglech and Fcgr2b (Supplementary Fig. 5c). These data highlight that brain region has a disproportionately large effect on expression of genes encoding microglial cell-surface proteins (compared to all genes) and that much of the heterogeneity affects receptors transducing microglial ‘off’ signals. Transmembrane pathways promoting microglial quiescence may therefore be active at differing levels depending on brain localization and contribute to regional differences in immune alertness.

Interregional microglial heterogeneity mirrors macrophage tissue diversity

We assessed the extent of interregional microglial heterogeneity in the wider context of macrophage diversity by comparing our regional microglial transcriptomes with selected purified macrophage data sets.
Aging of microglia occurs in a region-dependent manner

Aging is associated with altered inflammatory status systemically and in the brain, and involves marked changes in microglial morphology and phenotype. However, it is unclear whether the impact of aging on microglia is uniform throughout the brain. We first determined whether the gene networks defining young adult regional heterogeneity were equally sensitive to aging. Overall, ~50% of region-defining transcripts from mice at 4 months of age were differentially regulated during aging, but there was an unequal distribution across the major 4-month-old sample region-defining clusters of gene coexpression (Fig. 3). Notably, the majority (>80%) of transcripts from the 4-month sample immune regulation cluster were age-regulated, but fewer than 25% of transcripts in the 4-month sample

bioenergetics cluster were differentially expressed during aging (Fig. 7a). This shows that distinct modules of coordinated gene transcription that define microglial heterogeneity in the young adult brain are differentially sensitive to aging.

PCA showed that although the gross relative regional relationship identified at 4 months was generally preserved during aging, there was an age-dependent progression, suggesting an interaction between age and brain region (Fig. 7b). First, cerebellar microglia were relatively more distant from the remaining brain regions at 12 and 22 months compared to 4 months. Second, the intermediate hippocampal microglia profile at 4 months was preserved at 12 months but largely converged with cortical and striatal samples at 22 months. We substantiated these observations nonsubjectively using network analysis in BioLayout Express3D on the 13,741 transcripts regulated by age (FDR q < 0.05). Unbiased sample-to-sample correlation and clustering identified age-dependent unique cerebellar clusters and showed that hippocampal samples from mice at 4 and 12 months clustered independently, whereas at 22 months they formed a larger cluster together with striatal and cortical samples (Fig. 7c). These data show a region-dependent influence on microglial aging suggesting increased sensitivity of cerebellar microglia and a potential diminishing of the discrete hippocampal phenotype.

The kinetics of microglial aging were also region-specific. Changes in gene expression profile occurred relatively consistently during early (4−12 months) and 'late' (12−22 months) aging in both cerebellar and cortical microglia (Fig. 7d). In contrast, changes were most pronounced during early aging in the striatum and during late aging in the hippocampus. The hippocampal pattern was particularly interesting because, in contrast to other regions where only ~10% of gene alterations comprised decreased expression from 12 months to 22 months, >30% declined in expression in hippocampal microglia (Fig. 7e) supporting a diminishing distinction from other forebrain regions at the individual gene level. We also noted that twice the number of genes were differentially expressed (FDR q < 0.05, fold-change ≥ 1.5) at 22 versus 4 months in cerebellar microglia compared to other regions, reinforcing their greater sensitivity to age-related change (Fig. 7d).
Unsupervised hierarchical clustering and visualization of age-region interacting genes (FDR $q < 0.05$) demonstrated a number of striking patterns (Fig. 7f) likely underpinning the gross age-region relationships above. First, a large group of genes increased in expression during aging in all regions, but in the cerebellum this occurred earlier (i.e., by 12 months) and/or to a greater magnitude by 22 months. Second, there was a cluster of genes that increased during aging only in the cerebellum. Third, expression of a group of genes initially detected at relatively greater levels in hippocampal and cerebellar microglia at 4 months declined during aging selectively in the hippocampus.

Collectively, these data show that the microglial transcriptome ages in a non-uniform manner across brain regions. Key observations are an accelerated and more amplified aging trajectory in cerebellar microglia and a declining distinction of the hippocampal phenotype (relative to other forebrain regions).

Pathways underpinning region-specific microglial aging profiles

We next sought to establish the biological processes responsible for the age-region microglial interactions above. A correlation network graph of the age-regulated transcripts was clustered to nonsubjectively divide the graph into modules of highly coexpressed genes (Fig. 8a). We focused on clusters in which interactions between age and region were most evident. In general, expression of cluster 2 transcripts increased with age, but the most striking features were the greater and/or earlier age-regulated increased expression in cerebellar microglia (Fig. 8b). The majority of genes were involved in immunoregulatory function, and this was supported by GO analysis showing immune-related processes as the most overrepresented in cluster 2 (Supplementary Table 11). Multiple families of molecules were represented including those involved in sensing of self and foreign ligands, immune cell adhesion and chemotaxis, cytokine signaling and antimicrobial effector responses. A large group of coexpressed genes involved in several aspects of the interferon pathway was particularly prominent. This included transcriptional regulatory factors (for example, Irf7, Stat2 and Oasl1) and interferon-regulated genes including those encoding effector proteins involved in antiviral defense (for example, Sp100, Csprs, Isg20, Ifit families, Bst2 and Zbp1). Expression of the above genes was not only increased to a significantly greater extent (for example, fivefold) during aging in the cerebellum but increased expression was also evident earlier (12 months) (Fig. 8c). Further genes involved in the interferon pathway predominantly increased in cerebellar microglia but not until 22 months (for example, Stat1, Ifitm family and Gbp family) (Fig. 8d).

Figure 8 Biological pathways underlying region-specific microglial aging. (a) Transcript-to-transcript correlation network graph of transcripts differentially expressed according to age ($P < 0.05$ with FDR correction) and clustered using a Markov clustering algorithm. Nodes represent individual transcripts and edges the degree of correlation in expression pattern between them. Colors denote discrete clusters. Circled region includes clusters with greater expression in cerebellum and/or increasing with age; square region includes clusters with greater expression in forebrain regions and/or declining expression with age. (b) Cluster position and mean expression profile of transcripts from cluster 2 indicating greater and/or earlier age-related changes. (c,d) Interferon pathway genes showing earlier (c) and/or greater/selective increases in expression in cerebellar microglia compared to forebrain regions during aging (d). Data show mean ± s.d., $n = 4$ independent samples, each pooled from tissue from eight mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 4-month sample; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. 12-month sample, two-way ANOVA with Bonferroni correction. (e) Heat maps showing microarray expression patterns of selected immunoreceptor family genes during aging arranged according to activating or inhibitory classification. Row Z-score intensities represent the mean of four independent samples per region and age with red indicating higher expression and lower expression in blue. (f) Expression patterns of Cd300 family genes show interaction between brain region and age for activating but not inhibitory members. Data show mean ± s.d., $n = 4$ independent samples, each pooled from tissue from eight mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 4-month sample; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. 12-month sample, two-way ANOVA with Bonferroni correction. (g,h) Cluster position and mean expression profile of transcripts from cluster 14 indicating selective decline in expression during aging in hippocampal microglia. (h) Expression profiles of selected genes from cluster 14. Data show mean ± s.d., $n = 4$ independent samples, each pooled from tissue from eight mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 4-month sample; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. 12-month sample, two-way ANOVA with Bonferroni correction. Str, striatum; Hpp, hippocampus; Ctx, cerebral cortex; Cbm, cerebellum. $P$ values for all statistical comparisons are presented in Supplementary Table 13.

Genes more sensitive to greater and/or earlier age-related changes in cerebellar microglia included those already more highly expressed in cerebellar microglia and those expressed at negligible levels in all regions at 4 months.

Given the above data and regional differences in expression of ITAM and ITIM signaling immunoreceptors in microglia of young adult mice, we assessed whether there were region-dependent aging responses of specific immunoreceptor families. There was a notable contrast in the aging expression profile of amplifying and inhibitory immunoreceptors in all families examined (Fig. 8e). Expression of inhibitory receptors from each family in general remained stable (for example, Cd300a) or decreased in a largely region-independent manner (for example, Cd200 and Sirpa) during aging. In contrast, expression of amplifying receptors increased with age and in a mostly region-dependent manner, affecting the cerebellar microglia selectively (for example, Cd300ld, Trem1 and Sirpbl1a) or to a significantly greater extent (for example, Cd200r4 and Cd300b) than other regions. The Cd300 family is presented as an example (Fig. 8f). Thus, during aging, alterations in immunoreceptor expression across multiple molecular families support a regionally variable shift in balance toward immune amplification.

Genes significantly decreased in expression (FDR $q < 0.05$, fold change $\geq 1.5$) from 4 to 22 months in hippocampal microglia showed enrichment of GO processes related to cell adhesion/migration/motility, membrane organization/endocytosis, immune/inflammatory function and vascular development (Supplementary Table 12) suggestive of marked changes in the interaction of hippocampal microglia with their environment. Mining of these genes in the BioLayoutExpress3D network graph revealed their presence in several clusters each with a profile sharing reduced age-related hippocampal expression. Cluster 14 was particularly interesting because it contained genes expressed at relatively greater levels in both hippocampal and cerebellar microglia at 4 months of age that selectively declined in the hippocampus (and in the striatum for a subset) during aging (Fig. 8g,h). Among the genes in this cluster, many (for example, Cd36, Cd93, P4f and Lyve1) are involved in cell adhesion and motility pathways through interactions with matrix components and other extracellular ligands (Fig. 8h). Consistent with the above functions, genes coordinating cross-regulation of endocytosis/phagocytosis and cytoskeletal reorganization were present, notably Arkhef3, Dahb, Itsn1 and Vav3. Some of the above genes have overlapping roles in immune function through sensing and internalization of microbial ligands and involvement in antigen processing and presentation (for example, Cd36, Cd93 and P4f).
Together with further genes in this cluster such as the mannose receptor gene \textit{Mrc1} and the MHC-II genes \textit{H2-Aa} and \textit{H2-Ab1} (Fig. 8h), it was evident that gene networks involved in certain aspects of ligand recognition, processing and presentation are relatively selectively suppressed during aging in hippocampal microglia. More generally, the above data support a potential ‘disengagement’ of aged hippocampal microglia with their environment compared to their young adult counterparts.
Regionally variable depression of ‘homeostatic’ microglial signature during aging

Microglial heterogeneity in the young adult brain was superimposed on a core signature distinguishing microglia from systemic macrophages. Aging resulted in a modest decline in expression across all forebrain regions of key signature genes and a significantly greater effect (for example, 30% reduction in expression from 4 to 22 months old) in cerebellar microglia (for example, Tmem119, P2ry12, P2ry13, Fcris) (P < 0.05, two-way ANOVA with Bonferroni correction for multiple comparisons) (Supplementary Fig. 6a). This supports data above that the greatest deviation from the ‘baseline’ young adult homeostatic signature during aging occurs in cerebellar microglia. Reduced expression of TGFβ receptor genes in aged microglia (Supplementary Fig. 6b) may be important because expression of signature-defining genes is regulated by TGFβ12. In contrast, signature macrophage genes (for example, Serpinb2, Alox15 and Fhop4) were expressed at negligible levels at all ages (Supplementary Fig. 6c), and genes commonly expressed on macrophages and microglia and upregulated on overtly activated microglia (for example, Ptprc and Emr1) and Igaon were stably expressed during aging in all regions (Supplementary Fig. 6d).

These data suggest that the age-related lessening of the young homeostatic microglial signature is regionally variable but is not accompanied by the gain of a macrophage-like signature.

DISCUSSION

The data presented here provide compelling evidence of regional microglial phenotypic diversity in the healthy adult brain and the region-dependent impact of aging on microglial phenotype. To our knowledge, this is the first demonstration that regional localization of microglia influences their genome-wide expression profile across the adult lifespan, and we showed that this extends beyond their immunophenotype. Key findings include that (i) transcriptional networks controlling microglial bioenergetic and immunoregulatory functions contribute prominently to heterogeneity in the young adult, (ii) immunophenotypic variation suggests a more immune-vigilant state of cerebellar microglia, (iii) networks of gene coexpression underpinning heterogeneity in the young adult brain are differentially sensitive to aging, (iv) increasing distinction of cerebellar microglia and reduced distinction of hippocampal microglia (among forebrain regions) are key features of aging, (v) microglial diversity is superimposed upon a core profile that distinguishes all microglia from macrophages, and (vi) aged microglia display partial loss of the core young adult microglial identity in a regionally variant manner but do not adopt a macrophage-like signature.

In the young adult brain (4 months old), the general relationship among the brain regions analyzed showed that cerebellar microglia were the most distinct, that cortical and striatal microglia were similar to each other, and that hippocampal microglia had an intermediate profile. This pattern suggests a microglial relatedness correlating with the relative positioning of brain regions along the rostro-caudal neuroaxis. Recent studies have reported that microglial responses to injury, disease or inflammatory challenge also vary according to neuroaxis location36,37. Microglial morphology and density vary according to the relative composition of white and gray matter with a lower density of microglia reported in white matter of the adult mouse brain13. The extent to which white-to-gray matter ratios could influence regional differences in microglial transcriptomes is unclear, although the finding that the white matter–rich striatum and largely gray matter–dominant cerebral cortex have highly similar expression profiles suggests this may not be a major determinant. Forebrain microglia but not cerebellar microglia are dependent on IL-34, a ligand of the colony stimulating factor-1 receptor, for their maintenance15,18. Furthermore, the involvement of enhancers in controlling tissue and cell identity is increasingly recognized, including in microglia10, and a recent study indicated that expression of brain region–specific enhancer RNAs may be particularly important in cerebellum-specific gene expression18.

Heterogeneity in microglial phenotypic markers other than immunophenotype has received negligible attention previously. Our data provide new information on the transcriptional programs controlling metabolism in microglia and show that regional differences in expression of these networks is a core feature of microglial diversity in the healthy young adult brain. Coordinated differences in regional expression of genes involved in all phases of the energy-production pathway (mitochondrial biogenesis, glycolysis, TCA cycle, electron transport chain and ATP synthesis) was evident, emphasizing the integrated nature of regional bioenergetic variation in microglia. The cluster profile revealed that hippocampal and cerebellar microglia have relatively greater expression of these genes, suggesting greater energetic demands on microglia in these areas. Although there is little understanding of microglial bioenergetics, the routine cellular behavior of microglia (for example, process scanning and phagocytic activity) is likely to be metabolically demanding, and regional differences in these activities could therefore influence energy demands32. In addition, the lower density of microglia in some areas, notably in the cerebellum, requires each microglial cell to survey a larger volume of tissue and would be expected to increase energy demands on an individual cell basis.

Our data suggest that microglia in some regions of the young adult brain exist in a more immune-vigilant state but one that does not equate to a conventional activated or primed microglial/macrophage phenotype. Local differences in the physical and neurochemical environment, such as cellular and matrix composition, blood-brain barrier permeability, neurotransmitter profiles and heterogeneity in other cell types, may all be important. Consistent with this, we found that a substantial proportion of genes encoding the microglial-enriched cell surface sensing apparatus were differentially expressed. Previous studies have suggested white matter microglia exist in a relatively less quiescent basal state than their gray matter counterparts37, which could contribute to the more immune-vigilant profile of microglia in the white matter–enriched cerebellum. Another explanation is that the environment of some brain regions has evolved to support a more immune-vigilant phenotype as a result of genomic integration of endogenous retroviruses (ERVs) and other retrotransposons. ERVs comprise ~10% of the mouse genome40, and although normally inactive, deficiencies in innate immunity can predispose to ERV reactivation41. Cerebellum-specific expression of the murine leukemia virus (MuLV)-ERV has been shown previously42, and in mixed brain homogenates expression of the MuLV-ERV designated Mela (melanoma antigen) was restricted to the cerebellum (Supplementary Fig. 7). The hippocampus is also more susceptible to retrotransposition in the human brain43. Thus, microglia, which we showed have a more immune-vigilant phenotype, including higher expression of antiviral interferon networks, are found in areas where there may have been an evolutionary drive for development of greater immune vigilance.

Despite similarities in their regional expression profile in the young adult brain, the major transcriptional networks (bioenergetics and immune) underlying regional heterogeneity at 4 months of age were affected differently by aging. Genes in immune networks were particularly sensitive, whereas the majority of genes in the bioenergetics cluster were unaffected by age. This indicates that regional differences
are preserved during aging for some functional pathways alongside marked divergence in others. Given the similar regional profiles of the immune and bioenergetics clusters at 4 months of age and the close relationship between immune function and metabolism, there may be some degree of immune-metabolic decoupling during aging particularly in microglia from regions showing the greatest immunoregulatory deviation from the young adult (for example, cerebellum). However, further work will be needed to determine whether this divergence could allow for greater adaptation to the demands of aging or could predispose to functional dysregulation.

The sensitivity of the immune network to aging was largely responsible for the increasing distinction of the cerebellar phenotype in the aged brain. In contrast to aging and direct immune stimulation ex vivo, however, we observed regionally comparable responses to acute systemic inflammatory challenge with bacterial lipopolysaccharide (data not shown) indicating that microglial heterogeneity encodes region-specific sensitivities in a stimulus/stressor-dependent manner. A selective or significantly greater induction in expression of many immune amplifying genes occurred in cerebellar microglia compared to other brain regions during aging, whereas genes involved in restraining excessive immune activity were generally stable across all brain regions. This implies that the more immune-alert state of cerebellar microglia compared to other regions evident in the young adult is augmented in the aged brain. The functional consequences of this are important to consider but may be complex. More caudal regions of the CNS such as the cerebellum may be more vulnerable to age- or disease-related inflammatory degeneration if this heightened alertness is poorly controlled. In support of this, age-related increases in inflammatory marker expression were predominant in the cerebellum and were associated with functional cerebellar deficits57. However, the immune-alert phenotype may confer protective functions through increased vigilance and efficiency in removing potentially harmful agents. In this regard, it is pertinent to note the lower susceptibility of the cerebellum to amyloid deposition during aging44.

Selective age-related alterations in gene networks were also evident in hippocampal microglia, but in contrast to the cerebellum, these changes resulted in a declining distinction from other forebrain regions. Decreasing expression of genes involved in matrix interactions and sampling the extracellular environment were the most prominent cause, suggesting that a declining engagement with their environment during aging particularly affects hippocampal microglia. Although previous studies had not been performed on a region-specific basis, our data are consistent with reports of decreased process motility45 and reduced expression of cell surface sensing genes11 in aged microglia, indicative of compromised environmental sampling capabilities. In contrast to the cerebellum, the hippocampus is vulnerable to age- and disease-related deposition of misfolded proteins44, which could in part relate to the age-driven divergence in environmental and immune alertness of microglia we described in these brain regions. Dystrophic microglia in the aged human hippocampus have been described and postulated to represent a senescent state unable to carry out normal functions40; our data may provide a plausible transcriptional basis for this hypothesis. More broadly, the loss of distinction in the overall hippocampal microglial phenotype at 22 compared to 4 months of age among forebrain regions is consistent with the concept of age-related loss of differentiation in neural function, cognitive performance and reorganization of connectivity across brain regions47,48. Notably, the weighting of regional connections of the hippocampus changes markedly during aging48. Declining specialization among neuronal populations has also been described in the aged brain49. It is therefore possible that age-related changes in the regional diversity of local signals derived from other neural components, altered interregional communication and intrinsic microglial modifications in sensing pathways could all contribute to the diminished regional identity of hippocampal microglia in the aged brain.

Recent studies have revealed the distinctive transcriptional identity of microglia that distinguishes them from non-CNS tissue macrophages11,12,19. Our data show that, although microglia have multiple transcriptional identities dependent on brain region, a core signature differentiating them from macrophages is retained across regions. Hence interregional microglial heterogeneity is superimposed on a distinctive core profile. Microglial regional heterogeneity may be analogous in some respects to macrophage diversity observed in other tissues such as the spleen50. Despite reductions in expression of signature microglial genes with age, particularly in the cerebellum, highly macrophage-enriched genes (for example, Fabp4 and Alox15) were not expressed in any region at any age thus supporting that microglia in the aged brain also retain an overriding phenotypic individuality compared to macrophages in the aged brain.

In summary, regional microglial diversity we described may be important for meeting the location-dependent demands of brain tissue under steady-state conditions. The impact of aging on this diversity also suggests a basis for the regional variation in susceptibility to age-related neurodegenerative processes involving neuroinflammatory mechanisms. Further studies examining microglial diversity in the context of neurodegeneration are therefore warranted.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Gene Expression Omnibus: GSE62420 (microarray data).

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank members of Edinburgh Genomics, The University of Edinburgh, for performing microarray assays, B. Fleming for technical assistance with microglial purification and Y.-T. Lai for help optimizing regional brain dissection. This work was funded by a PhD scholarship from the Darwin Trust of Edinburgh to K Grabert and grants from the Biotechnology and Biological Sciences Research Council (BBSRC; BB/I004332/1) and the Medical Research Council (MRC; MR/J003384/1). The Roslin Institute and Edinburgh Genomics are partly supported through core grants from the Natural Environment Research Council (BB/H10/56), MRC (MR/K001744/1) and BBSRC (BB/I004243/1, BB/I004332/1).

**AUTHOR CONTRIBUTIONS**

B.W.M. and K.M.S. conceived the study; B.W.M., K.M.S., K.G. and T.C.F. designed experiments; K.G., B.W.M. performed experiments and analyzed data; T.M. advised on and performed analysis of transcriptional regulators; B.W.M., K.M.S. and K.G. wrote the paper; all authors contributed to data interpretation and editing of the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Prinz, M. & Mildner, A. Microglia in the CNS: immigrants from another world. *Glia* 59, 177–187 (2011).
2. Prinz, M. & Priller, J. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat. Rev. Neurosci.* 15, 300–312 (2014).
3. Paolicelli, R.C. et al. Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458 (2011).
4. Schaefer, D.P. et al. Microglia sculpt postnatal neural circuits in an activity- and complement-dependent manner. *Neuron* 74, 691–705 (2012).

© 2016 Nature America, Inc. All rights reserved.
5. Coulli, J.A.M. et al. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438, 1017–1021 (2005).

6. Neumann, H., Kotter, M.R. & Franklin, R.J.M. Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132, 288–295 (2009).

7. Lalancette-Hébert, M., Gowin, G., Simard, A., Weng, Y.C. & Kriz, J. Selective proliferation of microglial cells exacerbates ischemic injury in the brain. J. Neurosci. 27, 2596–2605 (2007).

8. Fenn, A.M., Hall, J.C.E., Gensel, J.C., Popovich, P.G. & Godbout, J.P. IL-4 signaling drives a unique arginase+/IL-1β+ microglia phenotype and recruits macrophages to the inflammatory CNS: consequences of age-related deficits in IL-4Rx after traumatic spinal cord injury. J. Neurosci. 34, 8904–8917 (2014).

9. Cardona, A.E. et al. Control of microglial neurotoxicity by the fractalkine receptor. Nat. Neurosci. 9, 917–924 (2006).

10. Biber, K., Neumann, H., Inoue, K. & Boddeke, H.W. Neuronal ‘On’ and ‘Off’ signals control microglia. Trends Neurosci. 30, 596–602 (2007).

11. Hickman, S.E. et al. The microglial sense is revealed by direct RNA sequencing. Nat. Neurosci. 16, 1896–1905 (2013).

12. Butovsky, O. et al. Identification of a unique TGF-β-dependent molecular and functional signature in microglia. Nat. Neurosci. 17, 131–143 (2014).

13. Lawson, L.J., Perry, V.H., Dri, P. & Gordon, S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39, 151–170 (1990).

14. de Haas, A.H. Boddeke, H.W.G.M. & Biber, K. Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. Glia 56, 888–894 (2008).

15. Wang, Y. et al. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat. Immunol. 13, 753–760 (2012).

16. Greter, M. et al. Stromal-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia. Immunity 37, 1050–1060 (2012).

17. Karch, C.M. & Goate, A.M. Alzheimer’s disease risk genes and mechanisms of disease pathogenesis. Biol. Psychiatry 77, 43–51 (2015).

18. Yao, P. et al. Coexpression networks identify brain region-specific enhancer RNAs in the human brain. Nat. Neurosci. 18, 1168–1174 (2015).

19. Chiu, I.M. et al. A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model. Cell Reports 4, 385–401 (2013).

20. Freeman, T.C. et al. Construction, visualisation, and clustering of transcription networks from microarray expression data. PLoS Comput. Biol. 3, 2032–2042 (2007).

21. Hume, D.A., Summers, K.M., Raza, S., Baille, J.K. & Freeman, T.C. Functional clustering and lineage markers: insights into cellular differentiation and gene function from large-scale microarray studies of purified primary cell populations. Genomics 95, 328–338 (2010).

22. Takaoka, A. et al. DAI (DL1/2BP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448, 501–505 (2007).

23. Ghersi-Bertrán, B., Wong, B.W., Kuchnio, A. & Carmeliet, P. Metabolism of stromal and immune cells in health and disease. Nature 511, 167–176 (2014).

24. Linnartz, B., Wang, Y. & Neumann, H. Microglial immunoreceptor tyrosine-based activation and inhibition motif signaling in neuroinflammation. Int. J. Alzheimer Dis. 2010, 587463 (2010).

25. Freilich, R.W., Woodbury, M.E. & Ikezu, T. Integrated expression profiles of miRNA and miRNA in polarized primary murine microglia. PLoS One 8, e79416 (2013).

26. Mathelier, A. et al. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic Acids Res. 42, D142–D147 (2014).

27. Frith, M.C. et al. Detection of functional DNA motifs via statistical over-representation. Nucleic Acids Res. 32, 1372–1381 (2004).

28. Huss, J.M., Torra, I.P., Staels, B., Girgure, V. & Kelly, D.P. Estrogen-related receptor α directs peroxisome proliferator-activated receptor α signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Mol. Cell. Biol. 24, 9079–9091 (2004).

29. Mootha, V.K. et al. Ermlph and Gabpa/b specify PGC-1α-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. Proc. Natl. Acad. Sci. USA 101, 6570–6575 (2004).

30. Pearen, M.A. & Muscat, G.E.O. Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease. Mol. Endocrinol. 24, 1891–1903 (2010).

31. Archer, M.C. Role of sp transcription factors in the regulation of cancer cell metabolism. Genes Cancer 2, 712–719 (2011).

32. Francis, G.A., Fayed, E., Picard, F. & Auwers, J. Nuclear receptors and the control of metabolism. Annu. Rev. Physiol. 65, 261–311 (2003).

33. Griffin, M.J. et al. Early B-cell factor-1 (EBF1) is a key regulator of metabolic and inflammatory signaling pathways in mature adipocytes. J. Biol. Chem. 288, 35925–35939 (2013).

34. Chen, B.-S., Yang, S.-K., Lan, C.-Y. & Chuang, Y.-J. A systems biology approach to construct the gene regulatory network of systemic inflammation via microarray and metabolomics. BMC Med. Genomics 1, 46 (2008).

35. Natoli, G., Ghisletti, S. & Barozzi, I. The genomic landscapes of inflammation. Genes Dev. 25, 101–106 (2011).

36. Schnell, L., Fearn, S., Klausing, H., Schwab, M.E. & Perry, V.H. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. Eur. J. Neurosci. 11, 3648–3658 (1999).

37. Hart, A.D., Wytenbach, A., Perry, V.H. & Teeling, J.L. Age related changes in microglial phenotype vary between CNS regions: grey versus white matter differences. Brain Behav. Immun. 26, 754–765 (2012).

38. Bosselin, D. et al. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. Cell 159, 1327–1340 (2014).

39. Bernhart, E. et al. Lysophosphatidic acid receptor activation affects the C13NJ microglia cell line proteome leading to alterations in glycosylation, motility, and cytoskeletal architecture. Proteomics 10, 141–158 (2010).

40. Stocking, C. & Kozak, C.A. Murine endogenous retroviruses. Cell. Mol. Life Sci. 65, 3383–3398 (2008).

41. Yu, P. et al. Nucelic acid-sensing Toll-like receptors are essential for the control of endogenous retrovirus viremia and ERV-induced tumors. Immunity 37, 867–879 (2012).

42. Lee, K.H., Hirouchi, M., Itoh, T., Greenhalgh, D.G. & Cho, K. Cerebellum-specific and age-dependent expression of an endogenous retrovirus with intact coding potential. Retrovirology 8, 82 (2011).

43. Baillie, J.K. et al. Somatic retrotransposition alters the genetic landscape of the human brain. Nature 479, 534–537 (2011).

44. Johnson-Wood, K. et al. Amyloid precursor protein processing and A beta42 deposition in a transgenic mouse model of Alzheimer disease. Proc. Natl. Acad. Sci. USA 94, 1550–1555 (1997).

45. Damani, M.R. et al. Age-related alterations in the dynamic behavior of microglia. Aging Cell 10, 263–276 (2011).

46. Streit, W.J., Braak, H., Xue, Q.-S. & Bechmann, I. Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer’s disease. Acta Neuropathol. 118, 475–485 (2009).

47. Steinem-Malkoun, R., Temprado, J.-J. & Hong, S.L. Aging induced loss of complexity and dedifferentiation: consequences for coordination dynamics within and between brain, muscular and behavioral levels. Front. Aging Neurosci. 6, 140 (2014).

48. Goh, J.O.S. Functional dedifferentiation and altered connectivity in older adults: neural accounts of cognitive aging. Aging Dis. 2, 30–48 (2011).

49. Park, D.C. et al. Aging reduces neural specialization in ventral visual cortex. Proc. Natl. Acad. Sci. USA 101, 13091–13095 (2004).

50. Davies, I.C., Jenkins, S.L., Allen, J.E. & Taylor, P.R. Tissue-resident macrophages. Nat. Immunol. 14, 986–995 (2013).
ONLINE METHODS

Mice. Experiments were performed using male C57Bl/6J mice (Charles River Laboratories) and Csf1r-Egfp reporter mice bred in-house. Mice were housed in individually ventilated cages (five mice per cage) maintained under specific pathogen–free conditions and a standard 12-h light-dark cycle with unrestricted access to food and water. All experiments using live animals were conducted under the authority of UK Home Office project and personal licenses; adhered to regulations specified in the Animals (Scientific Procedures) Act (1986) and Directive 2010/63/EU; and were approved by both The Roslin Institute’s and the University of Edinburgh’s Animal Welfare and Ethics Committees.

Microglial purification and mixed brain cell and homogenate preparation. At 4, 12 and 22 months of age, mice were perfused transcardially with physiologically saline, and brains were dissected into cerebellum, cortex, hippocampus and striatum. Tissue from eight mice was pooled for each regional replicate to obtain sufficient RNA for microarrays, and the experiment was performed in quadruplicate for each region. Brain tissue was finely minced by scalpel blade in ice-cold Hank’s balanced salt solution (HBSS; Sigma), centrifuged (400 g, 5 min, 4 °C), then resuspended and incubated for 1 h at 37 °C using an enzyme cocktail containing 50 U/ml collagenase, 8.5 U/ml dispase, 100 µg/ml N-tosyl-l-lysine chloromethyl ketone hydrochloride and 5 U/ml DNase I in 9.64 ml HBSS (Life Technologies). Tissue was dissociated manually using a Dounce homogenizer, and the enzymatic reaction was terminated by addition of equal volume HBSS containing 10% FBS. Homogenates were centrifuged (400g, 5 min, 4 °C), and pellets were resuspended in 35% Percoll (GE Healthcare), overlaid with HBSS and then centrifuged (800g, 45 min, 4 °C). The supernatant and myelin layers were discarded, and the cell pellet enriched with microglia was resuspended in separation buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS). The cell suspension was incubated with anti-CD11b microbeads (Miltenyi Biotec) for 15 min at 4 °C and then applied to a magnetic LS column (Miltenyi Biotec), and cells retained on the column (microglia) were flushed and resuspended in appropriate buffer for downstream applications (see below). Unretained cells were also collected during the initial validation for comparison. Mixed brain cell suspensions were prepared for flow cytometry according to the protocol, except the procedure was terminated after centrifugation on a Percoll gradient. For preparation of regional brain tissue homogenates for RNA extraction, mice were perfused, and brain tissue was dissected as above. Tissue was snap-frozen and stored at −80 °C. To validate that regional brain dissection did not result in cross-contamination of brain regions, expression profiles of established regionally enriched neuronal genes (Calb2, enriched in cerebellum; Rorb, enriched in cortical brain; Drd1a, enriched in striatum; and Sitr4, enriched in hippocampus) were assessed in the present regional brain homogenates. This showed the expected enrichment of Calb2 in cerebellum, Rorb in cerebral cortex, Sitr4 in hippocampus and Drd1a in striatum, and was comparable to the regional pattern reported in the Allen Brain Atlas51 (http://mouse.brain-map.org/) (Supplementary Fig. 1d). Images shown in Supplementary Figure 1 were available at: Calb2, http://mouse.brain-map.org/experiment/show/79556662 (image 78); Drd1a, http://mouse.brain-map.org/experiment/show/332 (image 293); Rorb: http://mouse.brain-map.org/experiment/show/79360296 (image 61); Sitr4: http://mouse.brain-map.org/experiment/show/73636037 (image 234).

Flow cytometry. For routine verification of purified samples, cells resuspended in FACS buffer (0.1% BSA in PBS) were incubated with 1 µg/ml anti-CD16/CD32 (BioLegend, 101301) to block Fc receptors and stained with anti-mouse CD11b-PE (BioLegend, 101207, clone: M1/70), CD45-Pacific Blue (BioLegend, 103125, clone: 30-F11) and F4/80-APC (BioLegend, 123115, clone: BM8). To assess overlap between microglial CD11b and EGFP expression in mixed brain cell suspensions from Csf1r-Egfp mice, samples were stained as above and microglia identified according to their characteristic CD11b+ F4/80+CD45− profile. To measure MHC-II expression on microglia from mixed brain cell suspensions, cells were stained with RPE–Alexa Fluor 750 anti-mouse CD11b (AbD Serotec, MCA47P570T), APC anti-mouse CD45 (BioLegend, 103111) and eFlour 450 anti-mouse MHC class II (eBioscience, 48-5321-80). Flow cytometry was performed using a FACs Aria Illu or LSR Fortessa (Becton Dickinson), and data were analyzed using FlowJo software (FlowJo).

Microglial culture and immunocytochemistry. 1 × 105 purified microglial cells were cultured for 7 d in an 8-well chambered coverslip µ-slide (Ibidi) with DMEM/F12 (Life Technologies) containing 10% FBS and 1% penicillin-streptomycin before fixation with 4% paraformaldehyde. The staining procedure was performed at room temperature. Fixed cells were permeabilised with 0.1% Triton X-100 for 5 min, washed and quenched by adding 0.25% NH4Cl for 5 min. Permeabilized cells were washed and blocked in PBS with 0.1% BSA and 5% donkey serum (Jackson ImmunoResearch) for 1 h before adding the primary antibodies prepared in 2.5% donkey serum in PBS. We used the following primary antibodies for staining: rabbit anti–Iba-1 (Wako Chemicals; 1:200) and biotinylated anti-mouse F4/80 (eBioscience, 1:200). Cells were washed, and secondary antibodies anti-rabbit Alexa Fluor 594 (Life Technologies; 1:1,000) and streptavidin Alexa Fluor 488 (Life Technologies; 1:1,000) were incubated for 1 h before counter staining with DAPI (1 µM) for 5 min. Imaging was performed using a Zeiss LSM 710 inverted confocal microscope. Maximum projection images of Z stacks at 400× magnification objectives are presented.

RNA extraction. Microglia purified from individual brain regions were immediately processed for RNA extraction using the RNeasy Plus Micro Kit (Qiagen). Preliminary experiments showed this method produced the highest yield and quality of RNA. RNA was extracted according to the manufacturer’s instructions, with the exception of the final step where RNA elution was repeated twice with 10 µl RNase-free water. RNA quantities were determined by Nanodrop 1000 (Thermo Fisher Scientific), and RNA quality was assessed using the Agilent Bioanalyzer (Agilent Technologies). RNA was also extracted from regional mixed brain cell homogenates using the RNeasy Midi Kit (Qiagen) following manufacturer’s instructions. All samples passed a quality control threshold (RIN > 8) to proceed to microarray analysis.

Transcriptional profiling using gene expression microarrays. Microarray assays were performed by Edinburgh Genomics, University of Edinburgh. Total RNA was labeled using the IVT Express Kit (Affymetrix). First-strand cDNA was synthesized and converted to double-stranded DNA template for transcription and synthesis of ARNA incorporating a biotin-conjugated nucleotide. aRNA was purified and fragmented before hybridization on Affymetrix arrays. Biotin-labeled aRNA was hybridized to the whole mouse genome HT MG-430PM array plate (Affymetrix) representing >39,000 transcripts, using the GeneTitan multichannel instrument (Affymetrix).

Quantitative PCR. 50 ng of total RNA remaining from the microarray samples was reverse-transcribed using Superscript III Reverse Transcriptase according to the manufacturer’s instructions (Life Technologies). qPCR was performed in a Stratagene MX3005P instrument (Agilent Technologies) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and primer pairs: B2m-f TGGCTCACAATGCTAATTCCCCCA, B2m-r TCTTGATTCACAGTACAGCAG, Gapdh-f TGCATACCTGTTGCTGTC, Gapdh-r GGTAGCCACCCAGCC, Pad6-f ACTGTGACGTTTCTCCAGGC, Pad6-r GATGTGCCAGGAACTT, Camp-r CACCTTTGGGAGAATGTCCA, H2-d1-f TCCGAGATTGTTAAG, H2-d1-r AAACCCCAAGTGCCCTATGAA, Gapdh-f GGTAGCCACCCAGCC, Gapdh-r GATGTGCCAGGAACTT. qPCR cycles were performed as follows: hot-start denaturation cycle 95 °C for 10 min, 40 cycles of amplification of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 1 min. ΔCt (Ct of housekeeping gene, Gapdh, minus Ct of gene of interest) was calculated for each brain region. Data are expressed as the reciprocal of this value.

Bacterial phagocytosis and replication assay. Purified cortical and cerebellar microglia (pooled from 8 mice, 4 months old) were infected with Escherichia coli (K-12 strain). Bacteria were grown in Luria Bertani (LB) broth at 37 °C, 190 r.p.m. for 16 h. Subsequently bacteria were subcultured at 1:1,000 ratio into fresh LB. Bacteria were grown to mid-exponential phase at 37 °C, 190 r.p.m. for 3 h, and growth was monitored at OD600. Bacteria were resuspended in DMEM/F12 (no FBS, penicillin or streptomycin) at 1 × 107 bacteria/ml. 4 × 104 purified microglia were infected with 4 × 107 bacteria, 4 × 107 cells only and 4 × 107 bacteria only served as control. After incubation of microglia with bacteria for 2 h to allow uptake, gentamicin was added to kill extracellular bacteria, enabling intracellular E. coli to be enumerated 1 h and 4 h later. Cells were then washed, lysed with 0.1% Triton X-100 and serial tenfold dilutions plated on
MacConkey agar. Bacterial colonies were counted after 17 h incubation at 37 °C. Data are from triplicate microglial samples with each replicate pooled from eight mice. The microglia-only control yielded no bacteria, and extracellular *E. coli* treated with gentamicin under the same assay conditions completely eliminated bacterial growth.

**Computational analysis and bioinformatics. Analysis of regional heterogeneity in the young adult (4 months old).** Microarray data sets were normalized by the robust multivariate averaging (RMA) method in Affymetrix Expression Console (Affymetrix) before analysis in BioLayout Express3D (ref. 20) or directly during import for analysis in Partek Genomics Suite (Partek Inc.). Analysis was performed using the ht_mg-430_pna.na33 annotation release (Affymetrix). 4 month old mouse sample data sets were first normalized and analyzed independently from samples of other ages. The gross interrelationships among regional microglial transcriptomes were assessed by PCA on the log-transformed and Z score–transformed data matrix where every transcript had mean value of 0 and s.d. of 1, using built-in functions of Matlab (MathWorks). Gross regional differences were also assessed in BioLayout Express3D by plotting a sample-to-sample correlation graph with the Pearson correlation threshold $r = 0.96$. Nodes represent individual samples (replicates), and edges between them show correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The resulting network was clustered using the Markov clustering algorithm (MCL) (inflation 2.2) to nonsubjectively sub-divide the graph into discrete clusters.

To assess whether there were transcripts differentially expressed by region overall and between each individual region, normalized data sets were compared in Partek by analysis of variance (ANOVA) with FDR correction ($q < 0.05$). Data were visualized by heatmap with transcripts and samples organized by hierarchical clustering using average linkage with the Euclidean distance metric. Heatmap visualization and hierarchical clustering were performed on the log-transformed and Z score–transformed data matrix using built-in functions of Matlab. To assess gene coexpression relationships across brain regions, a pairwise transcript-to-transcript matrix was calculated in BioLayout Express3D from the set of regionally differentially expressed transcripts using a Pearson correlation threshold $r = 0.80$. A network graph was generated where nodes represent individual probe sets (transcripts/genes), and edges between them correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The graph was clustered into discrete groups of transcripts sharing similar expression profiles using the MCL algorithm (inflation 2.2, minimum cluster size 10 nodes). The composition and functional representation of selected clusters were explored in more detail.

Enrichment analysis for Gene Ontology (GO) terms was performed in DAVID and visualized using the Enrichment Map plugin (http://www.baderlab.org/Software/EnrichmentMap)53 for Cytoscape (http://www.cytoscape.org/)54. In DAVID, gene lists were uploaded, and the GOterm_BP_Fat annotation category was selected. Default settings were used for analysis with enrichment based on $P < 0.05$ with Benjamini correction. Enriched GO terms were uploaded to Enrichment Map, and a network graph was constructed. Nodes represent enriched GO terms and edges the degree of similarity between them using the overlap coefficient. Visualization of GO enrichment was also performed using the GOrilla tool55 (http://cbi-gorilla.cs.technion.ac.il/) with default settings applied. Pathway analysis on individual clusters from the BioLayout Express3D transcript-to-transcript network graph was performed in DAVID using the KEGG tool and Ingenuity Pathway Analysis (Qiagen).

To compare immunophenotypes in the present data set with microglial activation profiles described previously, we mined published microarray data sets from microglia stimulated with either LPS or IL-4 (ref. 25). Raw expression data (.cel files) were downloaded from NCBI GEO Data Sets (GSE49329), imported from microglia stimulated with either LPS or IL-4 (ref. 25) and normalized independently from samples of other ages. The gross interrelationships among regional microglial transcriptomes were assessed by PCA on the log-transformed and Z score–transformed data matrix where every transcript had mean value of 0 and s.d. of 1, using built-in functions of Matlab (MathWorks). Gross regional differences were also assessed in BioLayout Express3D by plotting a sample-to-sample correlation graph with the Pearson correlation threshold $r = 0.96$. Nodes represent individual samples (replicates), and edges between them show correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The resulting network was clustered using the Markov clustering algorithm (MCL) (inflation 2.2) to nonsubjectively sub-divide the graph into discrete clusters. Nodes represent individual samples (replicates), and edges between them show correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The graph was clustered into discrete groups of transcripts sharing similar expression profiles using the MCL algorithm (inflation 2.2, minimum cluster size 10 nodes). The composition and functional representation of the three major clusters were explored in more detail.

Enrichment analysis for Gene Ontology (GO) terms was performed in DAVID and visualized using the Enrichment Map plugin (http://www.baderlab.org/Software/EnrichmentMap)53 for Cytoscape (http://www.cytoscape.org/)54. In DAVID, gene lists were uploaded, and the GOterm_BP_Fat annotation category was selected. Default settings were used for analysis with enrichment based on $P < 0.05$ with Benjamini correction. Enriched GO terms were uploaded to Enrichment Map, and a network graph was constructed. Nodes represent enriched GO terms and edges the degree of similarity between them using the overlap coefficient. Visualization of GO enrichment was also performed using the GOrilla tool55 (http://cbi-gorilla.cs.technion.ac.il/) with default settings applied. Pathway analysis on individual clusters from the BioLayout Express3D transcript-to-transcript network graph was performed in DAVID using the KEGG tool and Ingenuity Pathway Analysis (Qiagen).

To assess whether there were transcripts differentially expressed by region overall and between each individual region, normalized data sets were compared in Partek by analysis of variance (ANOVA) with FDR correction ($q < 0.05$). Data were visualized by heatmap with transcripts and samples organized by hierarchical clustering using average linkage with the Euclidean distance metric. Heatmap visualization and hierarchical clustering were performed on the log-transformed and Z score–transformed data matrix using built-in functions of Matlab. To assess gene coexpression relationships across brain regions, a pairwise transcript-to-transcript matrix was calculated in BioLayout Express3D from the set of regionally differentially expressed transcripts using a Pearson correlation threshold $r = 0.80$. A network graph was generated where nodes represent individual probe sets (transcripts/genes), and edges between them correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The graph was clustered into discrete groups of transcripts sharing similar expression profiles using the MCL algorithm (inflation 2.2, minimum cluster size 10 nodes). The composition and functional representation of the three major clusters were explored in more detail.

Enrichment analysis for Gene Ontology (GO) terms was performed in DAVID and visualized using the Enrichment Map plugin (http://www.baderlab.org/Software/EnrichmentMap)53 for Cytoscape (http://www.cytoscape.org/)54. In DAVID, gene lists were uploaded, and the GOterm_BP_Fat annotation category was selected. Default settings were used for analysis with enrichment based on $P < 0.05$ with Benjamini correction. Enriched GO terms were uploaded to Enrichment Map, and a network graph was constructed. Nodes represent enriched GO terms and edges the degree of similarity between them using the overlap coefficient. Visualization of GO enrichment was also performed using the GOrilla tool55 (http://cbi-gorilla.cs.technion.ac.il/) with default settings applied. Pathway analysis on individual clusters from the BioLayout Express3D transcript-to-transcript network graph was performed in DAVID using the KEGG tool and Ingenuity Pathway Analysis (Qiagen).

To assess the extent of microglial regional heterogeneity in the context of general tissue macrophage diversity, we mined published peritoneal and bone marrow macrophage microarray data sets from the GNF MouseAtlas V3 (GEO: GSE10246), which were generated on the same platform (MOE430 2.0) as the present arrays. All data sets were imported together and normalized in Partek as above. The gross relationships among microglia and macrophages were explored by PCA. Genes most highly expressed in microglia compared to macrophages (>10-fold, $q < 0.05$) were determined for each brain region and overlapping and unique genes among regions identified.

For the identification of transcriptional regulators that may act as contributing factors to the microglial regional diversity, we used Clover27 to detect statistical overrepresentation of known transcription factor binding motifs in the promoter regions of coexpressed genes. Refseq identifiers for each transcript on the Affymetrix ht_mg-430_pna.na33 array that was in the present immune regulatory and energy metabolism cluster were obtained from the NetAffx database (https://www.affymetrix.com/analysis/NetAffx/index.affx). Promoter sequences 300 bp upstream and 100 bp downstream of the transcription start site were extracted from the mouse genome sequence (version mm9). Transcription factor binding site motifs were identified using the JASPAR CORE motif set56 (http://jaspar.genereg.net/) and Clover ($P < 0.05$, score threshold = 6) was used to detect over-represented motifs in promoters for each expression cluster compared with a background set27.

Effect of aging and analysis of interactions between aging and brain region. Analysis was performed on data sets from all ages normalized together and performed as above. Gross interrelationships among regional microglial transcriptomes at different ages (4, 12 and 22 months of age) were assessed by PCA as above and in BioLayout Express3D. For BioLayout Express3D, a sample-to-sample correlation network graph (Pearson correlation threshold $r = 0.98$) was generated from the transcripts differentially expressed according to age (FDR $q < 0.05$) and clustered using the MCL algorithm (inflation 2.2) to nonsubjectively subdivide the graph into discrete clusters. Nodes represent individual samples (replicates), and edges between them show correlation of expression pattern with Pearson correlation coefficients above the selected threshold. To determine effects of aging and interactions between aging and brain region on individual transcript expression, normalized data sets were analyzed in Partek Genomics Suite using two-way ANOVA with FDR correction ($q < 0.05$) and appropriate post-hoc tests as indicated. Hierarchical clustering and visualization of the top 150 transcripts with significant interaction between age and region on expression levels was performed in Partek.

Transcript-to-transcript coexpression relationships were assessed using BioLayout Express3D. A pairwise transcript-to-transcript matrix was calculated from the set of transcripts differentially expressed according to age using a Pearson correlation threshold $r = 0.85$. A network graph was generated where nodes represent individual probe sets (transcripts/genes) and edges between them correlation of expression pattern with Pearson correlation coefficients above the selected threshold. The graph was clustered nonsubjectively into discrete groups of transcripts sharing similar expression profiles using the MCL algorithm (inflation 2.2, minimum cluster size 10 nodes). The composition and functional representation of selected clusters were explored in more detail using approaches as above (for example, GO and KEGG enrichment analysis).

Relative expression profiles of genes from selected immunoreceptor families were visualized by heat map using the log-transformed and Z score–transformed data matrix using built-in functions of Matlab. Genes were classified as activating or inhibitory according to known functional effects or prediction from presence of receptor ITAM-associating/ITIM domains.

**Experimental design and statistical analysis.** Experimental design, analysis and reporting followed the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) where possible. Mice were randomized to treatment group (age) at cage level using a computer-based random number generator (https://www.randomizer.org/). Microarray data are from $n = 4$ biological replicates with each replicate consisting of tissue pooled from 8 mice. No formal *a priori* statistical methods were used to predetermine sample sizes due to insufficient previous
data to enable this. However, sample sizes were chosen based on estimates of anticipated variability through previous general experience of microarray analysis and accounting for pooling of tissues reducing interreplicate variance. To avoid potential confounding cage effects during pooling of tissue, each separate pool contained tissue derived from mice housed in all cages for each age group selected in a randomized manner using a computer-based random number generator (https://www.randomizer.org/). Data collection and analysis were performed with the assessor unaware of allocation to treatment group. Statistical tests for computational analysis are described above. Flow cytometry and qPCR data were analyzed using one-way ANOVA with Bonferroni correction. Data from the bacterial uptake and replication assay were analyzed by two-way ANOVA with Bonferroni correction. Data were checked for compliance with statistical assumptions for each test, including normal distribution and equal variances across groups. Tests were two-tailed throughout. Statistical significance was considered at $P < 0.05$ (or equivalent corrected for multiple comparisons). Data show mean ± s.d. unless otherwise stated.

A Supplementary Methods Checklist is available.

51. Lein, E.S. et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature 445, 168–176 (2007).
52. Huang, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 (2009).
53. Merico, D., Isserlin, R., Stueker, O., Emili, A. & Bader, G.D. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. PLoS One 5, e13984 (2010).
54. Cline, M.S. et al. Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc. 2, 2366–2382 (2007).
55. Eden, E., Navon, R., Steinfield, I., Lipson, D. & Yakhini, Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 10, 48 (2009).