Single-cell RNA sequencing reveals time- and sex-specific responses of spinal cord microglia to peripheral nerve injury and links ApoE to neuropathic pain

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Abstract

Activation of microglia in the spinal cord following peripheral nerve injury is critical for the development of long-lasting pain hypersensitivity. However, it remains unknown whether distinct microglia subpopulations or states contribute to different stages of pain development and maintenance. We show, using single-cell RNA-sequencing, that nerve injury induces the generation of a male-specific inflammatory microglia subtype, and demonstrate increased proliferation of microglia in males as compared to females. We also show time- and sex-specific transcriptional changes in different microglial subpopulations following injury. Apolipoprotein E (*Apoe*) is the top upregulated gene in microglia at chronic time points after nerve injury in mice and polymorphisms in the *APOE* gene in humans are associated with chronic pain. Single-cell analysis of human spinal cord microglia reveals a subpopulation with a disease-related transcriptional signature. Our data provide a detailed analysis of transcriptional states of mouse and human spinal cord microglia, and identify a previously unrecognized role for ApoE in neuropathic pain.

Introduction

Peripheral nerve injury leads to neuropathic pain, a debilitating condition associated with spontaneous and light touch-induced pain, and accompanied by the activation and proliferation of microglia in the spinal cord dorsal horn^{1,2}. Microglia are resident immune cells in the central nervous system (CNS) that constantly survey the environment. Damaged primary afferents release multiple molecules such as chemokines (CCL2, CCL21, CXCL1), signaling molecules (CSF1), and proteases (MMP-9) to induce microglia proliferation and activation, which is characterised by a transition from a ramified homeostatic microglia phenotype with multiple fine processes to an

amoeboid-like cell³. The “activated” microglia release a wide range of bioactive substances that signal to neuronal and non-neuronal cells to facilitate pain transmission, critically contributing to pain hypersensitivity⁴⁻⁷. Microglia play important roles in both the development and maintenance phases of neuropathic pain as inhibition or depletion of microglia 1-2 weeks or three months post-nerve injury alleviate the hypersensitivity^{5,8,9}. The role of microglia in neuropathic pain is known to be sex-dependent^{10,11}. Microglia proliferation and morphological changes in response to peripheral nerve injury are present in both sexes; however, a functional role of microglia as critical drivers of neuropathic pain is observed in male but not in female animals¹²⁻¹⁶.

Progress in single-cell transcriptomic techniques has facilitated the study of microglia heterogeneity in different physiological and pathological processes, revealing that microglia in the brain exist in multiple transcriptional states tailored to different developmental stages, anatomical areas and pathologies¹⁷⁻¹⁹. Nine distinct transcriptional states of microglia have been described in the brain¹⁷, including several subpopulations of homeostatic microglia, microglia responding to injury (injury-responsive microglia; IRM), and to chronic pathologies including Alzheimer’s disease (AD) (disease-associated microglia; DAM)²⁰. The heterogeneity of microglia in the mouse and human spinal cord, however, remains ill-defined. Despite central roles of microglia in the development and maintenance of neuropathic pain³, it is unknown whether peripheral nerve injury leads to the generation of unique microglia transcriptional states that promote and maintain pain hypersensitivity.

In this study, we used single-cell RNA sequencing (scRNA-seq) to define transcriptional states of spinal cord microglia in mouse and human. In mice, by profiling a large number (188,787) of cells of both sexes, we observed that nerve injury-induced changes in microglia differ significantly in acute and chronic phases of neuropathic pain. We detected sex-specific changes in

gene expression, mainly in the acute phase, and identified a subpopulation of microglia that is selectively induced in males, but not in females three days after peripheral nerve injury. We identify *ApoE* as a top upregulated gene in microglia in chronic phases of neuropathic pain in mice. In humans, we show that polymorphisms in the *APOE* gene are correlated with distinct chronic pain conditions. In addition, we profile microglia from the human spinal cord and reveal the presence of cells with disease-related transcriptional signatures. Collectively, our studies identify the dynamic response of microglia to peripheral nerve injury in mice, link ApoE to chronic pain in mice and humans, and provide the first single-cell characterization of human spinal cord microglia.

Results

Microglia single cell transcriptional landscape in neuropathic pain

Recent single-cell RNA sequencing (scRNA-seq) studies of microglia have revealed the vast heterogeneity of microglia transcriptional states during physiological and pathological conditions, identifying distinct signatures of transcriptional responses to acute injury and chronic disease states such as AD^{17,18,20}. To reveal the heterogeneity of microglia at the single cell level in the development and maintenance of chronic pain, we studied microglia in the spinal cord of mice subjected to the spared nerve injury (SNI) assay of neuropathic pain^{21,22} (Fig. 1a). SNI is a common assay of peripheral nerve injury-induced pain, featuring mechanical hypersensitivity that persists for months in both male and female mice (Fig. 1b-c). We collected and analyzed single microglia cells from the lumbar spinal cord of male and female naive mice as well as mice of both sexes at day 3, day 14 and 5 months post-SNI (bilateral) and their age- and sex-matched sham controls (Fig. 1d). The day 3 time point represents an early (acute) pain development phase and corresponds

to the peak of microglia proliferation¹. At day 14, hypersensitivity is fully established, representing a sub-chronic phase of pain development. The 5-month time point was selected to study a chronic phase of neuropathic pain. Microglia from the lumbar spinal cord of naive, sham surgery, and SNI mice (2 replicates per condition, 4 mice pooled per replicate) were FACS sorted (Extended Data Fig. 1), gating for CD45^{low}CD11B^{high}CX3CR1^{high} cells. Purified microglia were processed using the droplet-based 10X Genomics Chromium system, generating high-quality single cell transcriptomic data (Extended Data Fig. 2a-f) from 188,787 sequenced microglia (~13,500 cells per condition after quality control) with a median number of ~1,900 genes per cell at an average sequencing depth of 43,000 reads/cell. Unsupervised clustering analysis for all cells under all conditions combined revealed 11 distinct clusters (Fig. 1e). Cells in all clusters expressed canonical microglia genes such as *Tmem119*, *Fcrls*, *P2ry12*, *Cx3Cr1*, *Trem2*, and *C1qa* (Fig. 1f).

Heterogeneity of spinal microglial transcriptional states

Gene expression analysis identified genes uniquely expressed in microglia in clusters 4, 5, 6, 7, 8, 10, and 11 (Fig. 1g and Supplementary Table 1) and generated a list of genes with the highest expression level in each microglia cluster (top 8 genes in Fig. 1h and Supplementary Table 2), allowing for the investigation of individual cluster identities. Clusters 1-6 grouped together within the uniform manifold approximation and projection (UMAP) plot. Cells in these clusters account for the majority of microglia in naive mice (~98%, inset in 1e). Clusters 7 and 8, together containing ~ 1% of all microglia in naive mice, showed proximity in UMAP plot. High expression of cell cycle-associated markers *Mki67* and *Top2a* in microglia in these but not in other clusters (Extended Data Fig. 3a) suggests that cells in clusters 7 and 8 are proliferating microglia. Gene Ontology (GO) analysis of cluster markers showed that microglia in clusters 7 and 8 are enriched

for DNA replication and cell cycle genes (Extended Data Fig. 3b). Microglia in cluster 9 exhibit a transcriptional signature characterized by increased interferon and cytokine-mediated signaling (Extended Data Fig. 3c). Cluster 9 microglia were absent in naive mice (0.035% of total) but their number was substantially increased 3 days post-SNI in males (4.9%) but not in females (0.03%) (Fig. 2a, b, d and e). Microglia belonging to cluster 9 were largely undetected at day 14 and 5 months post-SNI in both sexes (Fig. 2a-b), demonstrating their nerve injury-induced acute onset and male-specific origin. Cluster 9 microglia can be detected by the combination of two transcripts: the presence of *Lgals1* and absence of *Top2a*. Microglia in cluster 10 uniquely express *Cldn5* that encodes for the tight-junction, protein claudin-5. Claudin-5 is expressed in endothelial cells but also in vessel-associated microglia creating tight junction connections with endothelial cells to maintain the blood-brain barrier²³. Cells in cluster 10 express canonical microglia genes, along with *Cldn5*, indicating that this small population (0.19% of total) represents vessel-associated microglia. Cluster 11 (0.58% of total) was composed of cells expressing canonical microglia genes (*Fcrls*, *Trem2*, *Cx3Cr1*, *Tmem119*, *Clqa* and *P2ry12*) along with unique expression of macrophage/monocyte markers (*H2-Aa*, *Mrc1*, *Ccr2*, *Lyve1*, *Dab2*, *Mgl2*, *F13a1*) (Supplementary Table 1 and Extended Data Fig. 3d). This subpopulation of cells might be composed of perivascular macrophages that express both macrophage and microglia genes. Perivascular macrophages, which are resident brain macrophages, originate from the same pool of yolk sac hematopoietic progenitors, migrate to the brain early in development, and self-renew, similar to microglia^{24,25}. All together, our data show that the majority of microglia in naive spinal cord exist in six transcriptional states (clusters 1-6), in addition to small populations (< 2% of total) of proliferating (cluster 7 and 8), and vessel-associated (cluster 10) microglia.

Nerve injury induces greater proliferation of microglia in males as compared to females

Three days following SNI, the number of microglia in clusters 7 and 8 increased dramatically from 0.5% in male naive mice (5.4% in sham) to 20.8% of all microglia in SNI mice (Fig. 2a, c, and d). Surprisingly, the increase in females was smaller, from 1.7% in naive mice (2.9% in sham) to 13.2% in female SNI mice (Fig. 2b, c, and e), suggesting attenuated proliferation of microglia in females. To corroborate this finding, we labeled the proliferating microglia using a marker of proliferating cells, Ki67, co-stained with a microglia marker, Iba1. Quantification of Ki67-positive microglia in the dorsal horn spinal cord (Fig. 2f-g) showed that the number of proliferating microglia was significantly higher in males as compared to females (Ki67-positive microglia per section; males: 68.8 ± 5.9 ; females: 46.6 ± 1.8 , $p=0.011$, Fig. 2h), confirming the scRNA-seq result. Notably, microgliosis (total number of Iba1-positive microglia) was not different between male and female mice (dorsal horn microglia per section, males: 82.8 ± 5.5 ; females: 76.4 ± 1.8 , $p=0.31$, Fig. 2i), suggesting that other mechanisms compensate for reduced proliferation in females.

Peripheral nerve injury induces profound sex-specific changes in the microglia transcriptome

To study transcriptional changes in microglia following peripheral nerve injury, we identified differentially expressed genes (DEGs) in SNI versus corresponding sham groups in each microglia cluster at three time points in male and female mice (Fig. 3a-e and Supplementary Table 3). We detected a large number of DEGs in male and female microglia at day 3 post-SNI; this number decreased substantially on day 14 and 5 months in both sexes. Analyses of DEGs in each cluster

showed that the vast majority of changes in gene expression occurred in microglia from clusters 1-9 at day 3 post-SNI (Fig. 3c-e) and in clusters 1-6 at later time points.

Interestingly, we observed changes in the expression of numerous ribosomal proteins (small ribosomal subunit (Rps) and large ribosomal subunit (Rpl)) in microglia following SNI, an effect which was significantly more pronounced in males than females (Fig. 3f and Extended Data Fig. 4). Increased expression of ribosomal proteins might promote ribosomal biogenesis and protein synthesis in activated microglia. To obtain insights into the biological processes induced in spinal cord microglia by peripheral nerve injury, we performed GO analysis on DEGs (after removing Rp) in each cluster (Supplementary Table 4) and in clusters 1-6 combined (Fig. 3g). We found that in males, nerve injury induces a strong immune response at day 3 (major GO category: cellular response to cytokine stimulus, inflammatory response, apoptotic process), whereas in females inflammatory processes were less pronounced and top categories included ATF-6-mediated unfolded protein response and receptor-mediated endocytosis (Fig. 3g). At day 14 in males, mitochondrial ATP production and other metabolic processes, including lipid metabolism and trafficking, were the top categories, whereas genes involved in regulation of endocytosis/phagocytosis, cell adhesion, and lipid catabolism were affected in females. At 5 months post-SNI, male microglia showed changes in genes related to mitogen-activated protein kinase (MAPK) signaling, whereas female microglia exhibited changes in metabolic processes and responses to lipopolysaccharide (LPS). Microglia in male-specific cluster 9 at day 3 post-SNI exhibited strong inflammatory responses and showed changes in genes related to mitochondrial ATP production and phagocytosis (Fig. 3h).

Sexual dimorphism of microglia involvement in neuropathic pain has been extensively studied, revealing that microglia are necessary for the development of hypersensitivity in males

but not in females^{3,10,11}. To study the differences in responses of male and female microglia to peripheral nerve injury, we compared DEGs between two sexes. Microglia in males showed more DEGs than females at day 3 post-SNI (Fig. 3a-b), and only a fraction of DEGs was shared between the sexes at each time point (Fig. 3i). Surprisingly, correlational analysis between DEGs in male and female microglia in each of the first six clusters (1-6) on day 3 revealed no or negative correlations (Extended Data Fig. 5a), indicating highly different acute transcriptional responses in male and female microglia to peripheral nerve injury. At later time points (day 14 and 5 months), changes in DEGs in males and females by cluster showed weak to moderately positive correlations (Pearson correlations ranging from $r = 0.12$ – 0.55 on day 14 and from $r = 0.09$ – 0.45 at 5 months post-SNI, Extended Data Fig. 5b-c). Interestingly, we found that MAPK-specific phosphatases *Dusp1* and *Dusp6* are downregulated in clusters 3, 8 and 9 at early (day 3) but not late (day 14 or 5 months) time points post-SNI selectively in male but not female microglia (Extended Data Fig. 6). This finding is consistent with male-specific upregulation of p38 phosphorylation¹⁵, which is central to the activation of microglia following nerve injury^{16,26}. *Il1b* and *Tnf* also showed male-specific upregulation at day 3 post-SNI, whereas *Tspo* was increased in both sexes (Extended Data Fig. 6).

Previous studies have identified several populations of microglia exhibiting unique transcriptional responses to pathological and context-specific physiological conditions. Therefore, we compared, using Fisher's exact test, SNI-induced changes in the microglia transcriptome to previously described transcriptional signatures (Fig. 3j). IRM were identified in the subcortical white matter in response to lysolecithin (LPC)-induced demyelination, a common model of multiple sclerosis (MS)¹⁷. DAM were found in the brain of a mouse model of AD. Axon tract-associated microglia (ATM) were identified as a transient population detected in the pre-

myelinated developing brain in the axon tracts (corpus callosum)¹⁷. Our analysis revealed that the transcriptional landscape in male microglia in clusters 1-6 was similar to IRM at all time points post-SNI (day 3, day 14 and 5 months) and showed mild similarities to DAM and ATM at day 14 (Fig. 3j and Supplementary Table 5). In contrast, changes in gene expression in female microglia were less similar to the assessed transcriptional signatures, exhibiting similarities to IRM, DAM and ATM microglia mainly at day 14 post-SNI in clusters 1, 3, 4, 5 and 6 (Fig. 3j). Interestingly, microglia in male-specific cluster 9 at day 3 post-SNI exhibited a transcriptional profile which was highly similar to IRM as compared to other clusters (Fisher's exact test: $p=5.8 \times 10^{-68}$, Fig. 3j and Supplementary Table 5).

ApoE is upregulated in microglia in chronic phases of neuropathic pain

One of the top genes in both the IRM and DAM transcriptional signatures is that coding for apolipoprotein E (ApoE), which is involved in lipid metabolism and trafficking and implicated in AD pathophysiology²⁷. Our analyses revealed that *ApoE* is the most abundant transcript in cluster 5 spinal cord microglia in both sexes (Fig. 1h, Fig. 4a and Supplementary Table 2). Following SNI in males, *ApoE* mRNA levels showed no change in expression at day 3 after nerve injury but were significantly upregulated at day 14 (top upregulated gene in clusters 1 and 3 and significantly upregulated in clusters 2, 4 and 6) and 5 months (top upregulated gene in clusters 1-6) (Fig. 4a-b). Similarly, *ApoE* remained unchanged at day 3 post-SNI in females and was upregulated at day 14 and 5 months (top upregulated gene in clusters 1-6 at both time points) (Fig. 4c). To study if upregulation of *ApoE* transcript leads to changes in ApoE protein levels, we performed immunostaining against ApoE in the spinal cord after SNI. ApoE levels in microglia, labelled by Iba1, were low at day 3 post-SNI, but increased significantly at day 14 and 8 months (Fig. 4d-e).

These findings demonstrate that ApoE is persistently upregulated in microglia following peripheral nerve injury and suggest that ApoE might have a role in chronic responses of microglia to peripheral nerve injury.

Polymorphisms in *APOE* gene in humans is associated with chronic pain

Persistent upregulation of microglial ApoE after nerve injury in mice prompted us to study the link between *APOE* and chronic pain in humans. Polymorphisms in *APOE* are associated with several chronic diseases of the CNS; for example, it is a major genetic risk factor for late-onset AD²⁷. *APOE* has 3 major haplotype-based allelic variants: $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ (Fig. 5a). *APOE- $\epsilon 4$* confers increased risk²⁸, whereas *APOE- $\epsilon 2$* confers decreased risk²⁹ for AD, as compared to the common *APOE- $\epsilon 3$* allele. We assessed the association of *APOE- $\epsilon 2$* and *APOE- $\epsilon 4$* variants with the report of pain at several body sites in humans in the large UK Biobank cohort (Fig. 5b). Strikingly, we found that whereas *APOE* polymorphisms were not associated with acute pain (Fig. 5c and Supplementary Table 6), they were significantly correlated with distinct chronic pain reports (Fig. 5d and Supplementary Table 6). Carriers of *APOE- $\epsilon 4$* have reduced risk to develop back pain (odds ratio (OR) = 0.30, $p=3.1 \times 10^{-7}$), knee pain (OR = 0.61, $p=0.03$) and headaches (OR = 0.56, $p=0.003$). Conversely, *APOE- $\epsilon 2$* confers increased risk to develop back pain (OR = 1.71, $p=0.002$) and knee pain (OR = 1.48, $p=0.03$) (Fig. 5d). Interestingly, meta-analysis of conditions with a neuropathic component (back, hip, knee and neck/shoulder pain) showed a significant negative correlation with *APOE- $\epsilon 4$* (neuropathic; OR=0.64, $p=6.4 \times 10^{-5}$) and a positive correlation with *APOE- $\epsilon 2$* (neuropathic; OR=1.37, $p=1.4 \times 10^{-4}$), whereas no correlation was found with other conditions. Collectively, our data indicate that, in direct opposition to AD, carriers of *APOE- $\epsilon 4$* have a decreased risk whereas carriers of *APOE- $\epsilon 2$* have an increased risk to develop chronic pain.

Characterization of human spinal cord microglia at single cell level

To elucidate the role of spinal cord microglia in chronic pain and other spinal cord pathologies such as MS and amyotrophic lateral sclerosis (ALS)³⁰, and translate this knowledge to humans, it is imperative to study human spinal cord microglia heterogeneity and transcriptional states. Accordingly, we investigated microglia at the single cell level in human spinal cord samples from two females (53- and 68-years old) and one male (39-years old). Spinal cord tissues were obtained through a rapid autopsy organ donor program from individuals without documented history of chronic pain or neurological disease. Spinal cord tissue was dissociated and processed using droplet-based scRNA-seq, followed by isolation of microglia *in silico* (expressing either *TMEM119*, *ITGAM* (*CD11b* in mouse) or *CX3CR1*), resulting in high-quality transcriptional profiles from 3,823 cells (Extended Data Fig. 7a-b). Unsupervised clustering analysis identified 8 distinct clusters of microglia (Fig. 6a-b, Supplementary Table 7), all expressing canonical microglia markers *TREM2*, and *CIQA*. In addition to the main population of microglia, we observe a cluster of cells expressing mixed microglia-oligodendrocyte genes (cluster 9) and a minor population of T/NK cells (cluster 10). Cluster 1 is the largest microglia cluster, containing >15% of total cells analyzed. This cluster is characterised by high expression of genes associated with ‘homeostatic’ microglia³¹ such as *CSF1R*, *BHLE41*, *FCGR1A*, *CTSS* and *GPR34*. Microglia in cluster 3 are characterized by expression of chemokines *CCL3* and *CCL4*, and the zinc finger containing transcription factors early growth response protein 1 and 2 (*EGR1* and *EGR2*). A recent study of 1,180 human cortical microglia derived from surgically resected temporal lobe tissue identified a similar cluster of cells characterized by high expression of *CCL2*, *CCL4*, *EGR2* and *EGR3*, with the authors denoting these cells as ‘pre-activated’¹⁹. Prior to attaining a mature

surveillant phenotype, pre-microglia express high levels of *EGR1* and are closely associated with synaptic pruning and neural maturation³². Microglia in cluster 3 also express high levels of the complement component C3 which is associated with mediating synapse elimination and the developmental of precise synaptic connectivity^{33,34}. Collectively, the profile of spinal cord microglia in cluster 3 is that of cells interacting with and potentially modulating neuronal structure and function, as suggested by associated GO terms (Fig. 6c). Interestingly, gene expression in cluster 4 microglia overlapped with the IRM/DAM/ATM-like signatures (Fig. 6d and Supplementary Table 8), with a shared common transcriptional signature of 5 core genes: *LPL*, *SPPI1*, *APOE*, *CD63*, and *CTSB*. GO terms associated with cluster 6 highlight functions of ‘translation’, ‘peptide biosynthetic process’ and ‘translational initiation’, suggesting that cells in this cluster are actively generating new proteins (Fig. 6c). High expression of immunoproteasome subunit genes (*PSMA3/A4/A5/A7*, *PSME2*, *PSMB2/B3/B6/B8/B9*, and *PSMD9*) identify microglia in cluster 7 as actively processing antigen for presentation on MHC-I³⁵. Intriguingly, cluster 7 microglia also show expression of genes that cause familial forms of Parkinson’s disease, such as synuclein and protein deglycase DJ-1 (*SNCA* and *PARK7*). Finally, cluster 7 microglia show high expression of *TSPO*, which was shown to be upregulated in mouse microglia at day 3 post-SNI. GO analysis of DEGs in cluster 8 microglia suggest that cells in this cluster are primarily undergoing cell division events. Most significant terms include ‘mitotic cell cycle’ ‘cell division’ and ‘cell cycle phase transition’ (Fig. 6c). Cells in cluster 2 have acquired a transcriptomic signature dominated by immediate early genes, likely induced by the cellular dissociation process. This gene set has previously been identified in murine single cell sequencing studies and includes *DNAJB1*, *HSPA1A*, *DUSP1*, *FOS*, *FOSB*, *JUN*, *HSPA1B*, *HSP90AA1*, *HSP90AB1*, *HSP90B1*,

HSPA8, and *HSPB1*³⁶. These cells cluster together and make up ~15% of the total dataset; all other clusters are relatively unaffected by the processing.

This analysis represents the first report of primary human spinal cord-derived microglia transcriptomic signatures. We observe a strong correlation between human and mouse microglia (Fig. 6e), particularly in human clusters 1-3 and 6 (Fig. 6f). The most striking observation from these data is the identification of cluster 4, which displays remarkable similarity to previously identified transient developmental (ATM) and disease-associated signatures (IRM/DAM) in the mouse brain.

Discussion

In this study, we analysed the heterogeneity of microglia in the mouse and human spinal cord, and investigated microglial responses to peripheral nerve injury in mice at three different time points and both sexes by transcriptionally profiling a large number of microglia using scRNA-seq.

Single cell transcriptional analyses revealed that mouse and human spinal cord microglia exist in numerous heterogeneous subpopulations. Unsupervised clustering analysis of gene expression revealed that the majority of microglia in mice exist in six clusters (1-6), constituting ~98% of all microglia in naive animals. Small populations (less than 1% each) of vessel-associated microglia (cluster 10) and perivascular macrophages (cluster 11) were also detected. Peripheral nerve injury resulted in the appearance of three additional clusters: two subtypes of proliferating microglia (clusters 7 and 8) and IRM-like male-specific microglia (cluster 9). In human spinal cord, eight distinct microglia subpopulations were detected, consistent with a recent study showing that human microglia, collected from brain tissue, exhibit greater heterogeneity as compared to

other species¹⁸. Intriguingly, in the human spinal cord, we detected a large subpopulation of microglia (cluster 4) exhibiting a transcriptional profile highly similar to the previously described mouse disease (IRM/DAM)/early development (ATM) signatures. The identification of IRM/DAM/ATM in tissue isolated from 'healthy' individuals may be indicative of an undiagnosed neurological condition, or suggest that this population, while not observed in the mature mouse brain, may develop in the normally aged human spinal cord.

We found, in accordance with previous studies, a robust inflammatory response in male microglia at acute phase (day 3) following peripheral nerve injury. A large number of genes showed differential expression, many related to immune functions. At day 14 and 5 months post-SNI, the number of DEGs was significantly reduced, and metabolic processes, including energy production and lipid metabolism, were predominantly induced. These data indicate that following initial immune response to injury, there is a transcriptional shift from immuno-reactive microglia towards a metabolically altered state. This phenomenon has been observed in macrophages in response to damage³⁷ and in microglia in other chronic brain pathologies such as AD and MS³⁸, revealing that following prolonged activation, microglia shift from mitochondrial oxidative phosphorylation to glycolysis (Warburg effect) for ATP production^{39,40}, and upregulate genes involved in lipid, cholesterol and lipoprotein metabolism⁴¹.

In females, acute inflammatory responses were less pronounced as compared to males, and predominant processes included endocytosis, cellular metabolism, and cell adhesion. Interestingly, we found that a large number of ribosomal proteins (Rpl and Rps) showed increased expression selectively in male but not in female microglia at day 3 post-SNI. Induction of ribosomal proteins might enhance the microglia's capacity to produce new proteins, which are required during the activation state, via increased ribosomal biogenesis and mRNA translation⁴².

We found that changes in gene expression in response to peripheral nerve injury differ significantly between males and females at day 3 post-SNI but show similarities at later time points. Correlation analysis between DEGs in male and female microglia revealed negative or no correlation between males and female at day 3 post-SNI, but weak-to-moderate correlation at day 14 and 5 months. This is consistent with the divergent acute responses of male and female microglia, wherein microglia in males show stronger immune activation as compared to females.

We observed several additional sex differences of note. A subpopulation of microglia (cluster 9) with a marked inflammatory profile was detected exclusively in males at day 3 post-SNI, but not in females and not at later time points. These microglia, which constitute ~4.5% of all microglia at day 3 in males, show a pronounced IRM signature. We also found that microglia proliferate less in females as compared to males at day 3 post-SNI despite equal microgliosis. Altered expression of genes related to apoptosis in male microglia at day 3 post-SNI raises the possibility that apoptosis and loss of male microglia following their activation is one of the mechanisms accounting for equal microgliosis in males and females.

Our scRNA-seq analysis revealed a robust, across-cluster upregulation of *ApoE* mRNA levels in microglia at late (day 14 and 5 months) but not acute (day 3) time points after nerve injury (Fig. 4). *ApoE* was the top upregulated gene in numerous clusters at day 14 and 5 months. Remarkably, we found a strong link between *APOE* and chronic pain in humans by demonstrating that polymorphisms in *APOE* are associated with chronic, but not acute human pain states (Fig. 5). Interestingly, meta-analysis revealed an association between *APOE* polymorphisms and chronic pain states with a neuropathic component (back, hip, knee, and neck/shoulder pain), whereas no association was detected for pain without a neuropathic component. *APOE-ε4* confers

a decreased risk and *APOE-ε2* an increased risk to develop specific chronic pain conditions. This is opposite to the direction of associations found in individuals with AD, indicating that carriers of *APOE-ε4* have increased risk to develop AD, but decreased risk for developing chronic pain.

ApoE is the most abundant apolipoprotein in the CNS, and is involved in trafficking and metabolism of lipoproteins and cholesterol²⁷. ApoE may promote the efflux of intracellular cholesterol which accumulates in microglia following phagocytosis of dying cells and myelin debris. Lipids in microglia are used as precursors of many inflammatory mediators and as a source of energy, via oxidative metabolism. Therefore, the regulation of lipid metabolism by ApoE may have a direct effect on microglia inflammatory functions and energy production.

In summary, our datasets and analyses represent a comprehensive characterization of spinal cord microglia heterogeneity, providing the basis for elucidating the roles of specific microglia subpopulations and transcriptional states in distinct processes underlying chronic pain and other spinal cord pathologies (e.g., MS and ALS). Detection of a male-specific microglia subpopulation (cluster 9) with a marked inflammatory profile might lead to a better understanding of sex-specific mechanisms and development of targeted therapeutic approaches. The switch from an immune to an altered metabolic state of microglia is a potential mechanism underlying chronic and clinically relevant phases of neuropathic pain. Upregulation of *ApoE*, a gene that we show is strongly associated with human chronic pain conditions with neuropathic component, might be a central mechanism in this switch, and its further examination might generate important insights into the microglia-dependent mechanisms of neuropathic pain.

Online Methods:

Animals and environment

C57BL/6J mice (8-12 weeks of age) of both sexes were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at McGill University animal facility in standard shoebox cages (5 per cage) and maintained at a temperature-controlled environment on a 12:12 h light/dark cycle (lights on at 07:00 h). Food (Envigo Teklad 8604, Lachine, QC, Canada) and water were provided *ad libitum*. All mouse experiments were approved by the Animal Care Committee at McGill University and complied with Canadian Council on Animal Care guidelines. The experimenter was “blind” to the genotype/condition in all studies.

Spared Nerve Injury (SNI) Surgeries

Mice were deeply anesthetized with isoflurane and subjected to bilateral spared nerve injury (SNI) or sham surgeries. SNI surgery was performed as described previously^{21,22}. Briefly, the sciatic nerve was exposed after making an incision on the skin on the lateral surface of the mouse thigh and sectioning through the biceps femoris muscle. Two of the three terminal branches of the sciatic nerve, the tibial and common peroneal nerves, were tightly ligated with 7.0 silk (Covidien, S-1768K) and 2–4 mm of the nerve distal to the ligation were removed, avoiding any disturbance of the sural nerve. The muscle and skin were closed in separate layers using coated Vicryl (Ethicon, J489G). For the sham surgeries, the sciatic nerve, as well as its three branches, were exposed but left intact.

Behavioral studies

All experiments took place during the light cycle, no earlier than 09:00 h and no later than 16:00 h. Naive mice were placed in custom-made Plexiglas cubicles (5.3x8.5x3.6 cm) on a perforated metal floor and were habituated for at least 1 hour before testing. The up-down method of Dixon was used to estimate 50% withdrawal thresholds using calibrated von Frey nylon monofilaments (Stoelting Touch Test). Filaments were applied to the plantar surface of the hind paw for 3 seconds and responses were recorded. At least 2 consecutive measures were taken on each hind paw at each time point and averaged. Although data were collected on both hind paws, only data from the left hind paw (subjected to SNI) are presented, as no significant genotype effects on the contralateral paw were observed. Mice were tested for mechanical sensitivity using von Frey fibers at baseline and 3 days, 14 days, and 5 months after surgery to quantify mechanical allodynia.

Mouse microglia single-cell suspension

Fresh spinal cord samples (L4-L5 segment) were collected at day 3, day 14 and 5 months post-SNI or sham surgery. One biological sample consisted of lumbar segments pooled from four mice. The microglia single-cell suspension protocol was adapted from ¹⁷. Mice were deeply anesthetized and transcardially perfused with ice-cold Hank's balanced salt solution (HBSS). Then, the spinal cords were extracted using 3 mL of HBSS by inserting a 20G needle at the caudal end of the vertebral column. A 5 mm section of the spinal cord (lumbar region) was dissected and put into well plates containing 2 mg ml⁻¹ collagenase type IV (Gibco) in 3 ml DMEM. Spinal cords were minced using scissors and treated with 1 µl of DNase I (Thermo Scientific) for 45 min under incubation at 37 °C. After 20 min into the incubation, the tissue was Dounce homogenized 20 times with the pestle A while simultaneously rotating the pestle. The cell suspensions were then transferred back into the well plate and gently agitated by hand 10 min later. Then, the cell

suspensions were passed through a pre-wet (with DMEM) 70- μ m cell strainer, transferred to a prechilled 50 mL tube and centrifuged for 10 min at 22 °C (400 vcf, acceleration: 2, brake: 1). Samples were washed by gently decanting the supernatant and adding 15-20 mL 1X HBSS followed by centrifugation for 10 min at 22 °C (400 vcf, acceleration: 2, brake: 1). The supernatant was decanted, and the cell pellets re-suspended in 13 mL of 30% Percoll (Sigma) diluted in HBSS at room temperature. The 13 mL of cell suspension were transferred to a 15 mL tube and overlaid with 2 mL of HBSS. Cells were then centrifuged for 20 min at 22°C (400 vcf, acceleration: 1, brake: 0). The debris and Percoll were carefully removed without disturbing the pellet of immune cells at the bottom of the tube. The cell pellet was washed with 15 mL of ice-cold HBSS and centrifuged for 7 min at 4 °C (500 vcf, acceleration: 2, brake: 1). The HBSS was removed from the tubes and the samples were re-suspended in 500 μ l of ice-cold FACS buffer (0.4% BSA (non-acetylated) in 1x PBS, sterile) with CD11b (PE), CD45 (APC-Cy7) and CX3CR1 from Biolegend at a 1:200 dilution for 1 hour on ice and protected from light. Samples were washed in 14 mL of ice-cold HBSS and centrifuged for 7 min at 4 °C (500 vcf, acceleration: 2, brake: 1). Finally, the supernatant was carefully removed and the pellet was re-suspended in 500 μ l of ice-cold FACS buffer. The samples were kept on ice and protected from light until they were ready to sort. Samples were sorted using either a FACSAria III cell sorter equipped with 405 nm, 488 nm and 640 nm lasers and the appropriate filters or a FACSAria Fusion equipped with a 405 nm, 488 nm, 561 nm and 633 nm lasers and the appropriate filters (both from BD Biosciences). In both cases, live, single CD11b⁺, CD45 low, CX3CR1⁺ cells were sorted using a 70 μ m nozzle at 70 psi. Gates were determined using fluorescence minus one samples.

Single-cell RNA sequencing

Around 8,700 cells per sample were loaded into a Chromium Single Cell Chip (10x Genomics). Subsequent reverse transcription and library preparation was performed using the Chromium Single Cell 3' Library & Gel Bead Kit v3 (10X Genomics) following manufacturer instructions. Samples were sequenced to an average depth of 43,000 reads per cell on an Illumina NovaSeq sequencer.

Cell Ranger (v.3.0.1) (10X Genomics) was used for sample demultiplexing, barcode processing, unique molecular identifiers filtering, gene counting, and sample mapping to the reference transcriptome (mouse mm10 v.1.2.0).

scRNA-seq analysis of mouse spinal cord microglia

Raw sequencing data for each sample was converted to matrices of expression counts using the Cell Ranger software provided by 10X Genomics (version 3.0.2). Briefly, raw BCL files from the Illumina HiSeq were demultiplexed into paired-end, gzip-compressed FASTQ files using Cell Ranger's *mkfastq*. Using Cell Ranger's *count*, reads were aligned to the GRCm38 (mm10) mouse reference genome, and transcript counts quantified for each annotated gene within every cell. The resulting UMI count matrices (genes $\times$ cells) were then provided as input to Seurat suite (version 3.1.0). To filter out low-quality cells, we defined a window of a minimum of 500 and a maximum of 4000 detected genes per cell. Cells with more than 5% of the transcript counts derived from mitochondrial-encoded genes were further removed.

To identify sex-mediated differences in response to injury, all microglia samples were merged and analyzed using reciprocal PCA with reference-based integration as part of the Seurat single-cell analysis package^{43,44}. Here we specify one or more of the datasets as the 'reference' for integrated analysis, with the remainder designated as 'query' datasets. In short, PCA was

performed separately for each dataset, following normalization, variable feature selection, and scaling. Integration anchors were identified using both male and female naïve samples as reference (*FindIntegrationAnchors* function with parameter *reduction* = “*rpca*”).

Clustering and visualization of the integrated dataset were performed using Uniform Manifold Approximation and Projection (UMAP), with the first 15 principal components at a resolution of 0.3. Differential expression analysis between two conditions was performed using *FindMarkers* with parameter *min.cells.group* = 20 (minimum of 20 cells in any group being compared). Differentially expressed genes were identified using the cutoffs: $|\log \text{ fold-change}| > 0.25$ and Benjamini-Hochberg FDR < 0.05 . Cluster-specific marker genes were identified using similar cutoffs (*FindAllMarkers* function; up-regulated genes only).

Human spinal cord single-cell suspension

Human spinal cord tissue was harvested from organ donors through a collaboration with Transplant Quebec. All procedures are approved by and performed in accordance with the ethical review board at McGill University (IRB#s A04-M53-08B). Familial consent was obtained for each subject. The rapid autopsy spinal cord samples were processed within 2 hours of removal from the spinal column, tissue was kept on ice throughout. The samples were provided from the lumbar region of the cord (L1-L5) of three adults, two females and one male (see Extended Data Fig. 7). Tissue was processed as previously described⁴⁵. Briefly, the meninges were carefully removed, and the tissue crosscut into small pieces of 1–2 mm³. A single cell suspension was obtained following mechanical and enzymatic (trypsin) dissociation. Myelin was removed using an isotonic Percoll gradient. Glia cells were located between the myelin and red blood cell layers, this layer was collected, washed and resuspended in ice-cold PBS. Finally, a solution of cells (1000 cells per

microliter) was sent for library preparation and high-throughput sequencing through Génome Québec. Library preparation was carried out as follows. In accordance with the Single Cell 3' Reagent Kits v2 User Guide (CG0052 10x Genomics), a single-cell RNA library was generated using the GemCode Single-Cell Instrument (10x Genomics, Pleasanton, CA, USA) and Single Cell 3' Library & Gel Bead Kit v2 and Chip Kit (P/N 120236 P/N 120237 10x Genomics). The sequencing ready library was purified with SPRIselect, quality controlled for sized distribution and yield (LabChip GX Perkin Elmer), and quantified using qPCR (KAPA Biosystems Library Quantification Kit for Illumina platforms P/N KK4824). Finally, the sequencing was done using Illumina HiSeq4000 instrument. Cell barcodes and UMI (unique molecular identifiers) barcodes were demultiplexed and single end reads aligned to the reference genome, GRCh38, using the CellRanger pipeline (10X Genomics). The resulting cell-gene matrix contains UMI counts by gene and by cell barcode.

Cross-species analysis of single-cell transcriptomic data

To compare transcription between species, we extracted one-to-one orthologs using BioMart software suite⁴⁶. Human cells expressing either *TMEM119*, *ITGAM* or *CX3CR1* were included in the analysis. Mouse cells were subsampled to equal number of human cells, resulting in 5,456 cells for each species. Mouse and human microglia samples were analyzed separately using Seurat (version 3.1.0). Briefly, canonical correlation analysis (CCA) was performed to identify shared sources of variation to produce anchors across the datasets, following *SCTransform* normalization. Clustering and visualization of the integrated dataset were performed using UMAP, using *FindClusters* and *RunUMAP* functions.

In order to compare global expression profiles of microglia across species, we aggregated all cell counts within each scRNA-seq sample to generate a pseudo bulk sample, and then analyze the pooled data by using approaches designed for bulk RNA-seq. For all downstream analyses, we excluded lowly-expressed genes with an average read count lower than 10 across all samples, resulting in 10,672 genes in total. Raw counts were normalized using *edgeR*'s *TMM* algorithm⁴⁷ and were then transformed to log2-counts per million (logCPM) using the *voom* function implemented in the *limma* R package⁴⁸. We tested for significantly differentially expressed genes using the *lmfit* function and nominal p-values were corrected for multiple testing using the Benjamini-Hochberg method.

Correlation heatmap was created by obtaining the Pearson correlation of logCPM expression values of all genes between samples. Pearson correlation coefficients were calculated using the *cor* function in R. UMI count data for neurons in the mouse dorsal horn were obtained from the GEO data repository (accession number GSE103840). Gene ontology analyses were conducted using Enrichr^{49,50} for all datasets except Fig. 6, where gProfiler⁵¹ was used. Pearson correlation coefficients between males and females were calculated using the log fold change (logFC) values of genes that are differentially expressed in either sex.

Data availability

Single-cell RNA-sequencing data have been deposited in the Gene Expression Omnibus under the accession GSE162807.

Immunohistochemistry

Mice were anesthetized and perfused intracardially with PBS followed by 4% paraformaldehyde (PFA). Spinal cords were removed from SNI/SHAM Day 3, Day 14, and 5 month post surgery mice, incubated in 4 % PFA at 4 °C for 24 h and then transferred to PBS. Spinal cords were cut transversely into 30 µm thick sections on a Vibratome. Sections were rinsed with PBS and blocked with 10% normal donkey/goat serum and primary antibodies were applied overnight (1:500 Ki67 [Abcam, ab15580], 1:500 Iba1 [Synaptic systems, 234 004], 1:500 Iba1[Wako, 019-19741], 1:100 ApoE [Millipore Sigma,178479]). Secondary antibodies (1:500 Goat anti-guinea pig Alexa Fluor 647 [Thermo Fisher Scientific, A-21450] 1:500 Donkey anti-rabbit Alexa Fluor 568 [Thermo Fisher Scientific. A10042], 1:500 Donkey anti-goat Alexa Fluor 568 [Thermo Fisher Scientific, A-11057], 1:500 Goat anti-rabbit Alexa Fluor 405 [Thermo Fisher Scientific, A-31556] were subsequently applied and tissue was mounted using ProLong Gold Antifade reagent [Thermofisher, P36934]. Images were acquired on a Zeiss LSM 880 confocal microscope equipped with an Argon multiline, 405 diode and HeNe 594 and 633 lasers. Each group consisted of a total of four mice, with 5 sections of spinal cord dorsal horn (ipsilateral) being taken from each mouse. Confocal stacks were taken at 20x magnification (for Ki67), and 63x oil immersion (for ApoE), and processed as maximum intensity projections of confocal z stacks using ImageJ software (v.1.52). IMARIS 3D reconstruction was used alongside representative images of ApoE/Iba1 to show ApoE localization inside of microglia cytoplasm. ApoE images were analysed by calculating Mean Gray Value of respective channels using ImageJ. Ki67/Iba1 values were calculated by raw counting of Ki67 signal and Iba1 signal.

Human genetics

Genetic analyses for the *APOE* gene were conducted in the UK Biobank^{52,53}. Analyses were restricted to the “White British” ancestry, after genotyping quality control performed by the UKB detailed in the resource: https://biobank.ctsu.ox.ac.uk/crystal/crystal/docs/genotyping_qc.pdf. Alleles for SNPs rs7412 and rs429358 were extracted using bgenix (bioArxiv 308296; <https://doi.org/10.1101/308296>) and qctool (https://www.well.ox.ac.uk/~gav/qctool_v2/). The dosage data were converted into hard calls, using a threshold of 0.1 via PLINK⁵⁴. Allelic combinations were assigned one of three possible haplotypes: $\epsilon 2$ (rs7412-T, rs429358-T), the ancestral haplotype $\epsilon 3$ (rs7412-C, rs429358-T), or $\epsilon 4$ (rs7412-C, rs429358-C). Association tests between haplotypes and pain phenotypes were performed using the haplo.cc function in the haplo.stats R computer package (<https://cran.r-project.org/package=haplo.stats>), with minimum counts of haplotypes set to 100, in an additive genetic inheritance model, and relative to the ancestral haplotype. Age, age squared, sex, recruitment sites, genotyping array, and first 40 principal genetic components were used as co-variables. Meta-analyses used an inverse standard-error based analytical strategy, as suggested by METAL⁵⁵. Phenotype assignments for Alzheimer’s diseases derived from UK Biobank field 20002 “Non-cancer illness code, self-reported” code 1263 “dementia/alzheimers/cognitive impairment”, as well as main (field 41202) and secondary (field 41204) ICD10 diagnoses: G30 “Alzheimer’s disease” and sub-codes (G300, G301, G308, G309) for a total of 470 cases and 384054 controls. Cases for chronic pain at a body site were defined as participants answering “yes” to the following question: “Have you had [body site] pains for more than 3 months?”. Body sites included: headaches (UKB field 3799, n=36,381), facial (4067, n=3,495), neck/shoulder (3404, n=63,933), stomach/abdominal (3741, n=18,741), back (3571, n=70,634), hip (3414, n=35,634), knee (3773, n=68,237) and widespread (2956, n=5,259). Cases for acute pain at a body site were defined as participants that answered “no”, which implied that

they had answered “yes” for the question “In the last month have you experienced any of the following that interfered with your usual activities?” (UKB field 6159). Body sites included: headaches (n=43,310), facial (n=3,882), neck/shoulder (n=28,407), stomach/abdominal (n=14,918), back (n=32,209), hip (n=9,967), knee (n=18,316), and widespread (n=1,038). Control subjects were those that answered: “none of the above” at field 6159 (n=163,825).

Statistical analysis

All results are expressed as mean $\pm$ SEM. Statistical tests were made using a one-way ANOVA followed by between-group comparisons using Tukey’s post hoc test or unpaired t-test with $p < 0.05$ as significance criteria (GraphPad Prism 7.03). Fisher's exact test was performed in R, using *fisher.test* function, to evaluate the statistical significance of the overlap between two gene lists.

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Authors Contributions: S.T., S.U., A.U.G., L.H., J.S.M., and A.K. conceived the project, designed experiments and supervised the research. S.T., S.U., A.U.G., and J. T., performed mouse microglia analysis and M.Y., L.H., O.R., L.H., J.O and C. S., performed human microglia analysis. M.P., and L.D., conducted human genetics studies. J.Z., and M.W.S., assisted with study design and interpretation of results. S.T., S.U., A.U.G., L.H., J.S.M., and A.K. wrote the manuscript. All authors reviewed the manuscript and discussed the work.

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Supplementary Table 1. Unique gene markers for each mouse microglia cluster from Fig. 1e. Average log fold change, p-value, and adjusted p-value are shown.

Supplementary Table 2. Gene markers for each mouse microglia cluster from Fig. 1e. Average log fold change, p-value, and adjusted p-value are shown.

Supplementary Table 3. Differentially expressed genes (DEGs) in SNI versus corresponding sham groups, and sham versus naive groups, in each microglia cluster at three time points in male and female mice; as well as DEGs when comparing naive female vs naive male mice in each microglia cluster. Average log fold change, p-value, and adjusted p-value are shown.

Supplementary Table 4. Gene ontology for biological process and molecular function, as well as KEGG enrichment analysis, was conducted using EnrichR^{47,48} with gene identities of the top 40 genes either upregulated or downregulated in the DEG comparison SNI vs. sham group in each mouse microglia cluster after removing Rpl and Rps genes. If there were less than 40 genes in any condition, less were considered for the analysis as shown in the analysis description.

Supplementary Table 5. Statistical significance of gene list overlap, using Fisher's exact test, between DEGs from mouse microglia clusters 1 to 9 at three time points, and microglia transcriptional signature gene lists from Disease-Associated Microglia (DAM), Injury-Responsive Microglia (IRM), and Axon Tract-Associated Microglia (ATM) (¹⁷, Table S2), related to Fig. 3j.

Supplementary Table 6. Association of APOE-ε2 and APOE-ε4 variants with the report of pain, both acute and chronic, and with and without neuropathic component at several body sites (headaches, facial, neck/shoulders, stomach/abdominal, back, hip, knee, and widespread) in humans in the UK Biobank cohort, related to Fig. 5.

Supplementary Table 7. Gene markers for each human microglia cluster from Fig. 6a. Average log fold change, p-value, and adjusted p-value are shown.

Supplementary Table 8. Statistical significance of gene list overlap, using Fisher's exact test, between gene markers from human microglia clusters 1 to 8, and microglia transcriptional signature gene lists from Disease-Associated Microglia (DAM), Injury-Responsive Microglia (IRM), and Axon Tract-Associated Microglia (ATM) (¹⁷, Table S2), related to Fig. 6d.

Figure 1. Spinal cord microglia are present in several distinct subpopulations. **a**, A schematic illustration of the spared nerve injury (SNI) model of neuropathic pain. Paw withdrawal threshold (mean $\pm$ s.e.m.), as measured by von Frey filaments, was decreased following SNI in male (**b**, n=8-12/group) and female (**c**, n=8-12/group) C57BL/6 mice. **d**, Microglia were isolated from the lumbar spinal cord of male and female mice, followed by tissue dissociation, FACS purification and single cell RNA sequencing (scRNA-seq). **e**, UMAP plot reveals that microglia in the mouse spinal cord in all conditions are present in 11 distinct clusters. Inset shows proportion of cells in each cluster in naïve mice. **f**, UMAP plot showing the expression (log-normalized counts) of canonical microglial genes. **g**, Expression of a top unique gene in the indicated cluster and its UMAP plot are shown. **h**, Eight most abundant genes in each cluster are shown. See Supplementary Table 1 for a complete list of unique genes per cluster and Supplementary Table 2 for most abundant genes per cluster.

Figure 2. Peripheral nerve injury induces changes in proportions of specific microglia subpopulations. Proportion of microglia in each cluster in males (**a**) and females (**b**) is shown. Insets show the proportions of cells in clusters 7, 8 and 9. **c**, proportion of microglia in clusters 7 and 8, combined, at day 3 post-SNI. UMAPs of indicated conditions in males (**d**) and females (**e**), highlighting in color cluster 7, 8 and 9. Iba1 (microglia, red) and Ki67 (proliferating cells, green) immunostaining reveals proliferating microglia at day 3 post-SNI in males (**f**) and females (**g**). **h**, Quantification of proliferating microglia (n=4 mice/group). **i**, Quantification of total number of microglia (n=4 mice/group). An unpaired t-test was used to compare two groups. Data are plotted as mean $\pm$ s.e.m.

Figure 3. Changes in gene expression in microglia after peripheral nerve injury. **a**, Number of differentially expressed genes (DEGs) at day 3, day 14 and 5 months in males (left) and females (right). **b**, Number of upregulated and downregulated DEGs in each sex/direction condition. Number of DEGs per cluster at day 3 (**c**), day 14 (**d**), and 5 months (**e**) post-SNI. **f**, Number of upregulated and downregulated large (Rpl) and small (Rps) ribosomal protein subunits is shown. **g**, Gene Ontology (GO) analysis of DEGs (after removing ribosomal proteins) in males and females in each time point for clusters 1-6. The 20 top upregulated DEGs from each cluster were used for the analysis. **h**, GO analysis for cluster 9 at day 3 post-SNI in males. The 40 top upregulated genes were used for the analysis. **i**, Overlap between DEGs in male and female in each condition is shown. See Supplementary Table 3 for complete lists of DEGs per cluster per condition. **j**, Genes in Injury-responsive microglia (IRM), Disease-associated microglia (DAM), and Axon tract-associated microglia (ATM) transcriptional signatures (lists of genes taken from ¹⁷, full lists are in Supplementary Table 5) were compared with DEGs in each condition for cluster 1-6 using Fisher's exact test. Heatmap showing the enrichment for IRM, DAM, and ATM gene expression signature in each condition for cluster 1-6. White indicates no significant overlap ($p > 0.05$). Shades of blue (for p value < 0.05) indicate the significance of the overlap between two gene lists. **k**, Enrichment for IRM signature in cluster 1-9 at day 3 post-SNI in males (Fisher's exact test). See Supplementary Table 5 for numerical values of the analysis.

Figure 4. ApoE is increased in microglia in chronic phases of neuropathic pain. **a**, UMAPs of *Apoe* in males in different conditions. Color codes for *Apoe* log-normalized counts. Tables show fold change (LogFC) and rank of *Apoe* mRNA in the list of DEGs in males (**b**) and females (**c**). **d**, Representative images of immunostaining for ApoE and Iba1 in the spinal cord of male mice after SNI. Bottom images show 3-D reconstruction of ApoE signal (Imaris), demonstrating ApoE co-localization with Iba1. **e**, Quantification of ApoE immunostaining signal in microglia (n=4 mice/condition). Ipsi-D3: 104.8 ± 4.9, contra-D3: 106.1 ± 6.5, ipsi-D14: 179.5 ± 17.3, contra-D14: 111.6 ± 3.9, ipsi-8M: 217.5 ± 4.4, contra-8M: 149.5 ± 11.8. Data are plotted as mean ± s.e.m., one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.

Figure 5. Polymorphisms in *APOE* are associated with chronic pain in humans. **a**, Haplotypes in the *APOE*. The haplotypes are composed of specific alleles of SNPs rs429358 and rs7412, at distinct frequencies in the UK Biobank (UKB) cohort. ε3 is the ancestral haplotype. **b**, A schematic diagram of human body indicating reported pain sites. Haplotypic effects of *APOE* in human acute (**c**) and chronic (**d**) pain. The effects are depicted on the odds ratio (OR) scale, with 95% confidence intervals, for each pain sites (circles), for inverse standard-error weighted meta-analyzed results (meta, lozenges), and for Alzheimer's disease (AD, triangle). Meta-analyzed groups are: conditions with neuropathic component (neur; red, comprised of neck/shoulder, back, hip and knee pain sites), without neuropathic component (non-neur; blue, comprised of headaches, facial, stomach/abdominal and widespread pain sites), and all sites combined (comb, white). From left to right; effects of ε2 and ε4 in acute pain states, and ε2 and ε4 in chronic pain. Significant p values (*p≤0.05) for AD are: ε2 p=1.5x10⁻⁷ and ε4 p=1.4x10⁻⁸¹. Significant p values for meta-analyzed results of chronic pain states are: ε2 for neuropathic pain sites p=1.4x10⁻⁴, ε4 for combined pain sites p=4.5x10⁻³ and neuropathic pain sites p=6.4x10⁻⁵. Significant p values for individual sites in chronic pain are: ε2 back pain p=2.4x10⁻³ and knee pain p=2.6x10⁻², ε4 headaches p=3.1x10⁻³, back pain p=3.1x10⁻⁷ and knee pain p=3.2x10⁻². See Supplementary Table 6 for details of the analyses.

Figure 6. scRNA-seq analysis of human spinal cord microglia. **a**, UMAP plot reveals that cells in the human spinal cord are present in 8 unique clusters (#1-8), plus two additional non-microglia clusters (#9-10). Inset shows the proportion of cells in each cluster. Expression (log-normalized counts) of canonical marker TREM2 and C1QA shown on top. **b**, Heatmap showing expression of top 8 highly expressed genes in each cluster. Expression of genes is represented using a z-score value in which yellow indicates higher expression and purple indicates lower expression. See Supplementary Table 7 for a complete list of clusters markers. **c**, Alluvial plot depicting the most affected biological processes for each cluster. Upregulated genes of each cluster were used for the analysis. Ribbon thickness indicates the number of genes per biological term. P value for each term is shown in bracket. **d**, Heatmap showing the enrichment for IRM, DAM, and ATM gene expression signature in cluster markers for each cluster (Fisher's exact test). White indicates no significant enrichment ($p > 0.05$). Shades of blue (for p values < 0.05) indicate the significance of the overlap between two gene lists. See Supplementary Table 8 for numerical values and a list of shared genes. **e**, Heatmap showing correlation of transcriptomes (treated as bulk) of human microglia, mouse microglia and mouse neurons (neuron dataset taken from ⁵⁶). Pearson correlation coefficient (r) is color coded by shades of blue. **f**, Analysis of overlap between human and mouse microglia highly-expressed transcripts for each cluster (Fisher's exact test). The color-coded p -values indicate the significance of overlap. White indicates no significant overlap ($p > 0.05$).

Extended Data Fig. 1. Fluorescence-activated cell sorting of mouse microglia.

a, Forward scatter of cells (y axis is height and x axis is width) to determine singlets. **b**, DAPI staining on y-axis, singlets on x-axis to determine live cells. Negative staining of DAPI are live cells. **c**, Side-scatter plotted against forward scatter to determine similarity of size and complexity of singlets. **d**, Singlets are carried forward with CX3CR1⁺ (high) staining and CD45 (low) staining. **e**, Singlets are carried forward with CX3CR1⁺ (high) staining and CD11b⁺ (high) staining. Microglial cells are subsequently sorted with these criteria. **f**, Microglia are 13.3% of all events.

Extended Data Fig. 2

Extended Data Fig. 2. Sequencing metrics. Bar graphs showing metrics of all cells sequenced prior to filtering. **a**, Mean number of reads per cell by sample. **b**, Percentage of sequencing saturation by sample. **c**, Total number of genes detected per cell by sample. **d**, Median number of genes detected per cell by sample. **e**, Median number of UMI counts per cell by sample. **f**, Number of cells sequenced by sample estimated by Cell Ranger (10X Genomics).

Extended Data Fig. 3

Extended Data Fig. 3. Gene expression and GO analysis show heterogeneous transcriptional states in spinal microglia. **a**, Violin plots showing the expression of *Mki67* and *Top2a* transcripts across clusters. GO terms (biological process) enriched in markers from clusters 7 and 8 (**b**), and the top 40 markers from cluster 9 (**c**). The p-values were corrected for multiple testing using the Benjamini-Hochberg method. **d**, Violin plots showing the expression of *H2-Aa* and *Mrc1* transcripts across clusters.

		Upregulated Rpl genes						Upregulated Rps genes						Downregulated Rpl genes						Downregulated Rps genes					
Sex	Cluster / Time point	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Males	3 days post-SNI	7	0	15	10	3	14	5	2	17	5	3	16	0	1	0	0	0	0	1	2	0	0	2	0
	14 days post-SNI	0	0	0	0	2	6	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	1	
	5 months post-SNI	0	0	0	0	2	0	0	0	0	0	4	0	15	16	12	16	9	11	8	9	5	12	5	4
Females	3 days post-SNI	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	14 days post-SNI	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	5 months post-SNI	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Extended Data Fig. 4. Peripheral nerve injury induces changes in the expression of ribosomal proteins. Number of ribosomal proteins showing differential expression across conditions. Small ribosomal subunit (Rps), large ribosomal subunit (Rpl).

a

Day 3

Cluster 1: $r=0.04$

Cluster 2: $r=0.03$

Cluster 3: $r=0.03$

Cluster 4: $r=-0.14$

Cluster 5: $r=0.15$

Cluster 6: $r=-0.01$

b

Day 14

Cluster 1: $r=0.52$

Cluster 2: $r=0.12$

Cluster 3: $r=0.42$

Cluster 4: $r=0.28$

Cluster 5: $r=0.51$

Cluster 6: $r=0.55$

c

5 month

Cluster 1: $r=0.29$

Cluster 2: $r=0.09$

Cluster 3: $r=0.41$

Cluster 4: $r=0.45$

Cluster 5: $r=0.16$

Cluster 6: $r=0.33$

Extended Data Fig. 5. Correlation between changes in gene expression in male and female microglia. Correlational analysis between differentially expressed genes (DEGs) in male and female microglia in each of the first six clusters on day 3 **(a)**, day 14 **(b)** and 5 months **(c)** post-SNI. Pearson correlation coefficients (r) between males and females were calculated using the log fold change (logFC) values of genes that are differentially expressed in either sex.

Extended Data Fig. 6

Time point		Day 3		Day 14		Month 5	
Gene	Cluster	Males	Females	Males	Females	Males	Females
<i>Dusp1</i>	2			0.266 (3.16E-55)			
	3	-0.342 (1.069E-28)					
	5			0.368 (3.52E-10)			
	6			0.296 (0.007)			-0.269 (4.95E-03)
	9	-0.523 (0.015)					
<i>Dusp6</i>	1		0.490 (4.26E-93)				
	2		0.390 (2.04E-33)				
	3	-0.428 (1.07E-26)	0.354 (7.73E-21)				
	4		0.261 (5.16E-03)				
	7		0.341 (0.010)				
	8	-0.386 (1.141E-04)					
<i>Il1b</i>	1	0.861 (1.40E-59)	0.311 (5.46E-16)		0.280 (7.10E-14)		
	2	0.504 (1.57E-12)			0.282 (2.84E-03)		
	3	0.531 (3.74E-07)	0.449 (2.86E-04)				
	7	0.926 (1.61E-11)					
	8	0.847 (3.21E-06)					
	9	1.70 (3.78E-05)					
<i>Tnf</i>	1	0.471 (4.02E-77)			0.292 (2.81E-20)		
	2	0.280 (3.87E-09)					
	3	0.711 (6.77E-47)					
	6	0.552 (0.008)					
	7	0.537 (5.29E-15)					
	8	0.570 (1.63E-14)					
<i>Tspo</i>	9	0.879 (5.67E-08)					
	1	0.375 (1.29E-69)	0.295 (2.59E-47)				
	2		0.267 (1.02E-23)				
	3	0.419 (1.13E-30)	0.366 (4.63E-21)				
	4	0.376 (3.56E-09)	0.319 (5.16E-05)				
	5	0.366 (0.015)	0.405 (0.001)				
	6			0.486 (2.96E-06)			
	8	0.286 (1.07E-07)		0.698 (0.029)			
	9	0.639 (2.32E-06)					

Extended Data Fig. 6. Expression changes in MAPK and inflammation related genes. Table showing average log-transformed fold changes of MAPK-specific phosphatases *Dusp1* and *Dusp6*, pro-inflammatory genes *Il1b* and *Tnf*, and microglia activation marker *Tspo*. Upregulated genes are shown in red and downregulated in blue. Adjusted p-values are shown in brackets. The p-values were corrected for multiple testing using the Benjamini-Hochberg method.

Extended Data Fig. 7

Extended Data Fig. 7. Information on rapid autopsy human spinal cord material. **a**, Rapid autopsy human spinal cord tissue is provided from the level of the first lumbar nerve (L1) caudally to the *conus terminalis*. Meningeal layer is removed in preparation of tissue for digestion. **b**, Donor demographics and sequencing parameters.