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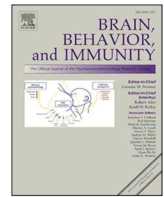

Full-length Article

Microglia undergo molecular and functional adaptations to dark and light phases in male laboratory mice

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ABSTRACT

Microglia are increasingly recognized to contribute to brain health and disease. Preclinical studies using laboratory rodents are essential to advance our understanding of the physiological and pathophysiological roles of these cells in the central nervous system. Rodents are nocturnal animals, and they are mostly maintained in a defined light–dark cycle within animal facilities, with many laboratories investigating the molecular and functional profiles of microglia exclusively during the animals' light (sleep) phase. However, only a few studies have considered possible differences in microglial functions between the active and sleep phases. Based on initial evidence suggesting that microglial intrinsic clock genes can affect their phenotypes, we sought to investigate differences in transcriptional, proteotype and functional profiles of microglia between light (sleep) and dark (active) phases, and how these changes are affected in pathological models. We found marked transcriptional and proteotype differences between microglia harvested from male mice during the light or dark phase. Amongst others, these differences related to genes and proteins associated with immune responses, motility, and phagocytosis, which were reflected by functional alterations in microglial synaptic pruning and response to bacterial stimuli. Possibly accounting for such changes, we found RNA and protein regulation in SWI/SNF and NuRD chromatin remodeling complexes between light and dark phases. Importantly, we also show that the time of microglial sample collection influences the nature of microglial transcriptomic changes in a model of immune-mediated neurodevelopmental disorders. Our findings emphasize the importance of considering diurnal factors in studying microglial cells and indicate that implementing a circadian perspective is pivotal for advancing our understanding of their physiological and pathophysiological roles in brain health and disease.

1. Introduction

Microglia are the main immune competent cells of the central nervous system (CNS) parenchyma (Borst et al., 2021). Besides their role in immunological defense, they are pivotal for brain circuit formation during development and maintenance of CNS functions in adulthood (Borst et al., 2021). The latter aspect has received increasing attention in recent years, as microglial cells have been shown to participate in a vast array of physiological brain functions in favor of proper circuit stability (Borst et al., 2021; Umpierre and Wu, 2021). Microglia constantly move

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their processes, and they can sense their environment via an asset of cell-surface receptors termed the sensome (Hickman et al., 2013; Li et al., 2012; Milior et al., 2020). Hence, they can sense and respond to neuronal activity and promote synaptic plasticity in an intricate neuro-immune crosstalk (Ferro et al., 2021; Marinelli et al., 2019; Umpierre and Wu, 2021).

Preclinical studies using laboratory rodents are essential to advance our understanding of the physiological and pathophysiological functions of microglia cells in brain health and disease. Rodents are nocturnal animals, and they are mostly maintained under in a defined light–dark cycle within animal facilities, with many laboratories performing analyses of microglial functions during the animals' light (sleep) phase. There is, however, evidence indicating that microglial functions differ between active and sleep phases (Deurveilher et al., 2021). For example, several studies found that clock genes such as *Per, Bmal1* (*Arntl*) and *Rev-erbα (Nr1d1),* follow a circadian expression pattern and can influence microglial immune responses and phagocytic functions in rodents (Fonken et al., 2015; Griffin et al., 2020, 2019; Wang et al., 2020). However, these studies only looked at selected microglial genes (Fonken et al., 2015), or they analyzed circadian differences in the response to the bacterial endotoxin, lipopolysaccharide (LPS), via mRNA-sequencing of bulk hippocampal tissue (Griffin et al., 2019), but not in a cell-specific manner. Other studies revealed that microglial morphology and process motility also changes between the light and dark phases in response to circadian fluctuations of noradrenaline levels, (Liu et al., 2019; Stowell et al., 2019). For instance, Stowell et al. (2019) observed that noradrenaline-mediated modulation of microglial process motility during the dark (active) phase is associated with a decrease in synaptic plasticity in the visual cortex of laboratory rodents. In line with these findings, Choudhury *et al.* demonstrated that microglia-mediated phagocytosis of synaptic material is higher during the light (sleep) phase in the prefrontal cortex of adult rats (Choudhury et al., 2020), while Griffin *et al.* found increased microglial synaptic pruning during dark (active) phase in the mouse hippocampus (Griffin et al., 2020). More recently, Corsi *et al.* demonstrated that hippocampal microglia alter their fractalkine receptor (*Cx3cr1*) expression between active and sleep phases, suggesting that circadian factors play a role in modulating microglial activity through neuron-derived signals (Corsi et al., 2022).

Taken together, accumulating evidence indicates that the molecular and functional properties of microglia differ between the active and sleep phases as a result of the influence of clock genes and circadian rhythms. However, a systematic investigation of the transcriptional, proteotype and functional profiles of microglia harvested during the active or sleep phase is still lacking. In the present study, we tested the hypothesis that microglial transcriptional and proteotype changes occur between the light and dark phases, which are accompanied by changes in functional readouts such as synaptic pruning and response to bacterial endotoxins. We further investigated in a diseases model whether microglia display different transcriptional deregulations depending on whether they are collected during the light or dark phase. Our rationale for the latter investigation was that most studies assess microglial functions in diseases models exclusively during the light (sleep) phase, and potential pharmacological strategies are formulated around deregulations observed during this single phase. Hence, investigating possible phase-dependent deregulations holds the potential to advance our understanding of microglia in disease and can open for more precise chrono-pharmacological targeting. Furthermore, a multi-modal comparison of microglial profiles during active and sleep phases would help explaining within- and between-study variability in microglial readouts, which may arise due to differences in sampling hours in relation to differing light-on/off schedules of animal facilities. For these reasons, the present study was designed to evaluate the transcriptional, translational, and functional adaptations of microglia to the active (dark) and sleep (light) phases in laboratory mice. To this end, we focused on hippocampal microglial cells, mainly because the hippocampus is a highly plastic brain region associated with learning and memory processes that are supported by microglial functions (Cornell et al., 2022).

2. Methods

Detailed information regarding the materials and methods used in the present study, including the statistical analyses, is provided in the Supplementary Methods file.

3. Results

3.1. Microglia undergo transcriptional adaptations to active and sleep phases

3.1.1. Changes in microglia-specific genes between the active and sleep phases are accompanied by altered levels of fractalkine and interleukins

First, we compared the expression of microglia-specific genes in freshly isolated hippocampal microglia along with hippocampal protein levels of cytokines and the chemokine fractalkine (CX3CL1) in samples that were collected at 1p.m. (i.e., Zeitgeber time (ZT)16, 4 h after lights were off; = active phase) and 1 a.m. (i.e. ZT4, 4 h after lights were on; = sleep phase) (Fig. 1A). We found that *P2ry6* and *P2ry12*, important for purine-mediated microglial phagocytosis and process motility (Bernier et al., 2019; Koizumi et al., 2007), were significantly increased during the active as compared to the sleep phase (Fig. 1B). On the other hand, *Siglech*, a microglial gene that is relevant for microglial activation and phagocytosis (Konishi et al., 2017), and *Spi1*, a key transcription factor for the microglial myeloid identity, were increased during the sleep phase (Fig. 1B). Next, we compared the hippocampal protein levels of CX3CL1 (fractalkine) between ZT4 and ZT16. CX3CL1 is a chemokine released by neurons to attract microglia, thereby facilitating synaptic pruning (Gunner et al. (2019)a; Paolicelli et al., 2014). We found a small but significant increase in hippocampal CX3CL1 during the active phase (Fig. 1C), consistent with previous findings of increased microglial fractalkine receptor (CX3CR1) expression during the dark phase (Corsi et al., 2022). To further elucidate potential differences in neuroimmune environment between ZT4 and ZT16, we compared the hippocampal protein levels of several cytokines, including IL-6, TNF-α, IL-1β, IL-10 and IL-4, which are relevant for microglial state polarization and important regulators of sleep (Chauhan et al., 2021; Krueger, 2008). We found a significant increase in the anti-inflammatory cytokine IL-4 during the active phase, whereas levels of the pro-inflammatory IL-6 were higher during the sleep phase (Fig. 1C). Hippocampal IL-1β, IL-10 and TNF-α were unaltered between ZT4 and ZT16 (Fig. 1C). Taken together, these results provide evidence suggesting that the basal immune microenvironment and microglia-related transcripts in the hippocampus of laboratory mice differ between light and dark phases.

3.1.2. Microglial cells undergo transcriptional adaptations between the active and sleep phases

Next, we performed unbiased, genome-wide RNA sequencing (RNAseq) using hippocampal microglia collected at ZT16 (i.e., 4 h after lights were off; = active phase) and ZT4 (i.e., 4 h after lights were on; = sleep phase) (Fig. 2A). This RNAseq analysis revealed marked differences between the transcriptomic profiles of microglia collected during the active or sleep phase. Specifically, we found 1328 genes to be upregulated and 231 genes downregulated at ZT4 as compared to ZT16 (Fig. 2B, Supplementary Table S1). The differentially expressed genes (DEGs) enriched at ZT4 were associated with several biological processes (BPs), including cell motility, cell adhesion, extracellular matrix (ECM) organization, and cytoskeleton organization (Fig. 2C, Supplementary Table S1). The DEGs enriched at ZT16 were associated with, amongst others, response to stress, response to cytokine, antigen processing, and toll-like receptor signaling (Fig. 2C, Supplementary Table S1).

In terms of individual DEGs, we found the sleep phase (ZT4) to be

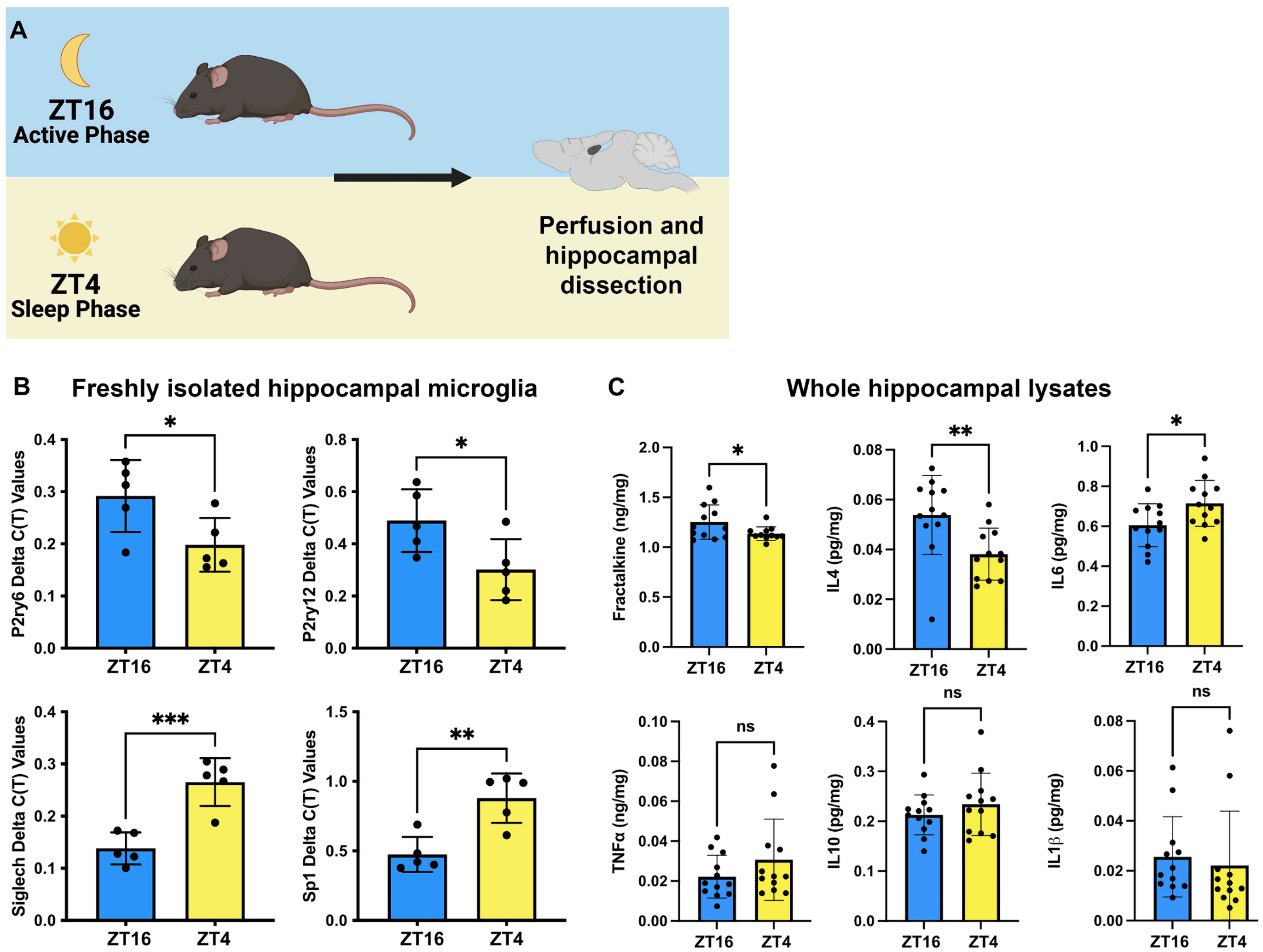


Fig. 1. Changes in microglia-specific genes between the active and sleep phases are accompanied by altered levels of fractalkine and interleukins. (A) Schematic representation of the experimental design. Whole hippocampi were collected from perfused male mice at ZT16 (i.e., 4 h after lights were off; = active phase) and ZT4 (4 h after lights were on; = sleep phase) for subsequent microglial isolation or hippocampal protein extraction. Microglial fractions were used for qPCR analysis of specific microglial genes, while hippocampal lysates were used for ELISA and mesoscale measurements of fractalkine and selected cytokines, respectively. (B) Gene expression analyses of the microglial genes *P2ry6* ($t_{(8)} = 2.44$, $p = 0.041$), *P2ry12* ($t_{(8)} = 2.5$, $p = 0.037$), *Siglech* ($t_{(8)} = 5.16$, $p = 0.0009$) and *Spi1*($t_{(8)} = 4.15$, $p = 0.0032$); $n = 5$ mice/group. (C) Protein levels of fractalkine ($t_{(20)} = 2.4$, $p = 0.026$, $n = 11$ mice/group) and interleukin (IL)-4 ($t_{(22)} = 2.87$, $p = 0.0089$), IL-6 ($t_{(22)} = 2.4$, $p = 0.025$), tumor necrosis factor-α (TNF-α), IL-10 and IL-1β between ZT4 and ZT16 (N = 12 mice/group). Error bars represent the mean ± standard deviation. Unpaired two-tailed *t*-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

associated with increased expression of several extracellular matrix (ECM) remodeling enzymes and proteases, including the matrix metalloproteinases *Mmp2 and Mmp9* (Supplementary Fig. S1). Moreover, we found *Adamts4* and *Mmp14* (Supplementary Fig. S1), which are relevant for ECM-remodeling and synaptic plasticity (Nguyen et al., 2020), to be enriched during the sleep phase (ZT4). *Adam10,* which encodes the enzyme responsible for fractalkine cleavage (Gunner et al. (2019)b), was enriched at ZT16 (Supplementary Fig. S1), concomitant with increased hippocampal fractalkine protein levels (Fig. 1C). Confirming our initial qRT-PCR analysis (Fig. 1B), we also found lower transcript levels of *P2ry12* in microglia collected during the sleep phase, accompanied by decreased expression of *Entpd1* (Supplementary Fig. S2), which encodes for CD39, a membrane bound enzyme responsible for converting extracellular adenosine triphosphate (ATP) and/or adenosine diphosphate (ADP) to adenosine monophosphate (AMP) (Illes et al., 2020). Moreover, the transcripts of various neurotransmitter receptors, including serotonergic, cholinergic, and adrenergic receptors (Supplementary Fig. S3), along with genes relating to cell adhesion, chaperone-mediated protein folding (Supplementary Fig. S4A-D), toll-like receptors (Supplementary Fig. S4E), and innate immune response (Supplementary Fig. S4F, Supplementary Table S2), also displayed phase-specific differences, with the latter two categories displaying enrichment during the active phase (ZT16). Genes crucial for microglial phagocytotic activity (Sierra et al., 2013), including *Dock*10, *Mertk, Rhob, Elmo1, Syk, Hck, Csk, Rac3* and phagocytic receptors (e.g., *Fcgr1*, *Fcgr*3, *Lrp1*) were enriched during the active phase, suggesting that the transcriptional correlates of phagocytotic activity follow a diurnal regulation, (Fig. 3B, Supplementary Fig. S4E-F). Finally, various genes characterizing the disease-associated microglia (DAM) signatures observed in β-amyloid plaque-associated microglia (Grubman et al., 2021) also showed significant transcriptional variation in gene expression between light and dark phases (Supplementary Table S2).

Of note, the transcriptional changes between ZT4 and ZT16 were accompanied by alterations in several microglial clock genes (Fig. 2D), confirming previous reports of circadian and diurnal effects on clock gene expression in microglia (Griffin et al., 2020, 2019; Wang et al., 2020). Taken together, these results demonstrate that hippocampal microglia undergo transcriptional adaptations between light and dark

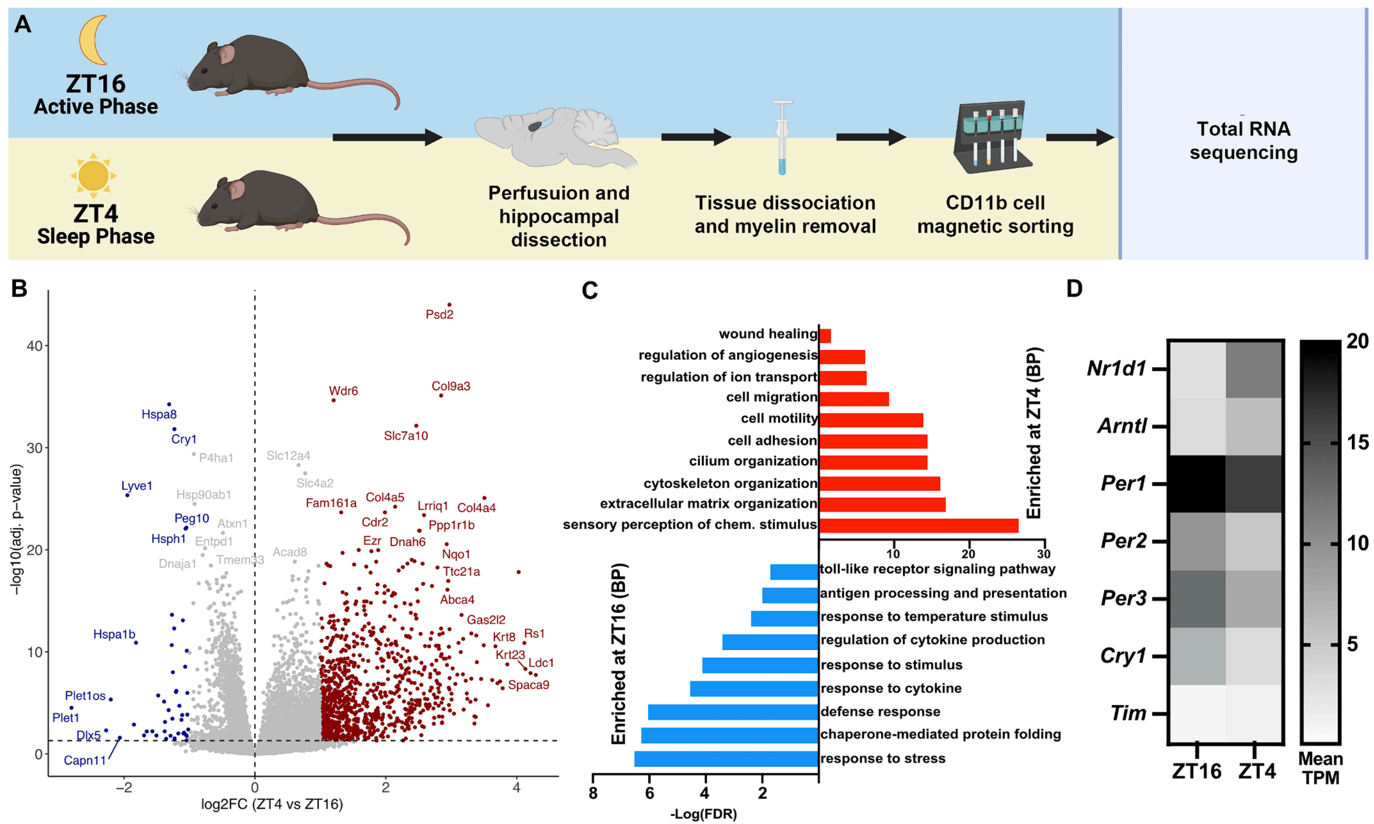


Fig. 2. Microglia undergo transcriptional adaptations between active and sleep phases. (A) Schematic representation of the experimental design. Schematic representation of the experimental design. Whole hippocampi were collected from perfused male mice at ZT16 (i.e., 4 h after lights were off; = active phase) and ZT4 (4 h after lights were on; = sleep phase) for CD11b cell enrichment and subsequent total-RNA-sequencing. (B) Volcano plot representing the differential expression of microglial genes. A total of 1559 genes were found to be differentially expressed between ZT4 and ZT16 using a Log2 fold change cut-off of 0.5 and adjusted p-value of 0.05; $n = 15$–16 animals/group. (C) −Log(FDR) values for selected biological processes (BPs) identified by the gene ontology analysis of up- and downregulated genes. (D) Heatmap displaying changes in clock gene expression in transcripts per million (TPM) between ZT4 and ZT16.

phases in laboratory mice.

3.2. Microglia display increased synaptic pruning during the dark phase

Fractalkine is a “find me” signal that can lead to microglial phagocytosis of synaptic material (Paolicelli et al., 2014). Our protein and gene expression analyses revealed increased levels of fractalkine during the active (dark) phase (Fig. 1C), which was accompanied by an enrichment in phagocytosis-associated genes (Fig. 3B). Based on these data, we hypothesized that mice would show a higher level of synaptic pruning during the active phase.

To test this hypothesis, we quantified the relative amount of synaptic pruning in hippocampal tissue collected during the active (dark) or sleep (light) phase, using a recently established protocol for detecting synaptic material within microglia cells via FACS analysis (Brioschi et al., 2020; Ugursu et al., 2024). Microglia were collected from adult mouse hippocampi 4 h after lights-on time (ZT4, sleep phase) or lights-off time (ZT16, dark phase), as displayed in Fig. 3A. We distinguished microglia cells from peripheral monocytes by the high expression of CD11b and low expression of CD45 (Supplementary Fig. S5). We observed a significant increase in the percentage of microglia that were positive for the excitatory presynaptic marker, vesicular glutamate transporter-1 (vGlut1), during the active (dark) phase (Fig. 3C), accompanied by a significantly higher vGlut1 mean fluorescence intensity (MFI, Fig. 3D-E; Supplementary Fig. S5).

3.3. The microglial response to bacterial LPS changes in intensity between active and sleep phases

3.3.1. Transcriptional response of microglia to LPS

Previous studies demonstrated a higher peripheral immune response to LPS during the sleep than active phase in rodents (Keller et al., 2009). Here, we examined whether the *in vivo* microglial transcriptional response to LPS changes as a function of time of sample collection in the dark and light phases. To this end, LPS was administered to adult male mice either two hours after lights-off time (ZT2; active phase) or lights-on time (ZT14; sleep phase). Hippocampal microglia were collected four hours after LPS or saline injection (i.e., at ZT6 and ZT18 respectively, Fig. 4A).

Total RNAseq revealed that peripheral LPS administration during the active (dark) phase induced a deregulation of 3286 protein-coding genes (Fig. 4B-C, Supplementary Table S3; 1675 up- and 1611 downregulated genes), as compared to vehicle control treatment during this phase. LPS administration during the sleep (light) phase caused a deregulation of 2799 protein-coding genes (Fig. 4D-E, 1674 up- and 1125 downregulated genes; Supplementary Table S3). Although the GO analysis revealed similar pathways to be deregulated at both time points (Fig. 4C, E), the transcriptional response to LPS during the sleep phase showed a stronger enrichment for immune-related pathways such as defense response, response to cytokines, and response to bacterium (Fig. 4C, E). This enrichment was observable also at different Log2FC cut-offs (Supplementary Table S3). These findings indicate increased innate immune reactivity of hippocampal microglia during the sleep phase as compared to the active phase.

To further investigate the influence of time of sample collection on

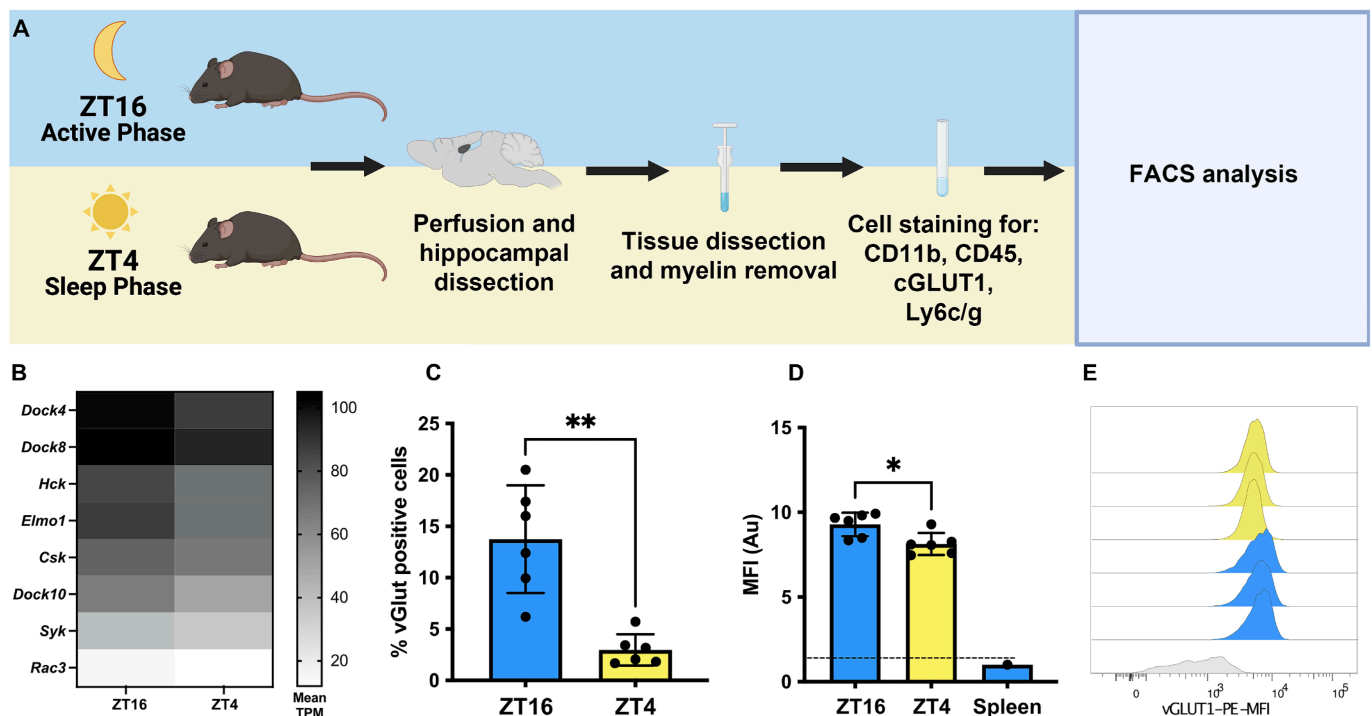


Fig. 3. Hippocampal microglia display increased synaptic pruning during the active phase. (A) Schematic representation of the experimental design. Schematic representation of the experimental design. Whole hippocampi were collected from perfused male mice at ZT16 (i.e., 4 h after lights were off; = active phase) and ZT4 (4 h after lights were on; = sleep phase) for CD11b cell enrichment and subsequent flow cytometry analysis of vGlut1-posive cells. (B) Heatmap showing the differential expression (in average transcripts per million, TPM), for selected genes (with significant differences) associated with microglial phagocytic functions, as analyzed using total-RNA-seq of hippocampal microglia collected at ZT16 or ZT4 (see Fig. 2). (C) Bar plot showing the difference in percentage of vGlut1-positive microglia cells between the active (ZT16) and sleep (ZT4) phases. $t_{(10)} = 4.84$, $p = 0.0031$, n = 6/group. (D) Bar plot showing the area under the curve (Au) for the microglial vGlut1-PE mean fluorescence intensity (MFI) normalized to spleen cells as negative control (represented in grey color). $t_{(10)} = 2.98$, $p = 0.014$, n = 6/group. (E) Histogram showing the area under the curve (Au) for the microglial vGlut1 mean fluorescence intensity (MFI) during the active (4 h after light-off) and sleep (4 h post light-on) phases, normalized to spleen cells as negative control (represented in grey color). Error bars represent the mean ± standard deviation. $^*p < 0.05$, $^{**}p < 0.01$.

the transcriptional response of hippocampal microglia to the peripheral LPS challenge, we contrasted the diurnal effects by subtracting the relative gene expression changes in animals that were treated with LPS during the active (dark) phase from the relative gene expression changes in animals that were treated with LPS during the sleep (light) phase. Hence, this additional analysis was conducted using the formula: Delta = (LPS_ZT18 vs. vehicle_ZT18)-(LPS_ZT6 vs. vehicle_ZT6) (Fig. 4F). We found 56 genes to be specifically regulated by the time of injection (Fig. 4G, Supplementary Table S3), including genes that are associated with the regulation of leukocyte migration, extracellular matrix remodeling and immune activation (Supplementary Fig. S6, Supplementary Table S3).

3.3.2. Proteotype response of microglia to LPS

We used LC–MS/MS analysis of isolated hippocampal microglia to examine whether the *in vivo* microglial response to LPS changes at the level of the proteome as a function of time of sample collection in the light and dark phases. Consistent with the transcriptomic analyses (see Fig. 4), LPS was administered to adult male mice either two hours after light-off time (ZT2; active phase) or light-on time (ZT14; sleep phase). Hippocampal microglia cells were collected four hours after LPS or saline injection (i.e., at ZT6 p.m. and ZT18 respectively, Fig. 5A).

Compared to corresponding vehicle control treatment, LPS administered at ZT2 (active phase) induced a deregulation of 875 microglial proteins (Fig. 5B-C, Supplementary Table S3; 372 up- and 503 downregulated), whereas LPS injection at ZT14 (sleep phase) led to the deregulation of 385 proteins (Fig. 5D-E, Supplementary Table S3; 106 up- and 279 downregulated). Consistent with our findings at the transcriptomic level (Supplementary Table S3), the GO analysis revealed that LPS injection during the sleep (light) phase caused a more extensive enrichment in proteins associated with immune responses, as compared to LPS treatment during active (dark) phase (Fig. 5C,E). Interestingly, however, LPS injection during the active phase induced a stronger enrichment in proteins that are associated with the translation and metabolic processes of RNA, as well as with chromatin remodeling and epigenetic enzymes (Fig. 5C,E, Supplementary Table S3 and S6).

3.4. Basal differences in the microglial proteome between the light and dark phases

To compare the basal proteotype profiles of hippocampal microglia between the active and sleep phases, we analyzed the data from vehicle-treated animals that were used as negative controls in the LPS injection experiment (Fig. 5A). At the proteome level, we identified 481 proteins that were differentially expressed when comparing hippocampal microglia harvested from vehicle-treated animal during active (ZT18) and sleep (ZT6) phases (Supplementary Fig S7A, Supplementary Table S4; 251 up- and 230 downregulated). Proteins enriched during the active (dark) phase annotated to several BPs, including cell motility, cell–cell recognition, response to stimulus, endocytosis, and cytoskeleton reorganization (Supplementary Fig. S7C, Supplementary Table S4).

In terms of individual proteins (summarized in Supplementary Table S5), we found increased levels of the complement component proteins C1QA and C1QC during the active (dark) phase, as were the corresponding transcripts of these complement components (Supplementary Table S5). Increased levels of complement components and fractalkine (Fig. 1C) during the active phase may contribute to the higher rate of synaptic pruning observed during this phase (Fig. 3).

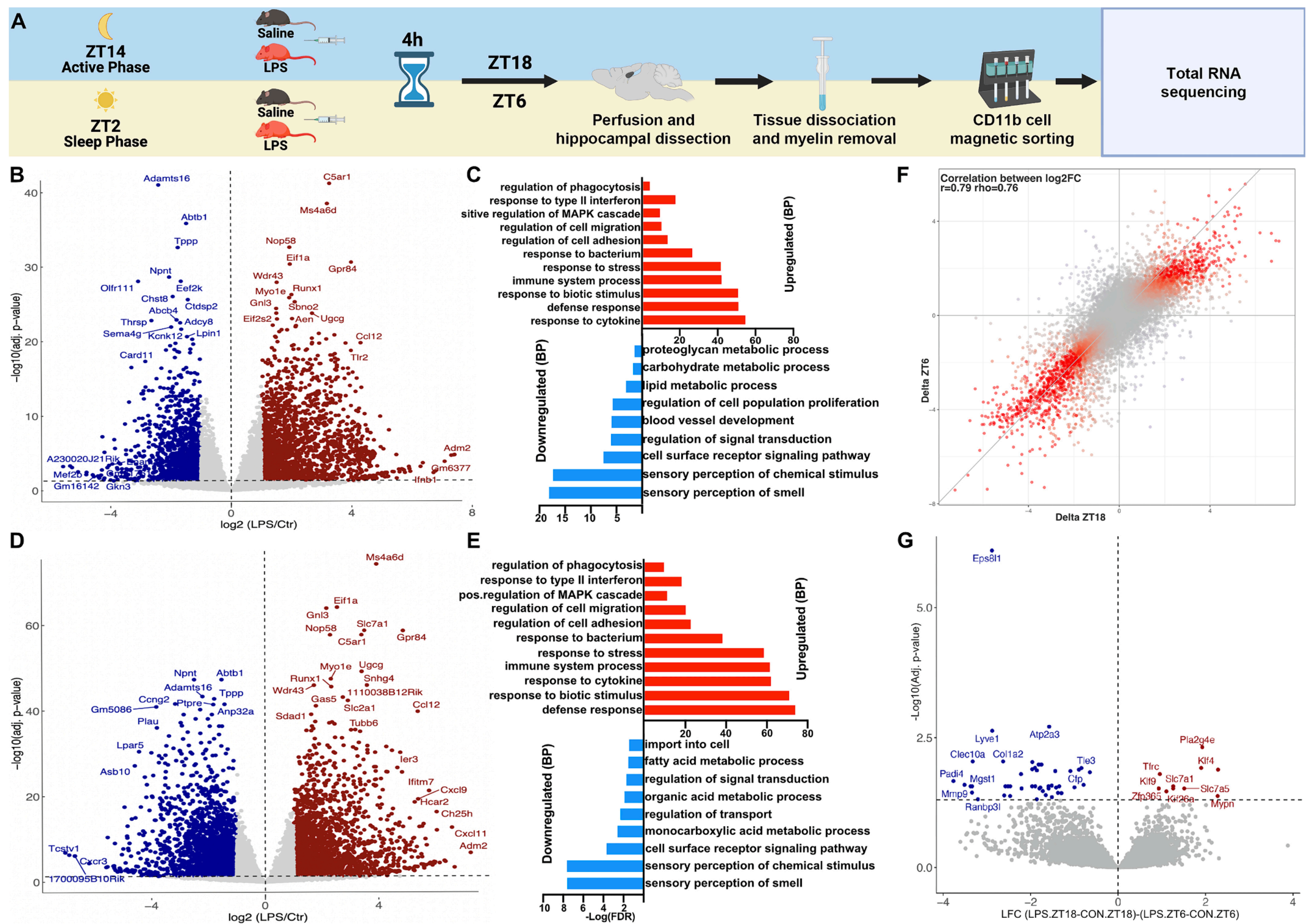


Fig. 4. Transcriptional response of hippocampal microglia to bacterial endotoxin during active and sleep phases. (A) Schematic representation of the experimental design. Adult male mice were injected intraperitoneally with either lipopolysaccharide (LPS) or saline solution at ZT2 (2 h post lights-off, active phase) or ZT14 (2 h post lights-on, sleep phase). Hippocampi were then collected 4 h after the injections for microglial isolation and subsequent total-RNA-sequencing. (B) Volcano plot displaying the differential gene expression of microglia from animals injected with LPS versus saline during the active phase (ZT18, $n = 3$ mice/group). Using a Log2 fold change cut-off of 1 and adjusted p-value of 0.05, a total of 3286 genes were found to be differentially expressed in response to LPS administered the active phase. (C) −Log(FDR) values for selected biological processes (BPs) identified by the gene ontology analysis of up- and downregulated genes in response to LPS during the active phase. (D) Volcano plot displaying the differential gene expression of microglia from animals injected with LPS versus saline during the sleep phase (ZT6, $n =$ 2–3 mice/group). Using a Log2 fold change cut-off of 1 and adjusted p-value of 0.05, a total of 2799 genes were found to be differentially expressed in response to LPS administered during sleep phase. (E) −Log(FDR) values for selected BPs identified by the gene ontology analysis of up- and downregulated genes in response to LPS during the sleep phase. (F) Disco plot for the correlation between the absolute Log2-fold changes in gene expression in response to LPS during the active (x-axis) and sleep (y-axis) phase. (G) Volcano plot showing genes significantly regulated as an effect of time of LPS injection (Adj. $p < 0.05$).

Consistent with this notion, we found that the phagocytic marker CD68 (Supplementary Table S5), as well as several proteins that are relevant for cytoskeletal rearrangements necessary for cell motility and phagocytosis such as paxillin, cofilin-2, CDC42, CD81, MYH10, MYH14, and CCT5 and DOCK10, (Feng et al., 2022; Gitik et al., 2014; López-Colomé et al., 2017; Martins et al., 2019; Porro et al., 2021; Srikakulam and Winkelmann, 1999), were enriched during the active phase, (Supplementary Table S5). Moreover, proteins pertinent to phagosome maturation and lysosomal acidification such as RAB1A *and* ATP6V1A (Prashar et al., 2017; Song et al., 2020) were upregulated during the active phase (Supplementary Table S5).

The corresponding transcripts for the above-mentioned proteins were also enriched during the active phase in most cases, except for *Myh10, Myh14 and Pfn2*, which showed transcriptional enrichment during the light-phase. Some genes displayed no transcriptional changes while being enriched at the protein level during the active phase (Supplementary Table S5). Notably, we also found an enrichment in several mitochondrial proteins along with proteins necessary for microglial glucose and lipid metabolism during the active phase, with most of the corresponding transcripts showing opposite enrichment direction (Supplementary Table S5), suggesting that changes in immunometabolism occur between the active and sleep phases. Finally, we also identified a concomitant upregulation of P2Y12R and CD39 proteins and their transcripts during the active phase (Supplementary Table S5), confirming a diurnal regulation of the microglial purinergic system (Fig. 1B, Supplementary Fig.S2).

On the other hand, proteins enriched at ZT6 (sleep phase) were associated with response to stress, chromatin remodeling, nucleosome organization, gene expression and cell differentiation (Supplementary Fig. S7B, Supplementary Table S4). Accordingly, we found that the light (sleep) phase is associated with protein enrichment for several components of two chromatin remodeling complexes (CRCs), namely the switch/sucrose non-fermentable (SWI/SNF) and nucleosome remodeling and deacetylase (NuRD) complexes (Supplementary Table S5 and S6). These complexes promote rapid changes in the chromatin architecture, enabling innate immune cells to swiftly alter their cellular states to changing microenvironments in a dynamic manner (Gatchalian et al., 2020; Ramirez-Carrozzi et al., 2006). CRC activity itself is guided by

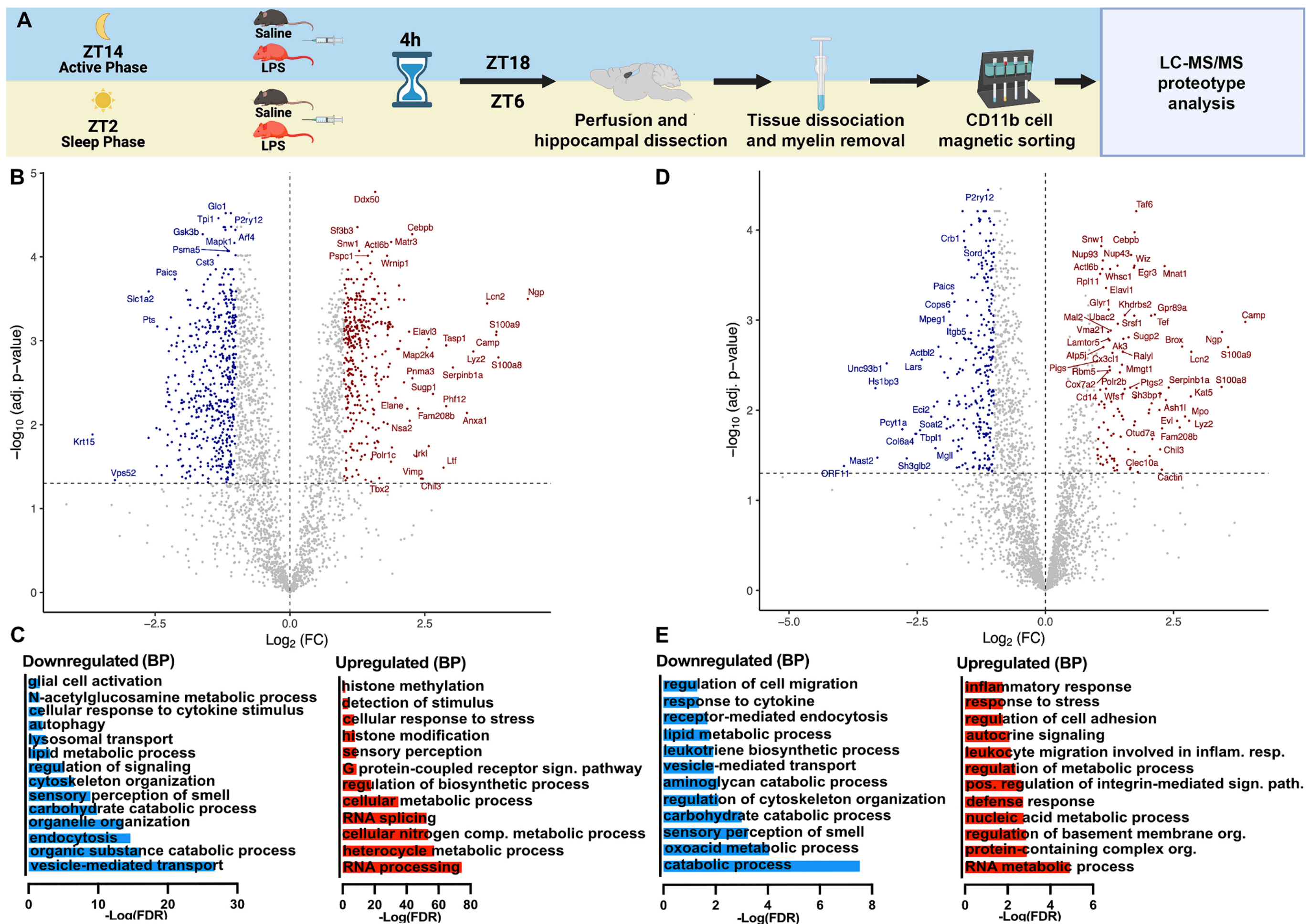


Fig. 5. Proteotype response of hippocampal microglia to systemic bacterial endotoxin during active and sleep phases. (A) Schematic representation of the experimental design. Adult male mice were injected intraperitoneally with either lipopolysaccharide (LPS) or saline solution at ZT2 (2 h post lights-off, active phase) or ZT14 (2 h post lights-on, sleep phase). Hippocampi were then collected 4 h after the injections for microglial isolation and subsequent liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. (B) Volcano plot showing the differentially expressed proteins in response to LPS administered during the active phase (ZT18, $n = 4$ mice/group). Using a Log2 fold change cut-off of 1 and adjusted p-value of 0.05, 875 proteins were differentially expressed 4 h after LPS injection during the active phase. (C) −Log(FDR) values for selected biological processes (BPs) associated with the up- and down-regulated proteins in response to LPS during the active phase. (D) Volcano plot showing the differentially expressed proteins in response to LPS administered during the sleep phase (ZT6, n = 4 mice/group). Using a Log2 fold change cut-off of 1 and adjusted p-value of 0.05, 385 proteins were differentially expressed 4 h after LPS injection during the sleep phase. (E) −Log(FDR) values for selected BPs associated with the up- and down-regulated proteins in response to LPS during the sleep phase.

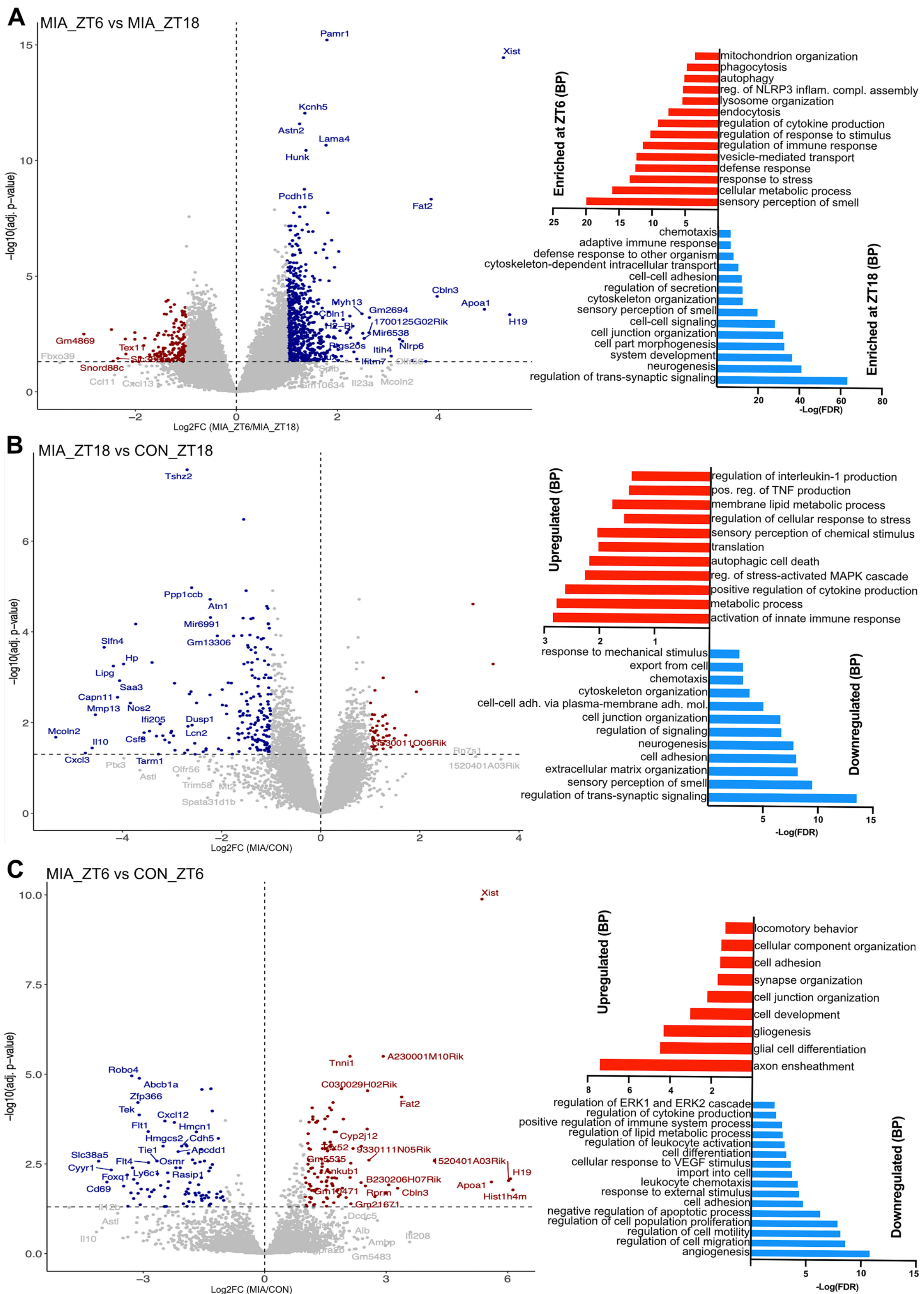
epigenetic readers, i.e., proteins that bind to specific histone and DNA epigenetic marks and recruit CRCs to chromatin. We further found that several epigenetic readers, including chromodomain/helicase/DNA-binding-2 (CHD2) and bromodomain-containing factor 4 (BRD4), which can be part of the NuRD and SWI/SNF complex, respectively, are also enriched in hippocampal microglia collected during the light (sleep) phase (Supplementary Table S5 and S6). Notably, the CRCs and epigenetic readers enrichment at baseline observed during the sleep phase was followed by a stronger immune response to LPS as compared to the active phase (Supplementary Table S6, Fig. 4E, Fig. 5E).

3.5. Influence of the time of sample collection on the microglial transcriptome after maternal immune activation

In a next step, we investigated whether the time of sample collection influences the nature of transcriptomic changes in hippocampal microglia in a mouse model of maternal immune activation (MIA), which is widely used in preclinical research of immune-mediated neurodevelopmental disorders (Brown and Meyer, 2018; Meyer, 2023, 2014). MIA was induced by administration of poly(I:C), whereas control dams received vehicle control (CON) solution only (Supplementary Fig. S8). Using hippocampal microglia collected from adult offspring of poly(I:C)- or vehicle-treated dams, we conducted a within-group comparison of poly(I:C)-exposed offspring in terms of their transcriptomic profile assessed during the light (sleep) and dark (active) phases (i.e., MIA_ZT6 vs. MIA_ZT18), as well as between-group comparisons between poly(I:C)- and vehicle-exposed offspring analyzed during the dark phase (MIA_ZT6 vs. CON_ZT6) or the light phase (MIA_ZT18 vs. CON_ZT18).

We identified 2527 DEGs (Fig. 6A, Supplementary Table S7; 1399 upregulated and 1128 downregulated) when comparing the microglial transcriptional profiles between the sleep and active phases in MIA-exposed animals (i.e., MIA_ZT6 vs. MIA_ZT18). GO-analysis revealed a strong increase in genes related to immune response and inflammation during the sleep phase, while the active phase was enriched in genes annotated to e.g., chemotaxis, cell adhesion, and cytoskeleton organization (Fig. 6A).

A notable influence of time of collection on the microglial transcriptome after MIA was also evident in the between-group comparisons between poly(I:C)- and vehicle-exposed offspring analyzed during the dark (active) phase (MIA_ZT18 vs. CON_ZT18) or light (sleep) phase (MIA_ZT6 vs. CON_ZT6). We identified 901 DEGs (298 upregulated and 603 downregulated) when contrasting the effects of MIA relative to CON



(caption on next page)

Fig. 6. Influence of the time of sample collection on the microglial transcriptome after maternal immune activation (MIA). A schematic representation of the experimental design is provided in Supplementary Figure S8. (A) The Volcano plot displays the differential gene expression of hippocampal microglia collected from MIA offspring during the sleep phase (ZT6) or active phase (ZT18). Using a Log2 fold change cut-off of 0.5 and adjusted *p*-value of 0.05, 2527 genes were found to be differentially expressed when comparing the microglial transcriptional profiles between the sleep and active phases in MIA-exposed animals ($n = 3$ mice/group). The bar plots show −log(FDR) values for selected biological processes (BPs) identified by the gene ontology analysis of up- and downregulated genes in hippocampal microglia that were collected from MIA offspring during the sleep phase (ZT6) relative to active phase (ZT18). (B) The Volcano plot displays the differential gene expression of microglia collected from MIA offspring during the active phase relative to microglia collected from control (CON) offspring during the active phase (ZT18). Using a Log2 fold change cut-off of 0.5 and adjusted *p*-value of 0.05, 901 genes were found to be differentially expressed in MIA relative to CON offspring during the active phase ($n = 3$ mice/group). The bar plots show −log(FDR) values for selected biological processes (BPs) identified by the gene ontology analysis of up- and downregulated genes in hippocampal microglia that were collected from MIA or CON offspring during the active phase (ZT18). (C) The Volcano plot displays the differential gene expression of microglia collected from MIA offspring during the sleep phase relative to microglia collected from control (CON) offspring during the sleep phase (ZT6). Using a Log2 fold change cut-off of 0.5 and adjusted *p*-value of 0.05, 250 genes were found to be differentially expressed in MIA relative to CON offspring during the sleep phase ($n = 2$–3 mice/group). The bar plots show −log(FDR) values for selected biological processes (BPs) identified by the gene ontology analysis of up- and downregulated genes in hippocampal microglia that were collected from MIA or CON offspring during the sleep phase (ZT).

during the dark phase (Fig. 6B, Supplementary Table S7). The upregulated genes were related to immune response, regulation of interleukin (IL)-1 and tumor necrosis factor (TNF) production, and lipid metabolism, whereas the downregulated genes annotated with cell adhesion, extracellular matrix organization, and regulation of synaptic signaling (Fig. 6B). On the other hand, we only identified 250 DEGs (157 upregulated and 93 downregulated) when contrasting the effects of MIA relative to CON during the light phase (Fig. 6C, Supplementary Table S7). During this phase, the upregulated genes were associated with cell development, glia cell differentiation and cell adhesion, whereas the downregulated genes were annotated with angiogenesis, cell motility, chemotaxis, and immune system processes (Fig. 6C).

In an additional analysis, we contrasted the effect of time of collection by subtracting the relative gene expression changes occurring in MIA compared to control offspring (CON) during the active (dark) phase from the transcriptional changes occurring in MIA compared to CON offspring during the light (sleep) phase. Hence, this analysis was conducted using the formula: Delta = (MIA_ZT18 vs. CON_ZT18)-(MIA_ZT6 vs. CON_ZT6). We found that prenatal exposure to poly(I:C) had a significant impact on the expression of microglial genes between phases in adult MIA offspring. Specifically, we identified 1420 protein-coding genes that were affected, with 574 genes upregulated and 845 genes downregulated (Supplementary Fig. S9, Supplementary Table S7). These genes annotated with immune response, phagocytosis, cell adhesion, cytoskeleton organization and neurogenesis, amongst other BPs, (Supplementary Fig. S9, Supplementary Table S7). Indeed, genes associated with innate immune functions, including cytokine production and phagocytosis, were enriched during the active phase (ZT18), whereas genes annotating with cell adhesion and motility were enriched during the sleep phase (ZT6), (Supplementary Fig. S9, Supplementary Table S7).

4.

4. Discussion

Transcriptional and proteomic profiling of microglia using laboratory rodents has become a key aspect of neuroimmunological research. Thus far, however, only a few studies have considered possible differences in microglial functions between the active and sleep phases of laboratory rodents. Based on accumulating evidence suggesting that microglial clock genes can affect their phenotype in a manner that is dependent on the circadian rhythm and diurnal cycles (Griffin et al., 2019; Stowell et al., 2019; Wang et al., 2020), we investigated how the time of sample collection influences the transcriptional, proteotype and functional profiles of hippocampal microglia in male laboratory mice. Consistent with previous findings highlighting an impact of circadian factors on microglial functions, including synaptic pruning, (Deurveilher et al., 2021; Fonken et al., 2015; Griffin et al., 2019; Stowell et al., 2019; Wang et al., 2020), we identified marked transcriptional and proteotype differences between hippocampal microglia that were harvested during the light (sleep) or dark (active) phase. These differences were apparent both under basal conditions and were mirrored by changes in functional readouts, such as phase-dependent alterations in synaptic pruning. Our data are also in agreement with recent studies on peripheral macrophages showing diurnal variation in immune and phagocytic gene and protein expression accompanied by changes in phagocytic activity and immune responsiveness (Blacher et al., 2022; Collins et al., 2021). Moreover, consistent with previous findings obtained in analyses of cultured hippocampal microglia (Fonken et al., 2015), we found that the time of sample collection influenced the molecular response of hippocampal microglia to acute *in vivo* LPS treatment. Finally, we also revealed that the time of sample collection influences the nature of transcriptomic changes in hippocampal microglia in a mouse model of poly(I:C)-based MIA, which is widely used in preclinical research of immune-mediated neurodevelopmental disorders (Brown and Meyer, 2018; Meyer, 2023, 2019, 2014; Meyer et al., 2011). Taken together, our findings emphasize the importance of considering diurnal cycles in studying microglia cells to advance our understanding of their physiological and pathophysiological roles in brain health and disease.

Our data suggest that microglia finetune their motility as a function of the diurnal cycle, which is accompanied by changes in the expression of genes regulating the sleep/wake state. This hypothesis has previously been supported by two studies demonstrating differences in microglial process surveillance between the active and sleep phases in association with phase-dependent changes in the noradrenergic tone (Liu et al., 2019; Stowell et al., 2019). The present work provides a transcriptional and proteotype resource underlying these changes at the molecular level. Intriguingly, we observed that the active phase was associated with an enrichment of transcripts and proteins for the purinergic receptor P2Y12 and the enzyme CD39, the latter of which converts extracellular ATP (or ADP) to AMP (Illes et al., 2020). ATP and ADP are endogenous ligands for P2Y12, prompting microglial processes to elongate towards regions with elevated levels of extracellular ATP/ADP (Entsie et al., 2023; Illes et al., 2020). This response is, for example, observed under conditions of heightened synaptic activity or brain injury (Haynes et al., 2006; Pankratov et al., 2006). Our data suggests that hippocampal microglia might be more responsive to ATP and ADP-mediated stimulation during the active phase, which is possibly linked to increased synaptic activity during wake and the surge in excitatory neuronal ATP occurring in the transition between sleep and wake states (Natsubori et al., 2020). Future studies are warranted to further address these potential diurnal variations in microglial purinergic functions.

Likewise, we found that genes and proteins associated with phagocytic functions were enriched during the active phase, including receptors and components of the complement system. These molecular changes were accompanied by increased hippocampal synaptic pruning during the active phase. Our findings are in accordance with Griffin et al. (2020), who found a higher number of synaptophysin-positive microglial inclusions during the active phase in the hippocampus of adult mice. It is also worth noting that the enrichment of molecular correlates of microglial motility and phagocytosis during the active phase was

paralleled by a concomitant enrichment in proteins related to mitochondrial functions as well as lipid and glucose metabolism. This association is, however, not unprecedented, given the high energy demand required for microglial motility and phagocytosis, (Hu et al., 2022). The microglial immunometabolism is gaining increasing attention for its role in shaping microglial phenotypes and responses in brain health and in disease (Borst et al., 2019). Importantly, while we found correspondence between changes in transcripts and protein levels for some genes between the dark and light phases, we also observed that e.g., metabolic genes followed opposite enrichments at the RNA and protein levels. This suggests potential post-transcriptional regulation of gene expression in microglial functional finetuning between phases, as previously observed in peripheral macrophages, (Collins et al., 2021).

Our study also highlights a potential diurnal regulation in microglia-mediated ECM remodeling. Genes like *Adamts4*, *Mmp14* and *Ctsc*, all of which are pivotal for ECM remodeling (Nguyen et al., 2020), showed altered expression between sleep and active phases. These genes are particularly important for the microglial response to IL-33, promoting ECM remodeling in favor of synaptic plasticity (Nguyen et al., 2020). Additionally, we observed a light/dark-phase difference in the expression of matrix metalloproteinases *Mmp2* and *Mmp9*, which are linked to various brain diseases, including glioma progression (Crapser et al., 2021). Together, our findings underscore the relevance of the diurnal context when studying the physiological and pathological roles of microglia in ECM remodeling.

Another main finding of our study is that the time of sample collection can markedly influence the molecular profiles of microglia in immune-mediated neurodevelopmental disruption models. As an example, we used a mouse model of poly(I:C)-based MIA, which is widely used in preclinical research of immune-mediated neurodevelopmental disorders, such as autism spectrum disorder, schizophrenia, and bipolar disorder (Brown and Meyer, 2018; Meyer, 2023, 2014). Like in any other model system, both planned and unplanned sources of variability exist in this animal model of MIA (Weber-Stadlbauer and Meyer, 2019), contributing to heterogenous short- and long-term outcomes even under stringent experimental conditions. As reviewed in detail elsewhere (Weber-Stadlbauer and Meyer, 2019) the specificity of the effects induced by MIA in laboratory animals is known to be influenced by a number of factors, including immunogen specificity and dosing, prenatal timing, genetic background, age and sex of the offspring, and rearing environment. On the other hand, only little attention has been given to the possible influence of diurnal cycle on the outcomes in MIA models. While our study is the first to demonstrate such an influence on the microglial transcriptome, a recent study from Delorme and colleagues found that prenatal poly(I:C)-induced MIA alters circadian rhythms and leads to altered locomotor activity depending on the prevailing light–dark conditions, (Delorme et al., 2021). Delorme et al. further demonstrated that male MIA offspring is sensitive to environmental circadian disruption in both behavioral and microglial readouts (Delorme et al., 2024, Delorme et al. (2023)).These observations parallel findings showing that the precise time of testing can significantly influence behavioral parameters and gene expression patterns in mice tested under basal conditions (Richetto et al., 2019). In a previous study, we found that hippocampal microglia isolated from adult mice of poly(I:C)-treated dams exhibited a transcriptional downregulation in genes associated with immune system processes, chemotaxis, and vascular endothelial growth factor (VEGF) signaling (Mattei et al., 2017). In that study (Mattei et al., 2017), the animals were maintained using a 12:12 hrs light–dark cycle, and microglia were isolated during the animals' light phase. Thus, our previous study (Mattei et al., 2017) only captured transcriptional differences between MIA-exposed and control offspring during their sleep phase. Importantly, the data presented here are consistent with our earlier findings reported in Mattei et al. (2017) and show a downregulation in genes annotating with immune system processes, chemotaxis and VEGF signaling pathways when microglia were isolated during the animals' light phase. At the same time, however, the present study shows that prenatal poly(I:C) exposure leads to an upregulation of genes annotating with glial cell differentiation, IL1 and TNF production, regulation of stress-activated kinases, and metabolic processes when hippocampal microglia were isolated and analyzed during the offspring's active (dark) phase. Hence, our study identified a notable influence of the time of sample collection on the microglial transcriptome after MIA, underscoring the need to consider diurnal cycles when interpreting and comparing transcriptomic data in this model of immune-mediated neurodevelopmental disorder.

Our study also highlights some molecular candidates that might be important for shaping the transcriptomic and proteotype profiles of microglia in phase-dependent manner. Specifically, we hypothesize that a diurnal regulation in NuRD and SWI/SNF chromatin remodeling complexes (CRCs) may contribute to the transcriptional, proteotype and functional changes between light and dark cycles. CRCs are recruited to open and close chromatin access at specific sites, and they are emerging as important players in immune cell state flexibility (Gatchalian et al., 2020; Ramirez-Carrozzi et al., 2006). A recent study demonstrated that loss of the ARID1A subunit of the SWI/SNF complex leads to a loss of the microglial homeostatic phenotype through changes in chromatin landscape in regions that influence microglial states (Su et al., 2022). Another study found a decrease in both NuRD and SWI/SNF subunit protein expression in aged microglia, which was further associated with reduced state-polarization fluidity observed in aged microglia (Flowers et al., 2017). Here, we found protein enrichment in several CRCs subunits during the sleep phase, when microglia were associated with a stronger immune response to LPS. We further noticed RNA and protein regulation in CRC subunit expression in response to LPS, which may contribute to differential microglial activation states, as shown by previous research (Ramirez-Carrozzi et al., 2006; Roger et al., 2011). Notably, this regulation was stronger during the active phase as compared to the sleep phase, where several CRC subunits were already enriched under basal (non-LPS) conditions. Other factors that could contribute to phase-dependent differences in microglial profiles and functions are regulatory RNAs. We recently found that hippocampal microglia display significant differences in circular RNA (circRNA) expression between ZT4 and ZT16 (Ivanov et al., 2022). One notable example is *Cdr1as*, which is known to act as sponge for microRNAs, thereby regulating gene expression (Ivanov et al., 2022). Importantly, *Cdr1as* has been shown to regulate the expression of several clock genes (Ivanov et al., 2022).

It is crucial to note that the majority of microglial studies are conducted during the light (sleep) phase. Consequently, current immune-pharmacological approaches are designed based on observations from this single phase. Our study suggests that examining microglia during both light and dark phases can enhance our understanding of their role in physiology and disease, paving the way for more precise chrono-pharmacological targeting of immune pathways that are active at specific times. Indeed, applying this concept could provide valuable insights for numerous brain disorders, including Alzheimer's disease (AD), where sleep abnormalities are relatively common (Kuang et al., 2021). In a recent study, Lee et al. (2023) showed that microglial expression of the clock gene *RevErb-α* influences their inflammatory profile and tau phagocytosis. (Lee et al., 2023). Additionally, Grubman et al. (2012) identified specific transcriptional signatures in AD-associated microglia referred to as disease associated microglia (DAM)-genes, which were, however, analyzed during one diurnal phase only (Grubman et al., 2021). In the present study, we found several DAM-genes to be differentially expressed between the light and dark phases. This suggests that microglial transcriptional deregulation in AD may vary depending on the phase of collection, similar to our findings in the MIA offspring model. Therefore, we propose that extending microglial analyses to different diurnal phases in various disease models could greatly enhance our understanding of microglial dysfunctions in brain diseases. The experimental design used in this study is detailed in Supplementary Fig. S11, providing a reference for future research on microglial

assessments in both physiology and disease contexts.

A clear strength of our study was that it included a comparison of the microglial transcriptome, proteome and cellular functions. At the same time, however, we acknowledge a number of imitations. First, in keeping with the high technical demands and costs of our study, we limited our analyses to one sex (males) only. Hence, our study was not designed to capture possible sex differences, which appears important for future studies in light of the known sex differences in microglia biology in health and disease (Hanamsagar et al., 2017). Another limitation of the present study is that we did not perform a complete circadian time-course, but rather had fixed time-points in the light and dark phase. A more detailed resolution of the circadian time-course may be needed to fully capture the influence of the diurnal cycle on the microglial transcriptome and/or proteome. Finally, it is worth noting that since all animals were hosted in a defined dark-light cycle, all tissues for cell isolation were collected separately for the time-points included in our study. This can potentially result in transcriptional and proteomic differences that may be caused by other factors than the time of sample collection *per se*. To minimize such influences, we used a brain dissociation technique that is associated with minimal technical and biological biases (Mattei et al., 2020).

Despite these limitations, we conclude that the time-of-sample-collection markedly influences the transcriptome and proteome of hippocampal microglia in laboratory mice, both under basal conditions and in the context of acute LPS or prenatal MIA exposure. Based on these findings, we believe that differences in timing of sample collection and/or in light schedules of animal facilities may readily contribute to within- and between-study variability in experimental analyses of the microglial transcriptome and/or proteome. Thus, our study underscores the need to consider and report the precise timing of tissue collection for obtaining reproducible results in molecular and functional investigations of microglia.

Author contributions

D.M., A.I., D.B., S.A.W., B.W., H.K. and U.M. conceived and designed the study. D.M., A.I., J.H., P.P., B.U., S.S. J.R., U. W.-S., and J.S. performed the experiments and analyzed the data. D.M., A.I. and U.M. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.07.007.

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