SUPPLEMENTARY METHODS

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

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Supplementary Methods

Animals

Nine to ten weeks old male C57BL6/N mice (Charles River Laboratories, Sulzfeld, Germany) were used throughout the study. They were caged 3-5 animals per cage in individually ventilated cages (IVCs). The animal vivarium was a specific-pathogen-free (SPF) holding room, which was temperature- and humidity-controlled (21 ± 3 °C, 50 ± 10%) and kept under a reversed light–dark cycle (lights off: 09:00 AM–09.00 PM). Hence, all animals were in their active and sleep phases during the experimenters' light (day) and dark (night) phases, respectively. All animals had *ad libitum* access to food (Kliba 3436, Kaiseraugst, Switzerland) and water throughout the entire study. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich, and all efforts were made to minimize the number of animals used and their suffering.

Breeding and maternal immune activation

The maternal immune activation (MIA) model was implemented as previously described (Scarborough et al., 2021). Briefly, successful mating was verified by the presence of a vaginal plug, considered as gestational day 0 (GD 0). Dams were housed individually throughout gestation. On GD 17, pregnant dams were randomly assigned to a single intravenous tail-vein injection of either poly(I:C) (Sigma–Aldrich P9582, Buchs, St. Gallen, Switzerland) to induce MIA, or pyrogen-free 0.9% NaCl (prenatal control). We previously ascertained the quality, molecular composition and immunopotency of the poly(I:C) batch (#086M4045V) used in this study (Mueller et al., 2019). Based on our previous dose–response studies (Mueller et al., 2018) and molecular composition of the poly(I:C) batch (Mueller et al., 2019), poly(I:C) was administered intravenously (i.v.) into the tail vein at a dose of 5 mg/kg. The gestational time point of poly(I:C) or vehicle administration (i.e., GD 17) was selected based on previous studies showing more extensive

abnormalities in hippocampal structure and function when MIA occurs in late gestation as compared to earlier time points (Giovanoli et al., 2015; Meyer et al., 2006, 2008). All injections were given using a volume of 5 ml/kg. Following the injection, the dams were placed back in their home cages and left undisturbed until the first cage change on postnatal day 7. Male offspring were weaned on postnatal day 21 and littermates of the same sex were caged separately and maintained in groups of 4–5 animals per cage.

In vivo LPS treatment

LPS (Enzo Lifesciences, serotype O55:B5, cat. Nr. ALX-581-013-L002) was diluted in sterile saline solution and injected intraperitoneally (i.p.) at a concentration of 1mg/kg. Control animals were injected with saline solution. To avoid batch to batch effects on the microglial immune response, all experiments with LPS were performed within 24h using the same batch of LPS.

Brain dissociation and cell isolation

Brain tissue dissociation and microglia cell isolation were performed according to a recently optimized mechanical dissociation (MD) protocol (Mattei et al., 2020). The protocol was carried out at 4°C to minimize microglial cell activation due to the isolation procedure (Mattei et al., 2020). Briefly, the animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories, North Chicago, IL, USA) and transcardially perfused with ice-cold, calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS). Hippocampi were dissected on a cooled petri dish and placed in ice-cold Hibernate-A medium. MD was carried out on ice with a Dounce homogenizer, using a loose pestle. Myelin debris were removed via percoll gradient centrifugation at 4°C. After washing of the percoll, total brain cell pellets were used for CD11b magnetic-activated cell sorting (MACS) using anti-mouse CD11b magnetic microbeads (Miltenyi) according to the manufacturer’s instructions. The MACS buffer consisted of 3% bovine serum albumin (BSA) diluted in DPBS from a 7.5% cell-culture grade BSA stock (Thermo Fisher Scientfic). Total

hippocampal cell pellets were re-suspended in 90 µl MACS buffer and 10 µl anti-mouse-CD11b magnetic beads. The cells were then incubated for 15 min at 4°C. Cells were washed with 1 ml MACS buffer. The cells were then passed through an MS MACS column (Miltenyi) attached to a magnet. After washing the columns three times with MACS buffer, microglia were flushed from the column with 1 ml MACS buffer and pelleted. Cell pellets were then snap-frozen in liquid nitrogen and stored at -80°C for further analysis. The duration of the entire procedure was on average 2.5 hours. It is therefore crucial for the initial perfusion to be carried out with ice-cold DPBS to ensure that brain cells quickly reach a metabolically inactive temperature, and all further steps are to be carried out on ice to further prevent cellular responses (Mattei et al., 2020) or continued circadian cycling throughout the isolation procedure. **Supplementary Fig. S10** shows the successful enrichment of microglia cells across batches.

Microglia from each individual animals' hippocampi were used in most experiments except for the proteomic analysis, where each sample represents a pool of microglia from the hippocampi of two animals.

Flow cytometry-based intracellular detection of synaptic particles in microglia

We used a recently established protocol (Brioschi et al., 2020) to detect and quantify the synaptic marker, vesicular glutamate transporter 1 (vGLUT1), in microglia by flow cytometry. Following percoll isolation (as described above, section 2.4), total cell pellets were sequentially stained with fixable live/dead staining (1/1000 in PBS, ThermoFisher, #L34969, 30 min) and for the microglial surface markers CD11b (1/100, ThermoFisher Scientific, **#**25-0112-82), CD45 (1/100, BD Biosciences, #559864), Ly6C (1/100, BD Biosciences, #553104) and Ly6G (1/100, BD Biosciences, #551460), and CD16/CD32(1/200, ThermoFisher Scientific, #14-0161-82). Subsequently, stained samples were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to manufacturer's instructions (#554714). Following fixation, intracellular staining for vGLUT1 (1/200, Miltenyi Biotec, #130-120-764, 1h in 1x BD Permeabilization Buffer) was

immediately performed. Samples were acquired on BD FACS Fortessa (BD Bioscience) flow cytometer and microglia population was defined as $CD11b^{++}$/ $CD45^{+}$/ $Ly6C^{-}$/ $Ly6G^{-}$/ viable cells. Splenocytes were used as negative control to set the threshold and gate on vGLUT1-positive microglia fractions. Raw data were analyzed with FlowJo v10 (BD Bioscience).

RNA extraction and quantification

RNA was extracted via the Lexogen Split-RNA extraction kit (cat. number 008.48) according to the manufacturer's instructions. RNA concentrations were measured via Qubit 4 fluorometer (Invitrogen), using the RNA HS Assay kit (Invitrogen).

Total RNA library preparation and sequencing

Before library preparation, RNA integrity was assessed on an Agilent TapeStation system 4150 using the RNA screen tape (Agilent). 50 ng total RNA were used as input for ribosomal RNA (rRNA) depletion using the NEBNext rRNA depletion kit (New England BioLabs inc., product code: E6350) according to the manufacturer's instructions. Following rRNA depletion, total RNA libraries were built using the NEBNext Ultra II library prep kit for Illumina (New England BioLabs inc., product code: E7775) according to the manufacturer's instructions. The yield of amplified libraries was measured on a Qubit 4 fluorometer using the Qubit high sensitivity DNA kit (HS DNA kit). Amplified libraries were further analyzed on the HS D1000 screen tape on a TapeStation system 4150 to assess library size and molarity prior to pooling. The libraries were sequenced using Illumina HISeq-4000 at a depth of 40 million reads/sample.

Computational analysis of RNA-sequencing data

RNA-Seq reads were mapped to the mouse genome (GRCm38, version M12 (Ensembl 87)) with STAR (Dobin et al., Bioninfomatics 2013, version-2.7.3a) using the following parameters –outSAMunmapped Within --outFilterType BySJout --outFilterMultimapNmax 20 --

alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000. Reads were assigned to genes using FeatureCounts (Liao et al., Bioinformatics 2014, version-v2.0.0) with the following parameters: -T 2 -t exon -g gene_id -s 0. For the differential expression analyses we used DESeq2 (version-1.32.0) with default parameters (Love et al., 2014). We filtered genes that had less than 5 counts in at least 3 samples. The model.matrix design was specified as "~0 + group", where "group" variable was defined as a combination of time and treatment (i.e. ctr.day [active phase], ctr.night [sleep phase], poly(I:C).day [active phase], poly(I:C).night [slpee phase], LPS.day [active phase], LPS.night [sleep phase], LPS. poly(I:C).day [active phase], LPS. poly(I:C).night [sleep phase]). The gene set enrichment analyses were carried out using CERNO algorithm from R tmod package (version-0.50.06), (Zyla et al., 2019) and the Panther (Mi et al., 2013) online tool.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iTaq™ Universal Probes One-Step Kit for probes (Bio-Rad Laboratories). The samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control. We chose *36B4* as internal standard for gene expression analyses based on its expression stability relative to the circadian rhythm (Giri and Sundar, 2022; Kosir et al., 2010). Furthermore, in the present microglial bulk-RNA-seq, the gene *36B4* (*Rplp0*) showed no significant change in expression between 1 a.m. and 1 p.m. (ZT4 and ZT16 respectively, **Supplementary Table S2**). Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Cutsom-made primers with probes for TaqMan were purchased from Thermo

Fisher: housekeeping gene: *36B4*, product code: NM_007475.5; *sialic acid-binding immunoglobulin-like lectin-H (Siglech)*, product code: Mm_00618627_m1: *purinergic receptor P2Y12* (*P2ry12)*, product code: Mm00446026_m1; *purinergic receptor P2Y6 (P2ry6)*, product code Mm02620937_s1; and *transcription factor PU.1 (Spi1)*, product code Mm00488140_m1. Relative target gene expression was calculated according to the Delta C(T) method.

Quantification of cytokines and fractalkine

Animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories, North Chicago, IL, USA) and transcardially perfused with 15 ml ice-cold DPBS. Hippocampi were quickly removed, snap-frozen in liquid nitrogen and stored at -80°C. Tissue lysis was performed in Roche cOmplete Lysis-M reagents (lysis buffer and protease inhibitor cocktail contained in the kit, Millipore Sigma cat. Nr. 4719956001). Hippocampi were placed in protein low-binding tubes mounted onto a Qiagen TissueLyser II (Qiagen cat. Nr. 85300). Lysates were span down at 20.000 rcf for 20 minutes at 4°C to remove debris, and the supernatant was stored at -80°C.

Cytokine protein levels in hippocampal lysates were quantified using a Meso-Scale Discovery (MSD) V-Plex electrochemoluminescence assay for mice according to the manufacturer's instructions (V-Plex proinflammatory panel 1 mouse kit, MSD cat. Nr. K15048D-1). The assay allowed for the detection and quantification of interleukin (IL)-1β, IL-6, IL-4, IL-10, and tumor necrosis factor (TNF)-α . Samples were run in duplicate, and the plate was read via a SECTOR PR 400 (MSD) imager and analyzed using MSD's Discovery Workbench analyzer and software package. Fractalkine protein levels were measured in hippocampal lysates via the R&D System's Mouse Fractalkine (CX_3CL1) Quantikine ELISA Kit (R&D Systems cat. Nr. MCX310) according to the manufacturer's instructions. Samples were measured in duplicate, and the plate was read on a microplate reader (SPARK® multimode microplate reader, Tecan Trading AG,

Switzerland). Cytokines and fractalkine concentrations were normalized to the total protein amount in each sample.

Proteotype analysis

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

For MS analysis, peptides were reconstituted in 5% acetonitrile and 0.2% formic acid containing standard iRT peptides (Biognosys AG, Switzerland). The peptides were analyzed in data-independent acquisition (DIA) mode and data-dependent acquisition (DDA) mode for spectral library generation. For spectral library generation, a fraction of the samples originating from the same condition were pooled to generate a mixed sample for each condition. Peptides were separated by reverse-phase chromatography on an 25-cm Easy Spray column (Thermo Fisher Scientific, USA) connected to an EASY-nLC 1200 instrument (Thermo Fisher Scientific). The HPLC was coupled to a Q Exactive HF mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific). For each injection, approximately 2 µg peptides were loaded onto the column and separated via a 180 min gradient from 100% buffer A (99% H2O, 0.1% formic acid) to an increasing percentage of buffer B (99.9% acetonitrile, 0.1% formic acid). The DIA method contained 24 DIA segments with varied m/z widths over a mass range of 350 to 1650 m/z with 30,000 resolution. Injection time (IT) was set to auto, an automatic gain control (AGC) of 3 x 106 was applied, and a survey scan of 120,000 resolution (54 ms maximum IT, 3 x 106 AGC target; default charge state of 3, loop count of 1, and 27 NCE [1]). For the DDA, a Top10 method was recorded with 60,000 resolution of the MS1 scan (54 ms max IT, AGC target of 3 x 106), followed by high-energy collisional dissociation (HCD)-MS/MS scans at 15,000 resolution of the MS1 scan (54 ms max IT, AGC of 5 x 104). To avoid multiple scans of dominant ions, the precursor ion masses of scanned ions were dynamically excluded from MS/MS analysis for 30 s. Single-charged ions and ions with unassigned charge states or charge states above 6 were excluded from MS/MS fragmentation. The covered mass range was identical to the DIA.

Data analysis DIA LC-MS/MS

LC-MS/MS DIA runs were analyzed using a spectral library generated from the pooled samples measured in DDA. The collected DDA spectra were searched against UniprotKB (UniProt Swissprot, Mus musculus retrieved in 2018) using the Sequest HT search engine within Thermo Proteome Discoverer (PD) version 2.3 (Thermo Fisher Scientific). The following PD parameters were applied: two missed cleavages, specific tryptic digestion, fixed carbamidomethylation of C and oxidation of M, variable deamidation of R and acetylation at the N-terminus. Monoisotopic peptide tolerance was set to 10 ppm, and fragment mass tolerance was set to 0.02 Da. The identified proteins were assessed using Percolator and filtered using the high peptide confidence setting in PD. Identification results were imported to Spectronaut Pulsar version 14.0 (Biognosys AG) for the generation of spectral libraries. Targeted data extraction of DIA-MS acquisitions was performed with Spectronaut with default settings using the generated spectral libraries.

Extracted features were exported from Spectronaut for statistical analysis with MSstats (version 3.18.0) using default settings, and iRT peptides to normalize across runs. Features were filtered for calculation of Protein Group Quantity as defined in Spectronaut settings; common contaminants were excluded. In MSstats, the model estimated fold change (FC) and statistical significance for all compared conditions. Significantly different proteins were determined by an adjusted p-value < 0.05 for the comparisons of control conditions (baseline microglial proteome ZT6 vs. ZT18). For the differential expression in response to LPS, the threshold was set at Log2-fold-change > 1 and adjusted p< 0.05. Benjamini-Hochberg method was used to account for multiple testing.

Statistical analysis

All statistical analyses of ELISA, electrochemiluminescence, qRT-PCR and flow cytometry data were performed using Graphpad Prism (Version 10.0.2; GraphPad Software, La Jolla, California), with statistical significance set at $p < 0.05$. Group-wise comparisons were analyzed using independent Student's *t* tests (two-tailed). For the transcriptional analysis of microglia between 1 a.m. and 1 p.m. (ZT4 and ZT16 respectively), and the transcriptional analysis of microglia from Poly(I:C) offspring at baseline, differentially expressed genes (DEGs) were defined by a Log2-fold change (Log2FC) cut-off of 0.5 and an adjusted (adj.) *p*-value cut-off of 0.05. DEGs presented in heatmaps in Fig.S1-S4 include genes that were up- or down-regulated based on an adj. *p*-value of 0.05. For the transcriptional and proteotype analysis of the LPS responses, DEGs were defined by a Log2FC cut-off of 1 and adj. *p*-value of 0.05. For the transcriptional and proteotype comparison of microglia cells between 3 a.m. and 3 p.m. (ZT6 and ZT18 respectively), DEGs and differentially expressed proteins were defined by an adj. *p*-value cut-off of 0.05.

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