alone accelerates cognitive decline during aging in conjunction with altered regulation of immune cells and microglia in the brain.

Keywords Circadian rhythm dysregulation, Activity rhythm, Barnes maze, Cognition, Adaptive immune cell, B cells, T cells, Microglia, Aging, Mice

Introduction

Materials and methods

Animals

ad libitum

Analysis of wheel-running activity

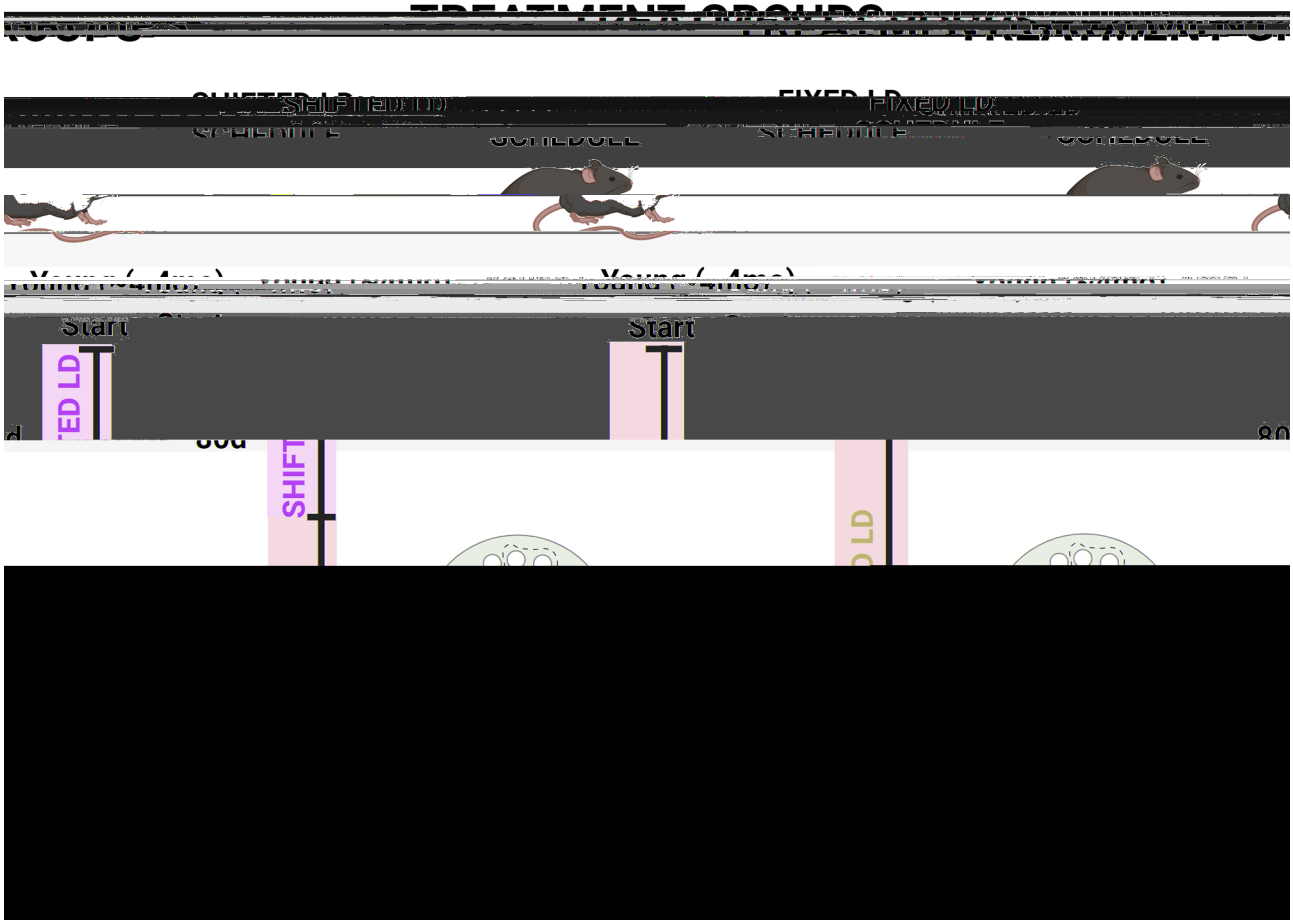
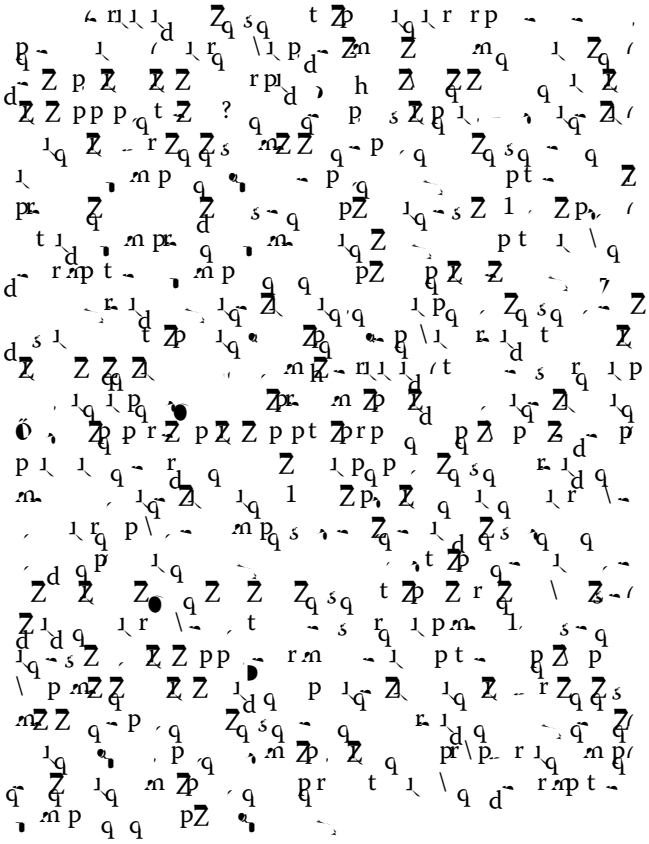
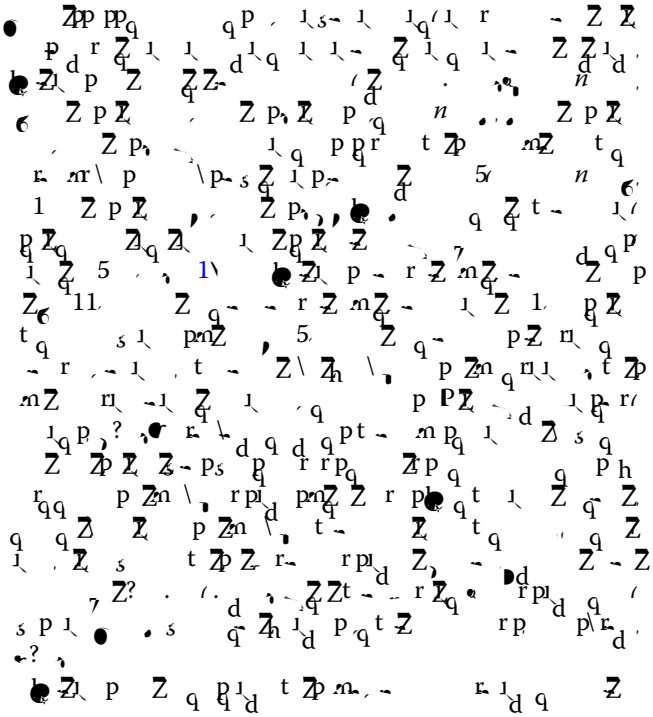


Fig. 1 LD treatment groups and experimental design. Mice were separated into two cohorts: (1) control group (left) was maintained throughout on a fixed LD 12:12 schedule and (2) experimental treatment group (right) was exposed to shifted LD cycles (12 h advance/5d) for 80 days and then was placed back on the same standard LD 12:12 cycle. At middle age (13mo), both groups were assessed in the Barnes maze and euthanized at 14 months for flow cytometry analysis. (Created with BioRender.com)



Behavioral assays

Barnes maze



IBA-1 immunohistochemistry and morphological analysis

Statistical analysis

Results

E□ect of shifted LD cycles on circadian rhythm of wheel-running activity

Fig. 2 E□ects of experimental LD cycles and aging on light-dark entrainment and other properties of the circadian rhythm in wheel-running activity. **(A)** Representative records of wheel-running activity in adult mice (≈3mo) that were maintained in a □xed LD 12:12 cycle (left) or exposed to a shifted (12 h/5d) LD 12:12 cycle (right). Actograms are plotted over a 24-hour period. The open and closed bars at the top respectively signify the timing of the light and dark phase in the □xed and shifted LD 12:12 cycles. Red arrows on the right denote the interval when exposure to the shifted LD cycles was initiated ("treatment" phase) and when shifted LD animals were returned to the same regular LD 12:12 schedule as the □xed LD group (post-treatment phase). **(B)** The phase angle (Ψ) between daily activity onsets and lights-o□, **(C)** absolute day-to-day variability, and **(D)** total daily wheel-running activity (wheel revolutions/24hr) were later analyzed during the post-treatment phase in □xed ($n = 7$) and shifted ($n = 7$) LD mice at middle age (13-14mo). Then these entrainment and qualitative parameters of the activity rhythm in middle-aged mice from both treatment groups were compared to similar published data obtained from aged mice on □xed LD cycles (Souza et al., [14]). In panel **B**, negative phase angle values (in minutes) indicate that daily onsets of activity occur after lights-o□. Bars (in **B**-**D**) depict mean values (+ SEM). Circles indicate individual data values for each mouse. (*$p < 0.01$; Tukey's multiple comparisons)

Comparison of the long-term e□ects of shifted LD cycles and aging on cognitive performance in the Barnes maze

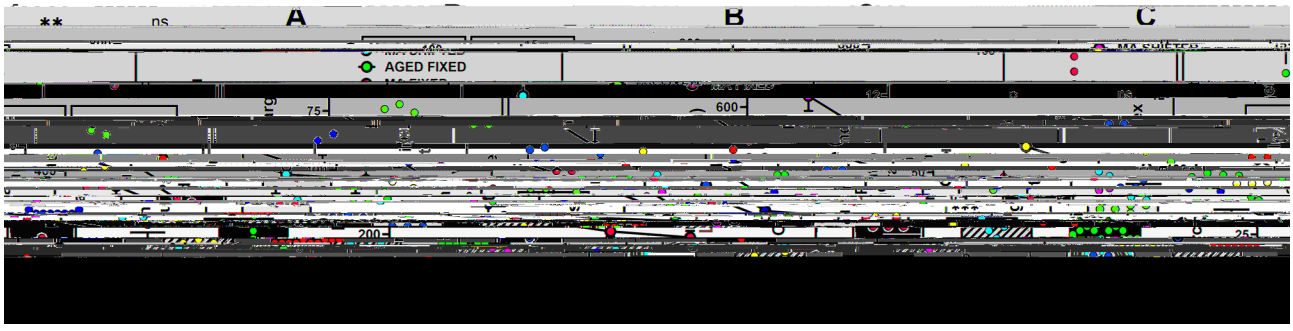


Fig. 3 E□ects of experimental LD cycles on cognitive performance in the Barnes maze. **(A)** Distance traveled (cm) to reach the escape is depicted across days of learning trials, **(B)** cognitive index for training trials, and **(C)** percent path in target quadrant (i.e., quadrant in which the escape was localized during training trials, during the □rst 30 s of the trial) were analyzed in □xed ($n = 20$) and shifted ($n = 16$) LD mice at middle age (13-14mo) and compared to similar data from aged mice on □xed LD cycles (Souza et al., [14]). Plotted values (in **A**-**C**) represent mean ± SEM. Circles (in **B**-**C**) indicate individual data values for each mouse. (*$p < 0.05$, **$p < 0.01$; ***$p < 0.0001$; Fisher's PLSD post hoc analysis)

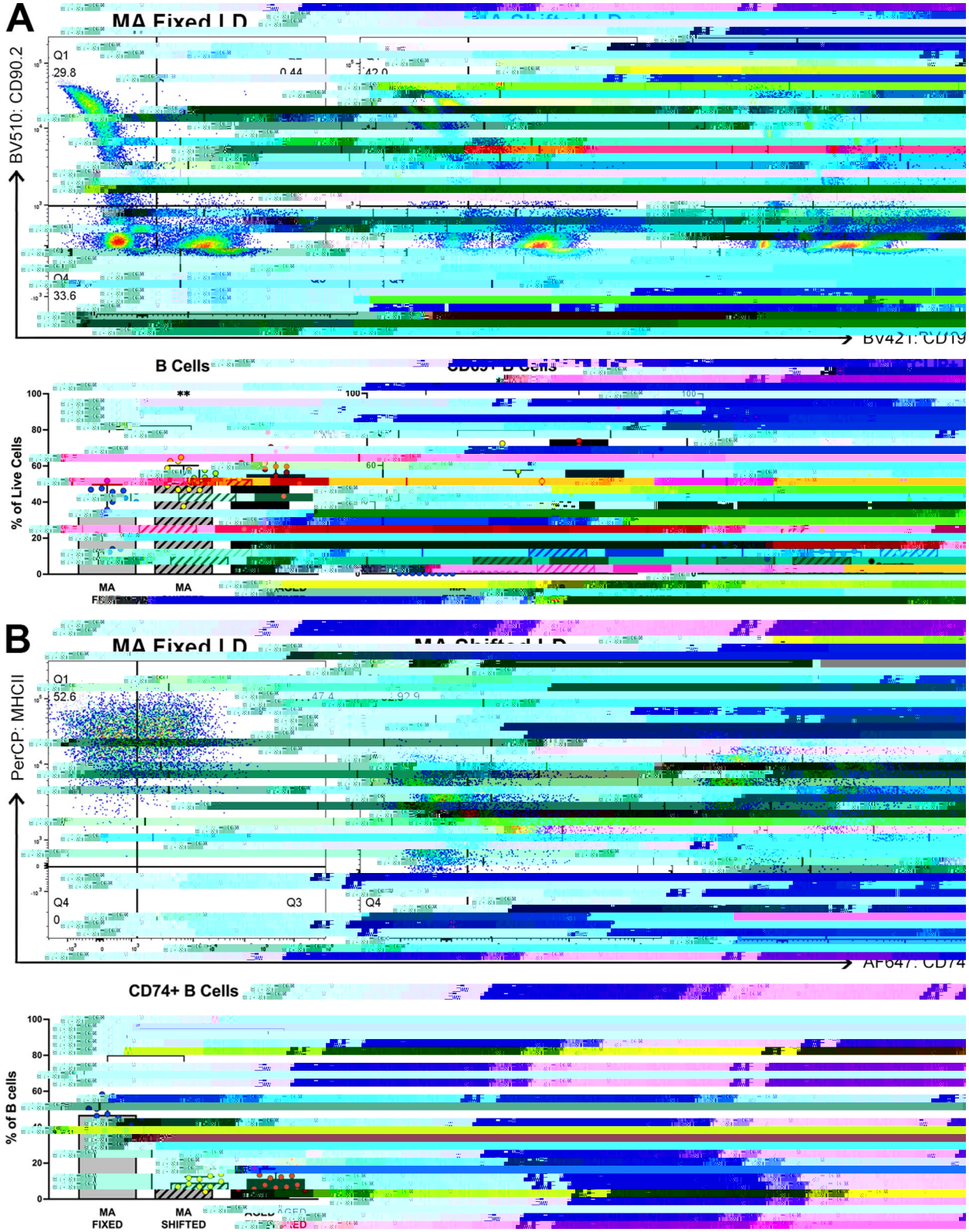


Fig. 4 (See legend on next page.)

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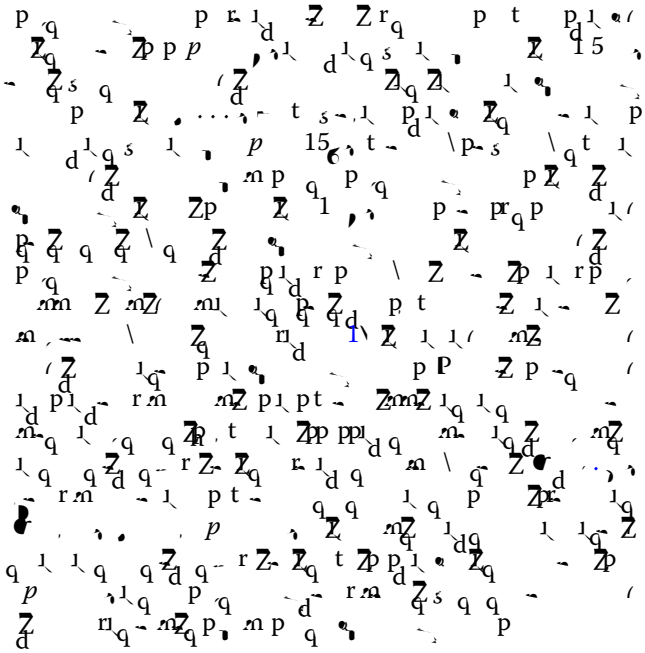
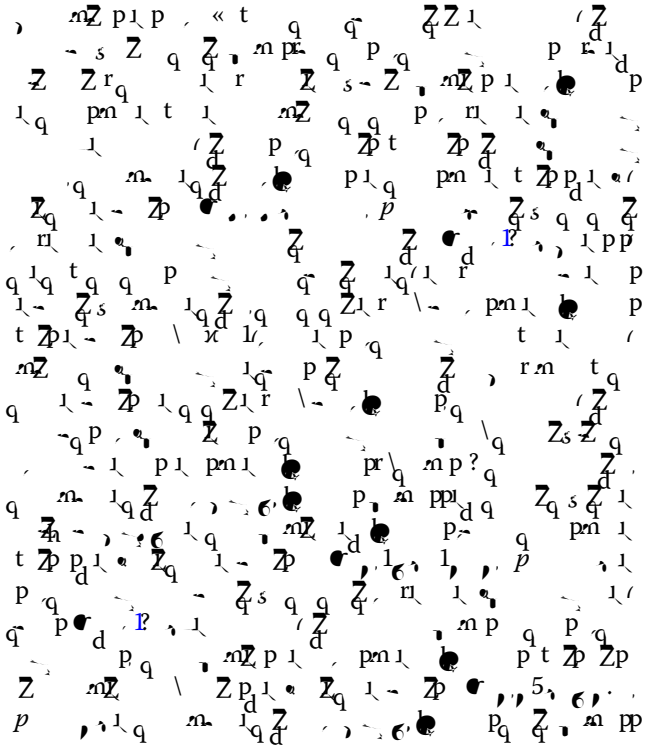
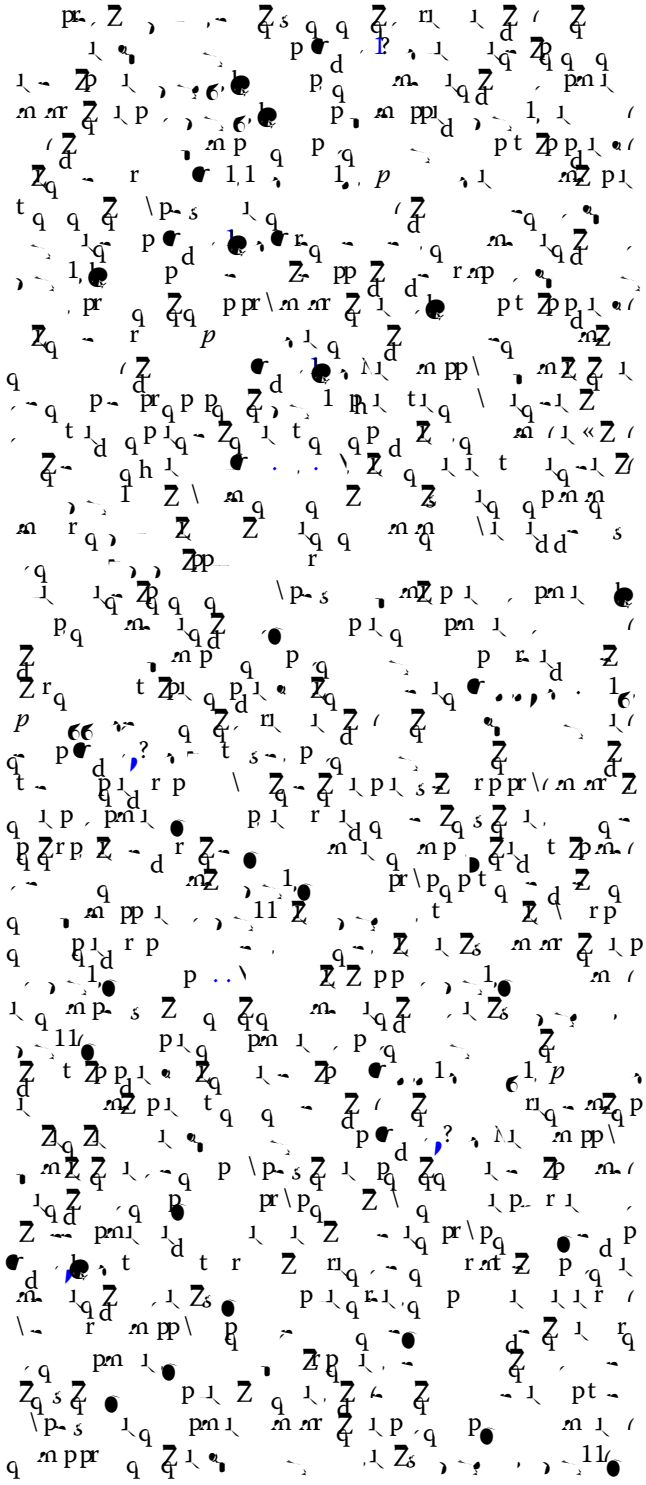
Fig. 4 E□ects of experimental LD cycles and aging on splenic B cell populations. (**A**) Representative dot plots (top) using CD19 (x-axis) as a B cell marker versus CD90.2 as a T cell marker to compare populations of B cells in isolated splenocyte samples from middle-aged (MA) mice on □xed (left) and shifted (12 h/5d, center) LD 12:12 cycles, and from an aged (18-22mo) animal (right) maintained on a □xed LD cycle. Bar graphs (bottom) depict group comparisons (MA □xed, $n = 12$; MA shifted, $n = 13$; Aged □xed, $n = 13$) of the percentage (mean ± SEM) of B cells identi□ed from Quadrant 3 (left), and of CD19+ B cell populations expressing the activation marker CD69 (center) or cell surface CLIP (right). (**B**) Representative dot plots (top) of live B cells gated from quadrant 3 (see panel **A**) displaying MHCII (y-axis) and CD74 (x-axis) expression. Bar graph (bottom) depicts group comparisons (MA □xed, $n = 9$; MA shifted, $n = 10$; Aged □xed, $n = 10$) of the percentage (mean ± SEM) of CD74+ B cells identi□ed from quadrant 2 and 3 (left). (*$p < 0.05$, **$p < 0.01$; Tukey's multiple comparisons)



Long-term e□ects of circadian dysregulation on adaptive immune cells in the spleen





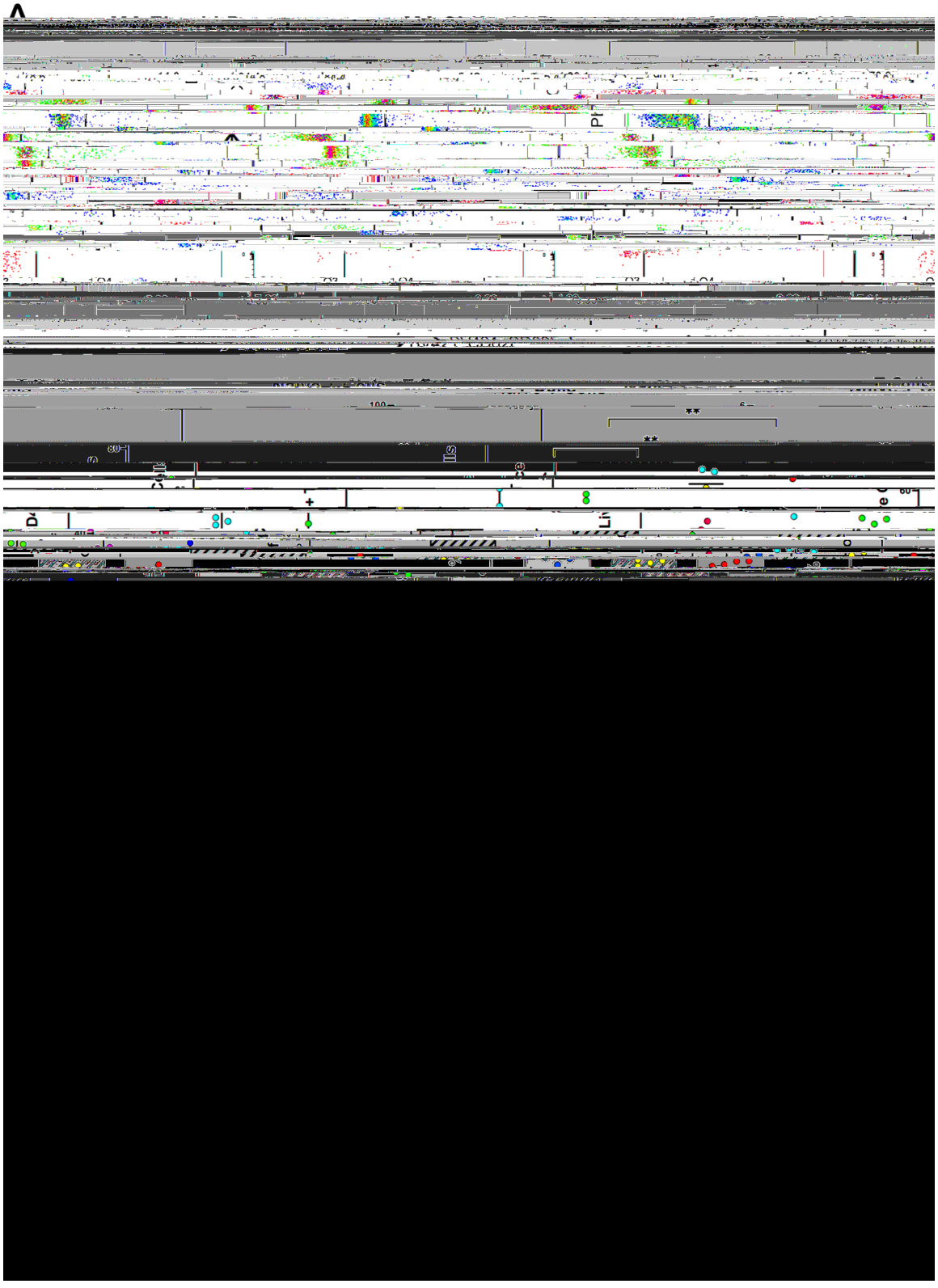
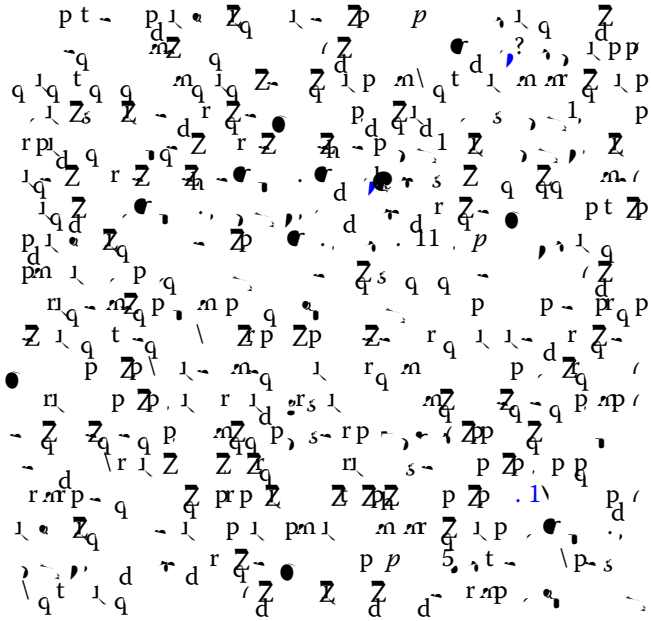
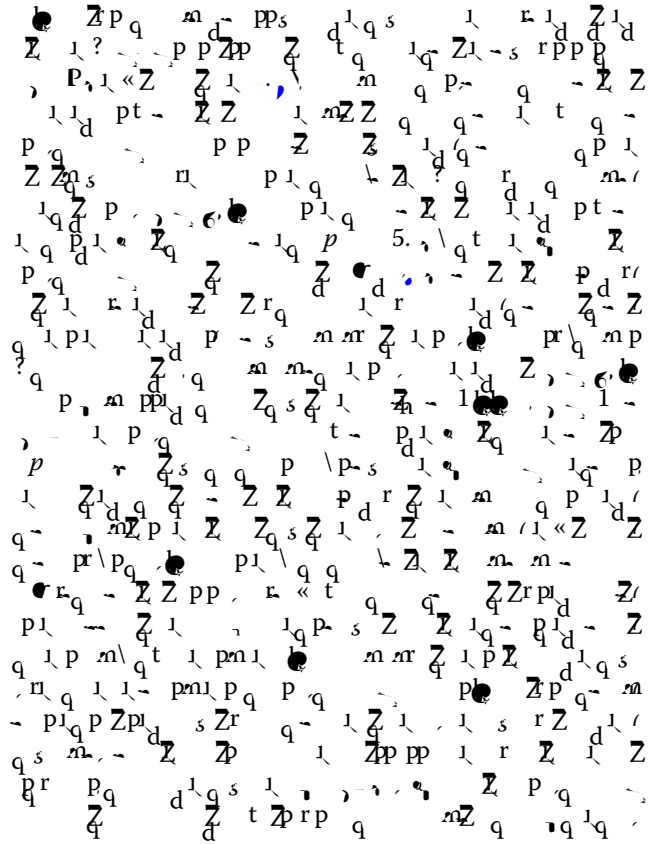
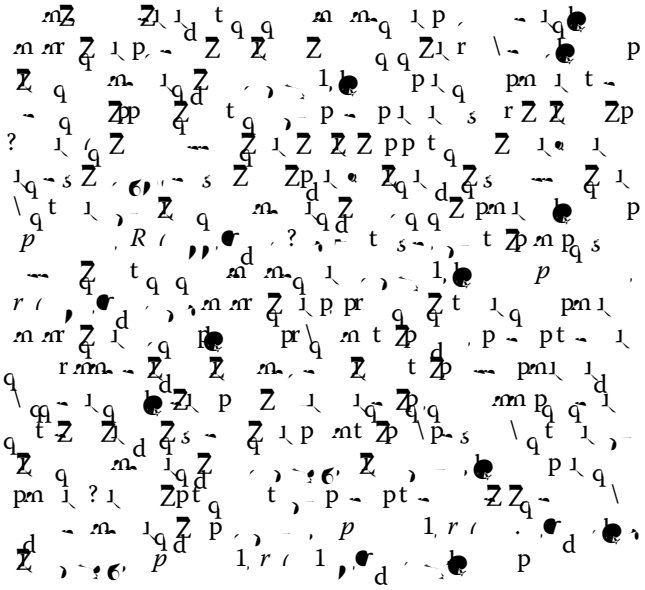


Fig. 5 (See legend on next page.)







Long-term e□ects of circadian dysregulation on hippocampal microglia

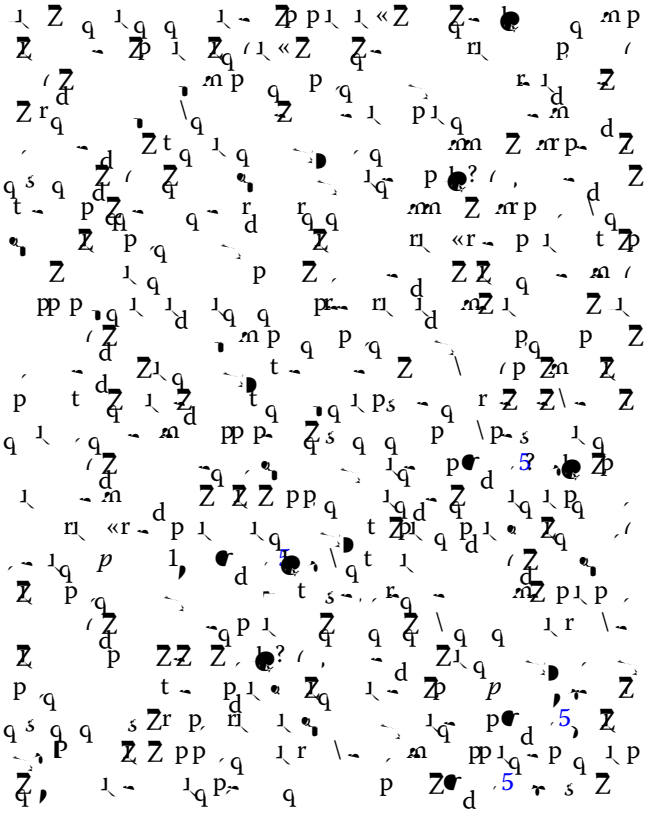


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Fig. 5 E□ects of experimental LD cycles and aging on splenic T cell populations **(A)** Flow cytometry analysis using CD90.2 (y-axis from Fig. 4A, quadrant 1) as a T cell marker to compare populations of T cells in isolated splenocyte samples from middle-aged (MA) mice on □xed (left) and shifted (12 h/5d, center) LD 12:12 cycles, and from an aged (18-22mo) cohort (right) of □xed LD mice. Representative dot plots (top) depict gated CD4+T cells exhibiting expression of CD44 (y-axis) and CD62L (x-axis). Bar graphs (bottom) depict group comparisons (MA □xed, $n = 10$; MA shifted, $n = 11–13$; Aged □xed, $n = 13–14$) of the percentage (mean ± SEM) of T cells (left) identi□ed in quadrant 1 of Fig. 4A and of CD4+ naïve T cells (right) that were gated from quadrant 3 as CD62L+/CD44-. **(B)** Representative dot plots (top) of CD4+T cells displaying expression of FoxP3 (y-axis) and CD25 (x-axis). FoxP3+ and CD25+ (high) expression was used to gate regulatory T cells, as shown in the outlined box. Bar graph (bottom) depicts group comparisons (MA □xed, $n = 8$; MA shifted, $n = 10$; Aged □xed, $n = 3$) of FoxP3+ and CD25+ regulatory T cells. (*$p < 0.05$, **$p < 0.01$; Tukey's multiple comparisons)

Fig. 6 E□ects of experimental LD cycles on meningeal B cell populations. (**A**) Representative dot plots using CD19 (x-axis) as a B cell marker versus CD90.2 as a T cell marker to compare populations of B cells in isolated meningeal samples from mice on □xed (left) and shifted (12 h/5d, right) LD 12:12 cycles. Bar graph depicts group comparisons of the percentage (mean ± SEM) of CD19+ B cells (right). **(B)** Bar graphs depict group comparisons (MA □xed, $n = 9–10$; MA shifted, $n = 16$) of the percentage (mean ± SEM) of CD19+ B cell subsets expressing 41BBL (left), CLIP (center), or CD74 (right) as indicated. (*$p < 0.05$, **$p < 0.01$; Mann-Whitney test)

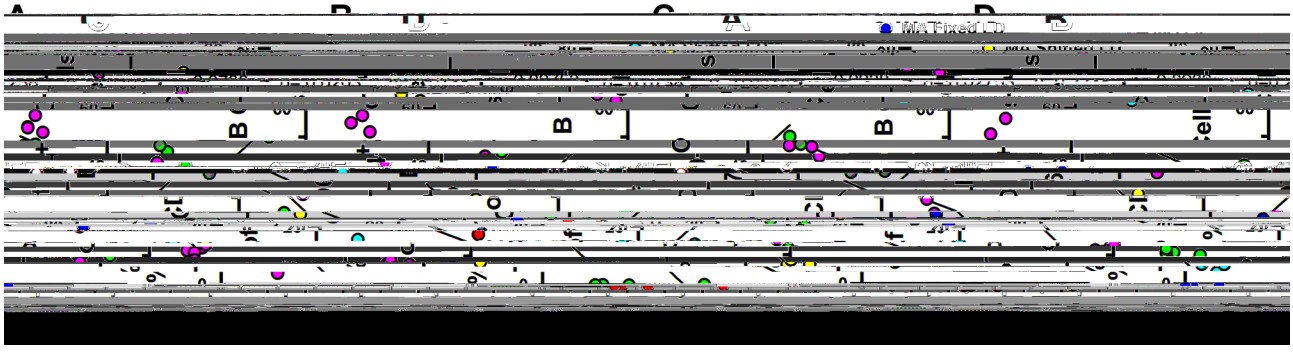

Fig. 7 Relationship between splenic B cell populations and cognitive performance in Barnes maze for middle-aged (MA) mice that were exposed to □xed or shifted LD cycles. Pearson correlation coe□cients comparing cognitive index with the percentage of: (**A**) B cells, (**B**) CLIP + B cells, (**C**) CD74 + cells and (**D**) CD69 + cells in the middle-aged cohorts of □xed ($n = 5$) and shifted ($n = 6–8$) LD mice. Circles represent individual data values for each mouse. Lines in each graph denote simple linear regression for the data set with corresponding p values

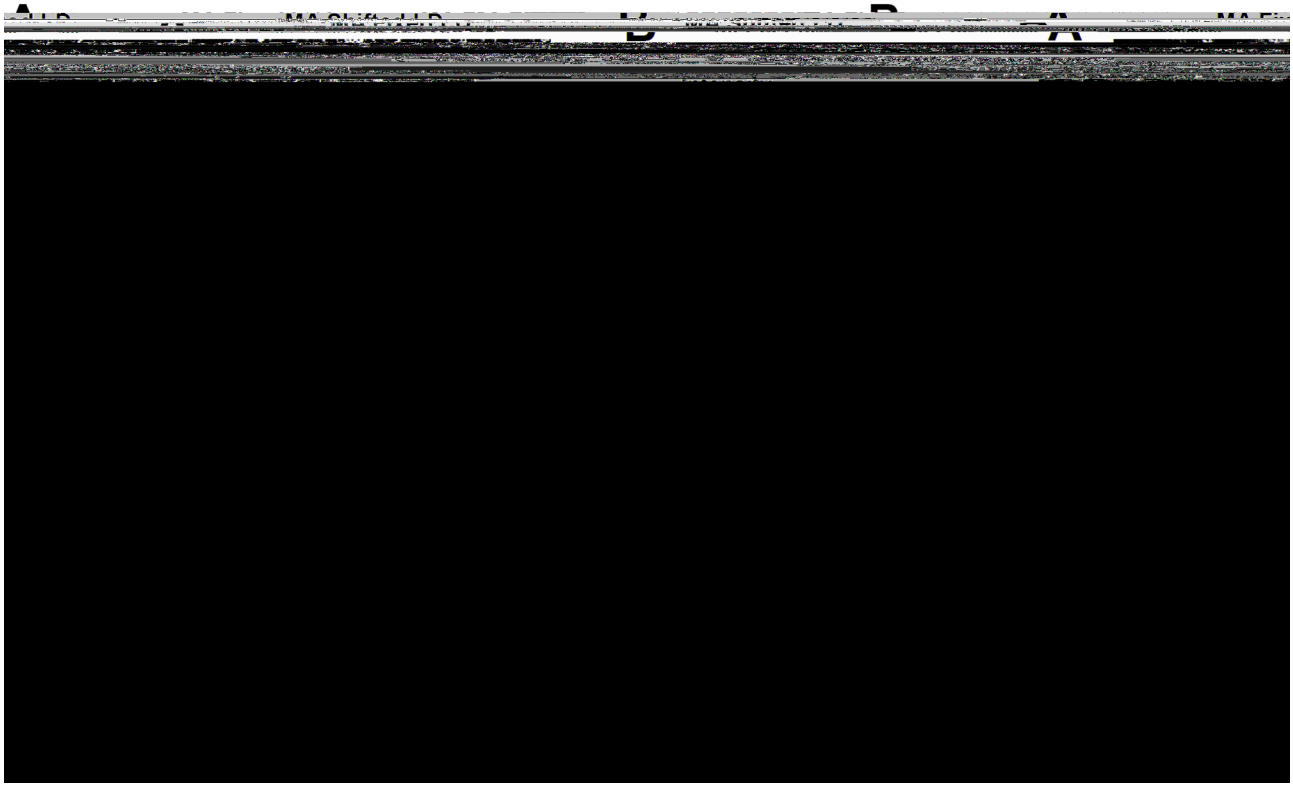

Fig. 8 E□ects of experimental LD cycles on the morphology of hippocampal microglia. (**A**) High-magni□cation (60X) confocal images of IBA1+ hippocampal microglia located within the dentate gyrus (DG) of representative □xed (left) and shifted (right) LD mice at middle age. Bar graphs depict quantitative analysis of the morphological pro□les of IBA-1+ microglia in the DG (mean ± SEM) from regions of interest (ROI) in □xed ($n = 5$) and shifted ($n = 5$) LD mice with regard to: (**B**) integrated □uorescent intensity, (**C**) number of immunopositive cells, (**D**) soma area, and (**E**) the number of primary immunopositive processes with Sholl intersections (at 5 µm intervals of the distance from the microglial soma). For all morphological analyses, three representative hemibrain sections through the hippocampus were analyzed from each animal and three randomized FOV within the dentate gyrus (DG) of the hippocampus were captured in each section. In turn, the data points represent individual IBA-1+ microglia (approximately 10–20/FOV) in the DG that were selected as ROI. (*$p < 0.05$, **$p < 0.01$; Mann-Whitney test)

Discussion

Acknowledgements
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Author contributions
Karienn A. de Souza: Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. Morgan Jackson: Conceptualization, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. Justin Chen: Formal analysis, Investigation, Data Curation, Project administration. Jocelin Reyes: Formal analysis, Investigation, Data Curation, Visualization, Supervision, Project administration. Judy Muyad: Formal analysis, Investigation, Data Curation, Project administration. Emma Tran: Formal analysis, Investigation, Data Curation, Project administration. William Jackson: Formal analysis, Investigation, Data Curation, Project administration. M. Karen Newell-Rogers: Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. David J. Earnest: Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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