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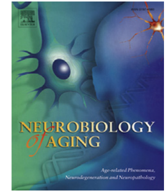

Diminished circadian rhythms in hippocampal microglia may contribute to age-related neuroinflammatory sensitization

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ABSTRACT

Aged animals exhibit diminished circadian rhythms, and both aging and circadian disruption sensitize neuroinflammatory responses. Microglia—the innate immune cell of the central nervous system—possess endogenous timekeeping mechanisms that regulate immune responses. Here, we explored whether aging is associated with disrupted diurnal rhythms in microglia and neuroinflammatory processes. First, hippocampal microglia isolated from young rats (4 months F344XBN) rhythmically expressed circadian clock genes, whereas microglia isolated from the hippocampus of aged rats (25 months) had aberrant *Per1* and *Per2* rhythms. Unstimulated microglia from young rats exhibited robust rhythms of *TNFα* and *IL-1β* mRNA expression, whereas those from aged rats had flattened and tonically elevated cytokine expression. Similarly, microglial activation markers were diurnally regulated in the hippocampus of young but not aged rats and diurnal differences in responsiveness to both ex vivo and in vivo inflammatory challenges were abolished in aged rats. Corticosterone is an entraining signal for extra–suprachiasmatic nucleus circadian rhythms. Here, corticosterone stimulation elicited similar *Per1* induction in aged and young microglia. Overall, these results indicate that aging dysregulates circadian regulation of neuroinflammatory functions.

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1. Introduction

Circadian programming of physiology and behavior is regulated by a hierarchical network of biological clocks (Mohawk et al., 2012), with the suprachiasmatic nucleus (SCN) at the top of this hierarchy. The SCN is synchronized by light information and entrains extra-SCN circadian clocks (in the central nervous system [CNS] and periphery) through both neural and humoral cues. At the molecular level, circadian rhythms are generated by an autoregulatory feedback loop composed of key transcriptional activators (e.g., CLOCK, BMAL1, RORs) and repressors (e.g., Per, Cry, Rev-Erb). These “clock genes” are expressed throughout the body and perform unique cellular- and tissue-specific functions, in addition to maintaining local oscillations.

For most organisms, the risk of encountering toxins, infection, and injury varies throughout the day (Curtis et al., 2014). This likely led to circadian regulation of the immune system. There are diurnal rhythms in central and peripheral immune responses in mammals, with peak immune activation occurring during the inactive phase [(Fonken et al., 2015; Marpegan et al., 2009; Spengler et al., 2012; Straub and Cutolo, 2007), although see (Rahman et al., 2015)]. Diurnal variations in immune responses are regulated in part by circadian rhythms in inflammatory cells (Arjona and Sarkar, 2006; Boivin et al., 2003; Bollinger et al., 2011; Fonken et al., 2015; Hayashi et al., 2007; Keller et al., 2009; Silver et al., 2012). For example, BMAL1 regulates diurnal oscillations in the trafficking of inflammatory monocytes in mice (Nguyen et al., 2013).

Circadian rhythms in the SCN and other central and peripheral tissues change dramatically across the life span, with aged animals exhibiting diminished molecular and behavioral circadian rhythms. In the SCN of aged animals, several functional properties are altered including neuronal spiking (Farajnia et al., 2012; Nakamura et al., 2011), neuropeptide production (Kallo et al., 2004), neurotransmitter signaling (Palomba et al., 2008), and responsiveness to melatonin (von Gall and Weaver, 2008). Light also has a diminished effect on the SCN of aged animals (Lupi et al., 2012), which may in turn contribute to age-related decline in the strength of SCN outputs. Indeed, circadian rhythms in extra-SCN clocks of aged rodents have decreased amplitude and slower re-entrainment, likely reflective of this loss of SCN output strength (Davidson et al., 2008; Sellix et al., 2012). Furthermore, clock gene expression in aged mice

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is significantly modified in peripheral oscillatory organs, such as the liver and heart (Bonaconsa et al., 2014). Maintaining circadian rhythmicity is evolutionarily advantageous: circadian disruption is associated with metabolic disturbances (Fonken and Nelson, 2014), altered inflammatory responses (Castanon-Cervantes et al., 2010; Fonken and Nelson, 2013), and cancer (Sahar and Sassone-Corsi, 2009). Importantly, the effects of circadian disruption are more pronounced in aged animals and may increase mortality (Davidson et al., 2006). The exaggerated effects of circadian disruption in the aged population may reflect more widespread changes in circadian function. Thus, understanding how aging disrupts circadian processes could lead to improved prevention and treatment of common age-related pathologies.

We previously demonstrated that microglia—the primary innate immune cell of the CNS—exhibit diurnal rhythms in clock genes and inflammatory potential (Fonken et al., 2015). Furthermore, neuroinflammatory responses to immune stimulation are blunted at the same time of the day at which isolated microglia are less reactive. This suggests that microglia may mediate circadian differences in the neuroinflammatory response. In the healthy young CNS, microglial rhythms appear to be entrained by rhythmic expression of glucocorticoids. Importantly, glucocorticoid rhythms are blunted in the hippocampus of aged rats (Barrientos et al., 2015).

Aging and circadian disruption are associated with sensitized neuroinflammatory processes (Barrientos et al., 2009a; Fonken and Nelson, 2013; Godbout et al., 2005). This led us to test whether sensitized neuroinflammatory responses in aged rats are associated with disrupted circadian function of hippocampal microglia. More specifically, we hypothesized that: (1) diurnal rhythms in hippocampal microglia may be disrupted with aging; (2) disrupted rhythms in aged microglia may be associated with heightened neuroinflammatory responses across the day; and (3) the microglial rhythm-entraining factor corticosterone may differentially affect microglia isolated from the hippocampus of young and aged rats. Our results suggest that aging disrupts intrinsic circadian rhythms in microglia, and that this may underlie aged microglial hypersensitivity to inflammatory stimuli.

2. Methods

2.1. Animals

Young (3 months) and aged (24 months) male Fischer-Brown Norway crosses (F344xBN F1) were received from National Institute on Aging (NIA) and pair housed (52 cm L × 30 cm W × 21 cm H) with an age-matched conspecific. Rats of this age and strain were selected based on previous work in our laboratory to study normal, non-neurodegenerative aging (these rats typically live for >30 months) (Barrientos et al., 2006, 2009a). Food and water were available ad libitum, and rats were maintained at an ambient temperature of 22 ± 2 °C. All rats were maintained on a 12-hour light–dark cycle with lights on either at 7 AM or 9 PM. Rats were given 1 month to acclimate to colony conditions before experimentation began (experimental manipulations occurred at ~4 and 25 months of age). All experimental procedures were conducted in accordance with protocols approved by the University of Colorado—Boulder Institutional Animal Care and Use Committee and The Guide. Efforts were made to minimize animal use and discomfort.

2.2. General methods

2.2.1. Tissue collection

Animals received a lethal intraperitoneal (IP) injection of sodium pentobarbital. After rats were completely unresponsive (as assessed by paw pinch), they were transcardially perfused with ice-cold saline (0.9%) for 3 minutes to remove peripheral immune cells from the CNS vasculature. Brains were then rapidly extracted, and the hippocampi were dissected out on ice. For the in vivo experiments, the hippocampi were flash frozen in liquid nitrogen and stored at −80 °C. For the ex vivo experiments, microglia were immediately isolated as described in the following section.

2.2.2. Microglia isolations and ex vivo treatments

Hippocampal microglia were isolated using a Percoll density gradient as described previously (Frank et al., 2006b). In brief, rats were saline perfused for 3 minutes, brains were removed, and the hippocampi were dissected out on ice. Each hippocampus was then homogenized in 3 mL of 0.2% glucose in 1× Dulbecco's phosphate-buffered saline (DPBS). The homogenate was passed through a 40-μm filter into a 50-mL conical, which was rinsed with an additional 2 mL of DPBS, and then, the homogenate was transferred to a 5-mL polystyrene falcon tube. Cells were pelleted at 1000 *g* for 10 minutes at 22 °C, and then, the supernatant was removed. A Percoll gradient was created by resuspending the pellet in 2 mL of 70% isotonic Percoll (GE Healthcare; isotonic Percoll is 10:1 Percoll with 10× PBS; 100% isotonic Percoll is then diluted with 1× DPBS), followed by a layer of 2 mL of 50% Percoll and topped with 1-mL DPBS. The gradient was spun at 1200 *g* for 45 minutes at 22 °C with no acceleration or braking. After the spin, myelin debris were removed from the DPBS/50% interface and then microglia were extracted from the 50/70% interface. Microglia were washed in DPBS and then pelleted at 1000 *g* for 10 minutes at 22 °C. In total, this isolation procedure takes <3 hours. After isolation, microglia were resuspended in media (filtered sterile high glucose Dulbecco's modified eagle medium [Gibco, 11960-044] +10% fetal bovine serum [Atlanta biological, S11050]), and the concentration of viable microglia was determined by trypan blue dye exclusion. Microglia were plated at a density of 6000 cells/100 μL in a 96-well v-bottom plate. To assess microglia cytokine responsiveness, cells were challenged ex vivo with lipopolysaccharide (LPS) (*E. coli* serotype 0111:B4; Sigma) at a final in-well concentration of 10 or 100 ng/mL or media alone at 37 °C, 5% CO_2 for 3 hours (Frank et al., 2010). The LPS concentrations and incubation time were based on previously published time courses and LPS concentrations curves (Frank et al., 2006b, 2010). In a separate experiment, microglia were treated with corticosterone (Sigma-C2505) at concentrations of 1, 10, or 100 nM for 2 hours (Fonken et al., 2016). Corticosterone was first diluted to a concentration of 10 mM in EtOH followed by serial dilution in media (0 nM control contains EtOH equivalent to 1000 nM EtOH concentration). After the incubations with LPS or corticosterone, plates were centrifuged at 1000 *g* for 10 minutes at 4 °C to pellet the cells and then the supernatants were removed. Cells were washed with 0.1 M 4 °C DPBS, centrifuged at 1000 *g* for 10 minutes at 4 °C, and then RNA was isolated (described in the following section). This isolation procedure yields highly pure microglia (Iba-1+/MHCII+/CD163−/GFAP−). The phenotype of these cells has been extensively validated in prior publications from our laboratory using flow cytometry, quantitative real-time polymerase chain reaction, and immunohistochemistry (Frank et al., 2006b, 2010).

2.2.3. Enzyme-linked immunosorbent assay

Hippocampal samples were sonicated on ice using a tissue extraction reagent (Invitrogen) supplemented with protease inhibitor cocktail (Sigma). The homogenates were centrifuged (14,000 *g* for 10 minutes at 4 °C), and the supernatants were collected and stored at −20 °C. Total protein was quantified using a Bradford assay and an enzyme-linked immunosorbent assay for rat IL-1β (R&D systems) was run in duplicate according to the manufacturer's instructions.

2.2.4. Quantitative real-time polymerase chain reaction

RNA was isolated from microglia and reversed transcribed to cDNA using SuperScript III CellsDirect cDNA Synthesis System (Life Technologies) per the manufacturer's instructions. RNA was extracted from hippocampal homogenates using TRIZOL reagent and 2 μg of RNA was reverse transcribed to cDNA using Superscript II (Life Technologies) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of cDNA was performed in duplicate using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) with a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA, USA).

Primers were previously designed using Genbank at the National Center for Biotechnology Information, the Operon Oligo Analysis Tool, and the Basic Local Alignment Search Tool at National Center for Biotechnology Information and obtained from Invitrogen. Primers span exon/exon boundaries, and primer specificity was verified by melt curve analysis. Primers included β-actin (F: TTCCTTCCTGGGTATGGAAT and R: GAGGAGCAATGATCTTGATC), BMAL1 (F: AAAATGCAAGGGAGGCCCAC and R: TCTAACTTCCGGGACATCGC), CD200 (F: CTCTCTATGTACAGCCCATAG and R: GGGAGTGACTCTCAGTACTAT), *CD200R* (F: TAGAGGGGGTGACCAATTAT and R: TACATTTTCTGCAGCCACTG), *CD68* (F: CAAGCAGCACAGTGGACATTC and R: CAAGAGAAGCATGGCCCGAA), *CLOCK* (F: CTGCTGACAAAAGCCAAGAT and R: GACTTTCTTGAGCTTCTGGA), *CX3CL1* (F: ATCATCCTGGAGACGAGACAGC and R: CCACACGCTTCTCAAACTTGCC), *CX3CR1* (F: TCAGGACCTCACCATGCCT and R: CGAACGTGAAGACAAGGG), *GFAP* (F: AGATCCGAGAAACCAGCCTG and R: CCTTAATGACCTCGCCATCC) *Iba1* (F: GGCAATGGAGATATCGATAT and R: AGAATCATTCTCAAGATGGC), *IL-1β* (F: CCTTGTGCAAGTGTCTGAAG and R: GGGCTTGGAAGCAATCCTTA), MHCII (F: AGCACTGGGAGTTTGAAGAG and R: AAGCCATCACCTCCTGGTAT), *Per1* (F: GTGCAGGCTAACCAGGAATA and R: GCGGAGAGTGTATTCAGATG), *Per2* (F: ACAAGCGGCTGCAGTAGTGA and R: TTCAAGGTTGCCAGCGTGCT), and *TNFα* (F: CAAGGAGGAGAAGTTCCCA and R: TTGGTGGTTTGCTACGACG). There was no group difference in *β-actin* in experiments described in Sections 2.3.1, 2.3.2, and 2.3.4, and it was used as the housekeeping gene in all mRNA comparisons (Cleal et al., 2014). There was a significant difference in β-actin in the experiment described in Section 2.3.3: β-actin amplified later in samples collected after the 15-hour incubation as compared to the 3-hour incubation; this likely reflects fewer surviving cells over time in culture.

2.2.5. Statistical analyses

All data are presented as mean ± standard error of the mean. Data were analyzed with Prism 5 (GraphPad Software) using 2-way analysis of variance with age and Zeitgeber time (ZT) serving as the between-subjects factors. F values are reported for each analysis of variance and serve as the criteria for post hoc analysis (Tukey's honestly significant difference). Rhythm data were further tested for a fit to a 24-hour cosinor curve using CircWave analysis software. The threshold for statistical significance was set at $p < 0.05$ in all cases.

2.3. Experimental design

2.3.1. Experiment 1: are diurnal rhythms in clock gene expression intact in hippocampal microglia isolated from aged rats?

Hippocampal microglia were isolated from young and aged rats at 6-hour intervals. Following isolations, microglia were plated in 100 μL of media alone or, in some cases, with 10 or 100 ng/mL of LPS (*E. coli* serotype 0111:B4). These doses were selected based on previous work in our laboratory and reflect physiologically relevant concentrations (Fonken et al., 2015; Frank et al., 2010). After 3 hours, RNA was extracted and the expression of clock genes and inflammatory cytokines was quantified using quantitative real-time polymerase chain reaction (as detailed previously).

The hippocampus (and hippocampal microglia) was used in these experiments for several reasons: (1) our laboratory has previously demonstrated that the potentiation of the inflammatory response in the aged CNS is particularly robust in the hippocampus (compared to other brain regions—e.g., prefrontal cortex, parietal cortex, and hypothalamus) (Barrientos et al., 2006, 2009a, 2009b); (2) Corticosteroid rhythms are specifically disrupted in the hippocampus of aged animals (Barrientos et al., 2015); (3) The hippocampus is a discrete and large-brain structure that enables isolation of a sufficient number of microglia; other potentially interesting brain structures (e.g., hypothalamus) do not yield enough cells for ex vivo manipulations; and (4) It is unclear if microglia from different brain structures would have comparable rhythms in clock and inflammatory genes. Combining microglia from multiple brain structures (Grabert et al., 2016) could preclude effective evaluation of circadian and inflammatory phenotypes. Thus, we evaluated microglia from a single brain region rather than pooling microglia from several areas.

2.3.2. Experiment 2: does corticosterone induce Per1 expression in microglia isolated from aged rats?

Previous work indicates that glucocorticoids specifically induce expression of *Per1* in isolated microglia (but not other clock genes) (Fonken et al., 2015). This implicates glucocorticoids as a potential entraining signal for microglia rhythms. Microglia from aged rats have disrupted *Per1* and *Per2* gene expression. Thus, here we evaluated whether hippocampal microglia isolated from aged and young rats had similar *Per1* response to corticosterone stimulation. Hippocampal microglia were isolated from young and aged rats during the light phase and plated for 2 hours with 0, 10, or 100 nM corticosterone (Sigma-C2505). RNA was then isolated to assess corticosterone-evoked *Per1* induction.

2.3.3. Experiment 3: are diurnal oscillations in microglial clock gene expression sustained ex vivo?

In our first experiment, microglia isolations required approximately 3 hours, and then microglia were cultured for 3 hours prior to RNA isolation. Although we expect that microglial clock genes sustain oscillatory expression in vitro [based on work in other immune cells; e.g., (Keller et al., 2009)], it is possible that the isolation procedure disrupts ongoing clock gene rhythms. Clarifying this issue is important to establish whether the cells are one interval (6 hours) behind the point of tissue collection. Unfortunately, microglia isolated from aged F344xBN rats are not viable for an entire 24-hour cycle, which limits the experimental approaches that can be used to test the question. Thus, to determine whether microglial clock genes continue to oscillate ex vivo, microglia were isolated from young and aged rats during the middle of the light phase and either plated for 3 hours (as described in our previous experiment) or for 15 hours (12 hours longer than the 3-hour time point) and then RNA was extracted to evaluate clock gene expression.

2.3.4. Experiment 4: are circadian rhythms in microglia apparent in whole hippocampus?

Next, we determined whether circadian rhythms in microglia and astrocyte activation markers are apparent in the hippocampus of young and aged rats. Hippocampal tissue was collected from young and aged male rats either in the middle of the light (ZT 6) or dark phase (ZT 18), and mRNA expression for genes related to glial activation was evaluated.

2.3.5. Experiment 5: do aged rats demonstrate diurnal differences in inflammatory response?

To determine whether diurnal differences in microglia responsivity result in physiologically meaningful changes in the neuroinflammatory response, aged and young rats were challenged with *E. coli*, and hippocampal IL-1β induction was examined. Young and aged rats received a 250-μL IP injection of *E. coli* (2.5×10^9 colony forming units; ATCC#15746) or saline in the middle of the light (ZT 6) or middle of the dark (ZT 18) phase (n = 6–8 per group) [as described in (Barrientos et al., 2006)]. Hippocampal tissue was collected 3 hours following the injection to evaluate IL-1β protein expression. *E. coli* was used in this experiment because: (1) *E. coli* affects a broad range of immune targets (LPS mostly acts through TLR4); (2) *E. coli* is more ecologically relevant; and (3) this maintains consistency with our previous studies on aging (Barrientos et al., 2006, 2009a).

3. Results

3.1. Hippocampal microglia from aged rats display comparable rhythms in some but not all clock genes

First, we sought to determine whether microglia isolated from the hippocampus of aged rats have altered clock gene expression. Hippocampal microglia were isolated from young and aged rats at 6-hour intervals (ZT 0/24, ZT 6, ZT 12, and ZT 18), and the expression of several circadian clock genes (*Per1*, *Per2*, *BMAL*, and *Rev-Erbα*) was assessed. Both *BMAL1* and *Rev-erbα* were expressed rhythmically in young and aged hippocampal microglia. There was a main effect of time on *BMAL1* expression in microglia isolated from aged and young rats (main effect of time: $F_{3,30} = 5.8$, $p < 0.01$, Fig. 1A). Furthermore, cosinor analysis revealed a significant 24-hour rhythm in *BMAL1* expression with the acrophase (cycle peak) occurring at ZT 4.3 ($F_{3,30} = 4.1$, $p < 0.05$). There was also a main effect of time on *Rev-Erbα* mRNA expression (main effect of time: $F_{3,30} = 4.9$, $p < 0.01$, Fig. 1B) and a significant 24-hour rhythm in *Rev-Erbα* expression with an acrophase of ZT 9.6 ($F_{3,30} = 3.9$, $p < 0.05$). The pattern and levels of gene expression were comparable between microglia isolated from the hippocampus of young and aged rats with respect to *BMAL1* and *Rev-Erbα* (there was no effect of age or interaction between time and age). In contrast, there were time-of-day differences in *Per1* and *Per2* mRNA in hippocampal microglia isolated from young but not aged rats (*Per1*—interaction of age × time: $F_{3,30} = 4.6$, $p < 0.01$, Fig. 1C; *Per2*—interaction of age × time: $F_{3,30} = 5.0$, $p < 0.01$, Fig. 1D). Indeed, there were main effects of time on *Per1* and *Per2* mRNA in microglia isolated from young (*Per1*: $F_{3,16} = 7.3$ and *Per2*: $F_{3,16} = 31.3$, $p < 0.05$) but not aged animals ($p > 0.05$). Similarly, there was a 24-hour rhythm in *Per1* mRNA in microglia isolated from young ($F_{3,16} = 10.9$, $p < 0.001$; acrophase at 6.4) but not aged rats ($p > 0.05$). Although there was not a main effect of time with respect to *Per2* expression in microglia isolated from aged rats, there was a significant 24-hour rhythm ($F_{3,15} = 4.5$, $p < 0.05$). The rhythm in *Per2* expression was shifted in microglia isolated from aged as compared to young rats ($F_{3,16} = 43.7$, $p < 0.001$): the acrophase was at ZT 12.9 in aged as compared to ZT 10.1 in young rats. Thus, aged hippocampal microglia show altered and/or dampened diurnal rhythms of the core clock genes *Per1* and *Per2*.

Disrupted *Per* rhythms in aged microglia could reflect altered responsiveness to and/or expression of corticosterone, a proposed microglia entraining signal. Indeed, previous work suggests that glucocorticoid rhythms are suppressed in the aged hippocampus (Barrientos et al., 2015). To determine whether a diminished microglial-intrinsic capacity to respond to glucocorticoids also contributes to disrupted *Per* expression in aged cells, we next isolated microglia from hippocampus of young and aged rats and stimulated them ex vivo with corticosterone for 2 hours. Consistent with previous work (Fonken et al., 2015), corticosterone treatment induced *Per1* expression in microglia and there was no effect of age on corticosterone-elicited *Per1* expression ($p > 0.05$; Fig. 1E), suggesting that aged microglia maintain typical entrainment sensitivity to glucocorticoids.

3.2. Hippocampal microglia sustain clock gene oscillations ex vivo

Microglial isolations take several hours, and it is unclear whether the cells continue to oscillate throughout the isolation procedure and during the subsequent incubation. Thus, we next sought to establish whether microglia maintain diurnal oscillatory gene expression patterns ex vivo. Hippocampal microglia were isolated from young and aged rats during the middle of the light phase (ZT 6) and then plated for either 3 or 15 hours. There was an interaction in *Per1* mRNA expression ($F_{1,28} = 7.0$, $p < 0.05$) and an effect of time in microglia isolated from young (post hoc, $p < 0.01$) but not aged rats ($p > 0.05$; Supplementary Fig. 1). This is consistent with rhythmic expression of *Per1* in microglia isolated from young but not aged rats (refer to Fig. 1C; RNA expression assessed immediately after microglial isolation). Furthermore, *BMAL1* expression was suppressed in microglia 15 hours following isolation ($F_{1,28} = 5.3$, $p < 0.05$; Supplementary Fig. 1B), consistent with reduced *BMAL1* noted in microglia isolated during the middle of the dark phase. There was an overall upregulation in cytokine mRNA expression in microglia maintained in vitro for 15 hours, which is consistent with increases in reactivity with time in culture (data not shown) (Caldeira et al., 2014). These results indicate that microglial clock gene expression likely continues to oscillate ex vivo.

3.3. Hippocampal microglia from aged rats lack diurnal rhythms in inflammatory cytokine expression

In addition to displaying diurnal clock gene rhythms, microglia from young rats show rhythmic expression of several inflammatory cytokines (Fonken et al., 2015). Since microglia from aged rats show disrupted rhythms in *Per1* and *Per2* expression, these aged cells may also show altered cytokine expression rhythms. In agreement with previous findings, *IL-1β* mRNA was differentially expressed over the course of the day in microglia isolated from the hippocampus of young rats ($F_{3,16} = 8.3$, $p < 0.05$, Fig. 2A) and there was a significant 24-hour rhythm with an acrophase of ZT 7.3 ($F_{3,16} = 12.3$, $p < 0.001$). In contrast, there was not a significant 24-hour rhythm or diurnal difference in *IL-1β* mRNA in hippocampal microglia isolated from aged rats ($p > 0.05$, Fig. 2A). There was also a main effect of age, such that microglia isolated from aged rats demonstrated an elevation in average *IL-1β* mRNA expression collapsed across the course of the day ($F_{1,30} = 5.6$, $p < 0.05$, Fig. 2B). *TNFα* was similarly regulated (interaction between age × time: $F_{3,30} = 3.0$, $p < 0.05$), with 24-hour rhythmic expression observed in microglia isolated from the hippocampus of young ($F_{3,15} = 10.6$, $p < 0.05$) but not aged rats ($p > 0.05$, Fig. 2C). Furthermore, there was an average upregulation in *TNFα* mRNA expression in microglia isolated from aged as compared to young rats ($F_{1,30} = 16.6$, $p < 0.05$, Fig. 2D). Thus, whereas microglia from young rats showed diurnal rhythms in *IL-1β* and *TNFα* expression (lower during the dark phase), microglia from aged rats expressed these proinflammatory cytokines at high levels throughout the day.

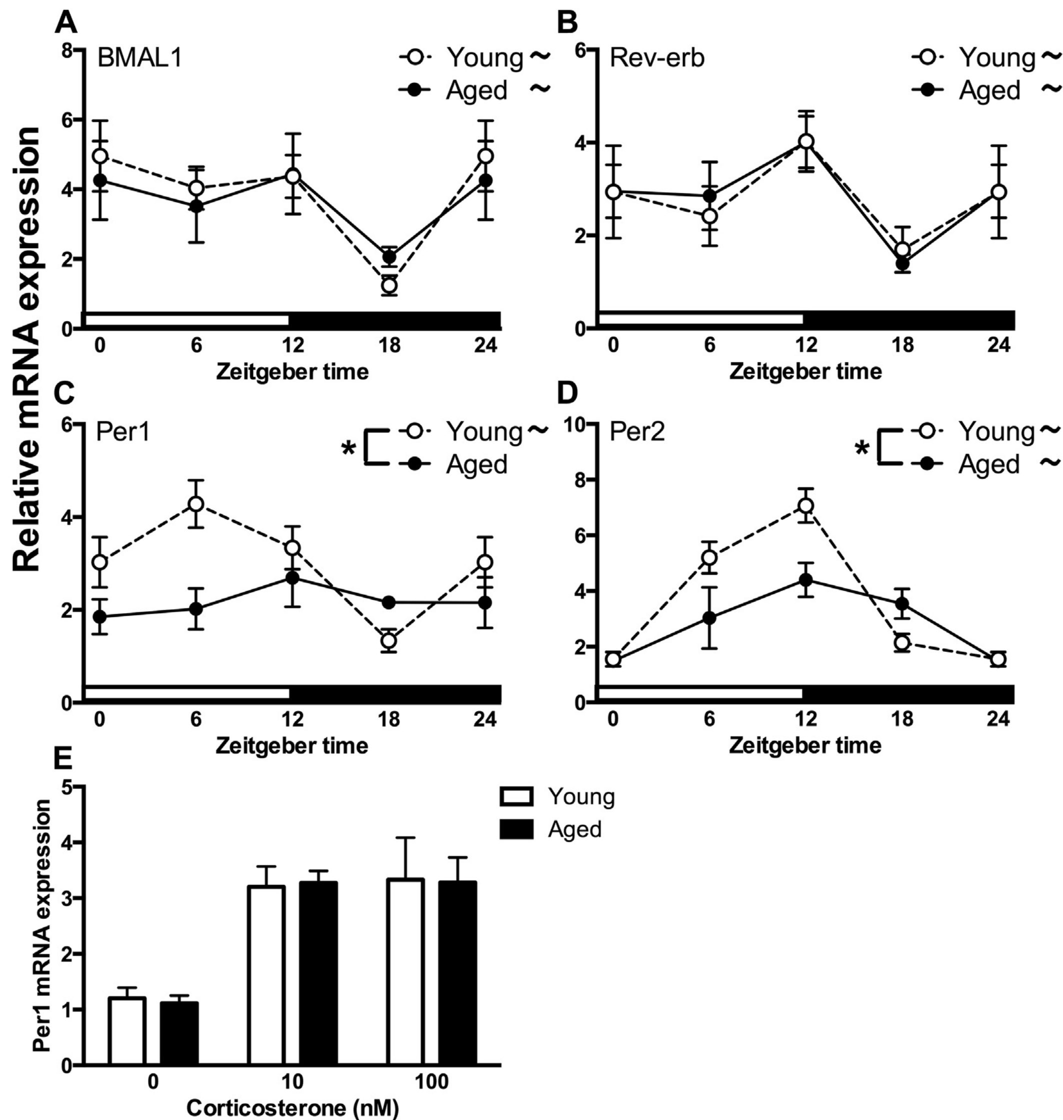

Fig. 1. Hippocampal microglia isolated from aged rats display aberrant diurnal clock gene rhythms, yet remain responsive to entrainment by glucocorticoids. Microglia were isolated from the hippocampus of young and aged rats at 6-hour intervals across the day (n = 5 per age and time). Gene expression for (A) *BMAL1*, (B) *Rev-Erbα*, (C) *Per1*, and (D) *Per2* was evaluated in unstimulated microglia. There was a 24-hour rhythm in *BMAL1* and *Rev-erbα* expression, and there were no age-associated differences. In contrast, *Per1* and *Per2* were differentially expressed in microglia isolated from young but not aged rats. A 24-hour rhythm in *Per1* was not detectable in microglia isolated from aged rats. These data were analyzed using a 2 × 2 ANOVA with age and time as the between-subjects factors; results were further analyzed using CircWave for fit to a 24-hour cosinor curve. (E) In a separate experiment, hippocampal microglia were isolated from young and aged rats at ZT 6 and plated for 2 hours with corticosterone; then, *Per1* mRNA expression was evaluated to determine whether microglia from young and aged rats exhibit a similar response to a proposed entrainment factor. Glucocorticoid-treated young and aged microglia both upregulated *Per1* indicating they are responsive to this entrainment signal. Data were analyzed using a 2 × 2 ANOVA with age and time as the between-subjects factors All data are expressed relative to *β-actin* and presented as mean ± SEM. ~Significant 24-hour rhythm, *interaction between time and age; in all cases, $p < 0.05$. Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean; ZT, Zeitgeber time.

3.4. There are diurnal differences in response to an ex vivo LPS challenge in hippocampal microglia isolated from young but not aged rats

Hippocampal microglia isolated from aged rats expressed higher levels of the proinflammatory cytokines *IL-1β* and *TNFα* throughout the day, suggesting that these cells may display an exaggerated inflammatory response—particularly during the dark phase (e.g., ZT 18). To determine whether microglia are more sensitive to an inflammatory challenge at different times of the day, microglia were isolated from the hippocampus of aged and young rats at 6-hour intervals and treated ex vivo with LPS (Fig. 3). In microglia isolated from the hippocampus of young rats, LPS-induced *IL-1β* expression differed over the course of the day (interaction between ZT × LPS in young microglia: $F_{6,48} = 4.5$, $p < 0.005$, Fig. 3A). Hippocampal microglia isolated from young rats had an increased LPS-elicited *IL-1β* response during the light phase (ZT 6) (compared to microglia isolated from young rats during the dark phase [ZT 18]; main effect of time $F_{3,48} = 24.4$ and post hoc, $p < 0.05$). In contrast, microglia isolated from aged rats had comparable LPS-elicited *IL-1β* mRNA increases at all 4 circadian time points ($p > 0.05$). Comparing the response of aged and young hippocampal microglia to LPS challenge at individual ZTs revealed that microglia isolated from aged rats exhibited an enhanced *IL-1β* response at ZT 18 (vs. young rat microglia at ZT 18; interaction of age × LPS at ZT 18: $F_{2,24} = 18.8$, $p < 0.05$).

Similar diurnal time- and age-dependent patterns of LPS-induced *TNFα* expression were observed. Microglia isolated from

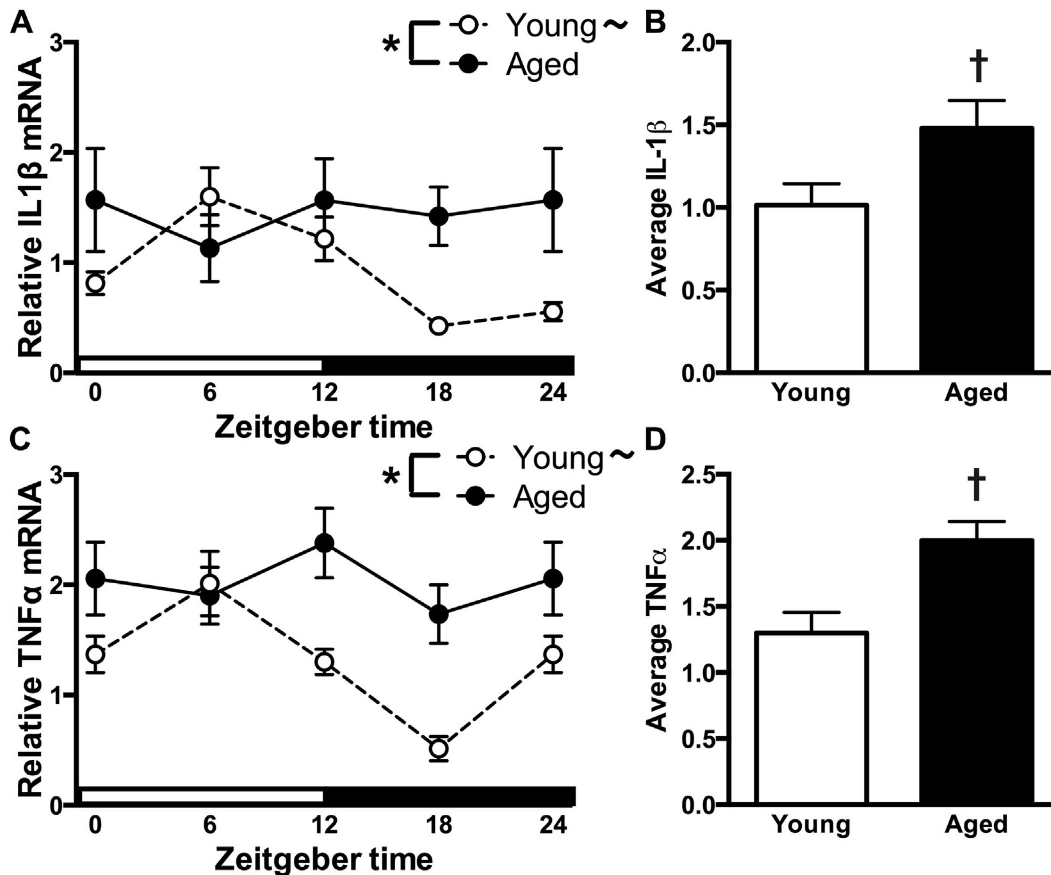

Fig. 2. Cytokine expression is diurnally regulated in hippocampal microglia isolated from young but not aged rats. Unstimulated microglia were isolated from the hippocampus of aged and young rats at 6-hour intervals across the day (n = 5 per age and time). Microglia isolated from young rats displayed diurnal variation in basal *IL-1β* (A) and *TNFα* expression (C). In contrast, microglia isolated from aged rats had comparable, relatively high cytokine expression throughout the day. Furthermore, average *IL-1β* (B) and *TNFα* (D) values collapsed over the course of the day were elevated in microglia isolated from aged as compared to young rats. Results were analyzed using a 2 × 2 ANOVA with age and time as the between-subjects factors and using CircWave for fit to a 24-hour cosinor curve. Data are expressed relative to *β-actin* and presented as mean ± SEM. ~Significant 24 hours rhythm, *interaction between time and age, †main effect of age; in all cases $p < 0.05$. Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean.

the hippocampus of young rats exhibited differences in LPS-induced *TNFα* mRNA at different times of day (interaction between ZT × LPS in young microglia: $F_{6,46} = 5.5$, $p < 0.001$). Hippocampal microglia isolated from young rats during the light phase (ZT 6) had heightened LPS-induced *TNFα* mRNA expression (compared to microglia isolated from young rats during the dark phase [ZT 18]; $F_{3,46} = 26.8$, $p < 0.0001$; Fig. 3B). There were no time-of-day differences in *TNFα* response in microglia isolated from aged rats ($p > 0.05$). Comparing the response of aged and young microglia to LPS challenge at individual ZTs revealed that microglia isolated from aged rats exhibited an enhanced *TNFα* response at ZT 0 and ZT 18 (vs. young rat microglia at these times; interaction of age × LPS at ZT 0 and ZT 18, respectively: $F_{2,24} = 3.4$ and $F_{2,24} = 10.9$, $p < 0.05$). Together, these results suggest that aged hippocampal microglia do not display typical (young like) diurnal sensitivity differences to LPS. Hippocampal microglia from aged rats have an exaggerated inflammatory profile, particularly during the dark phase.

3.5. There are diurnal differences in microglia activation markers in the hippocampus of young but not aged rats

The first several experiments presented here demonstrate that hippocampal microglia isolated from young rats display robust daily rhythms in the expression of clock and inflammatory genes. In contrast, the rhythmic expression of these factors appears disrupted in hippocampal microglia isolated from aged rats. However, it is unclear whether a similar disruption in diurnal expression of inflammatory factors persists in the intact hippocampus of aged rats. To evaluate potential rhythmic expression of glial activation markers in young and aged hippocampus, whole hippocampus was collected from young and aged rats during the middle of the light (ZT 6) or middle of the dark (ZT 18) phase (Fig. 4). *Iba1* mRNA expression, a marker of microglia activation, was significantly elevated in the aged hippocampus ($F_{1,38} = 17.0$, $p < 0.001$; Fig. 4A). Furthermore, *Iba1* mRNA expression was elevated during the middle of the light as compared to the dark phase ($F_{1,38} = 13.6$, $p < 0.005$). However, post hoc analysis revealed this time-of-day difference was specific to hippocampal tissue from young rats. *CD68* mRNA, another marker for microglia activation, was similarly elevated in the hippocampus of aged rats ($F_{1,38} = 31.2$, $p < 0.0001$; Fig. 4B) and was regulated by time-of-day in young but not aged rats ($F_{1,38} = 4.3$ and post hoc, $p < 0.05$). As expected, *GFAP* (an astrocyte-specific RNA that increases with astrocyte activation) ($F_{1,38} = 25.1$, $p < 0.0001$; Fig. 4C) and *MHCII* (an antigen-presenting protein associated with microglial activation) ($F_{1,36} = 53.4$, $p < 0.0001$; Fig. 4D) were also elevated in the aged hippocampus; however, there were no time-of-day differences in GFAP or MHCII mRNA expression. We also evaluated expression of genes involved in neuronal inhibition of microglia, including *CD200*, *CD200R*, *CX3CR1*, and *CX3CL1* (Fig. 4E–F). There was no effect of time-of-day on any of these genes. Consistent with previous research

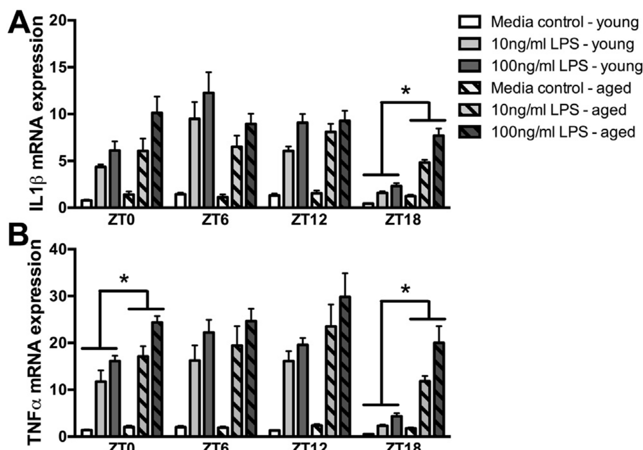

Fig. 3. LPS-induced cytokine responses are dampened during the dark phase in hippocampal microglia from young but not aged rats. Hippocampal microglia were isolated from aged and young rats at 6-hour intervals across the day (n = 5 per age and time). To assess microglial inflammatory potential, microglia were stimulated for 3 hours ex vivo with media alone (0 ng/mL LPS control), 10 ng/mL of LPS, or 100 ng/mL of LPS. (A) *IL-1β* and (B) *TNFα* mRNA expressions were evaluated and were expressed relative to β-actin. During the dark phase (ZT 18), LPS-induced cytokine increases were dampened in microglia from young rats. In contrast, LPS-induced cytokine responses remained elevated in microglia from aged rats. Data are expressed as mean ± SEM. *Main effect of age, $p < 0.05$. Abbreviations: LPS, lipopolysaccharide; SEM, standard error of the mean; ZT, Zeitgeber time.

(Frank et al., 2006a), aged hippocampi had reduced *CX3CL1* and increased *CD200R* mRNA expression ($F_{1,38}$ = 4.9 and 5.5, respectively, $p < 0.05$). These results corroborate previous studies showing that aged rat hippocampi have an overall increase in markers of microglial and/or astrocyte inflammatory potential (increased Iba1, CD68, MHCII, GFAP). Furthermore, microglial activation markers (Iba1 and CD68) that are expressed rhythmically in young rat hippocampus are tonically elevated (in both light and dark phase) in the aged hippocampus.

3.6. *Hippocampal IL-1β response is abrogated in young but not aged rats during the dark phase*

To determine whether differences in ex vivo microglia responsivity correspond with more functionally relevant in vivo changes, *E. coli* was administered to young and aged rats either during the middle of the light or dark phase and tissue collected 3 hours later. Hippocampal IL-1β protein expression was increased 3 hours following a light-phase IP *E. coli* injection in both young and aged rats (main effect; $F_{1,23} = 18.8$, $p < 0.05$; Fig. 5). There was not a significant interaction between age and *E. coli* at this time point ($p > 0.05$), although there was a trend for elevated hippocampal IL-1β in aged rats ($F_{1,23} = 3.5$, $p = 0.07$). In contrast, there was an interaction between *E. coli* and age on hippocampal IL-1β expression during the middle of the dark phase ($F_{1,23} = 6.3$, $p < 0.05$): *E. coli* caused a significant elevation in IL-1β in aged but not young rats (post hoc, $p < 0.05$). Thus, similar to the results with isolated hippocampal microglia, whole aged rat hippocampus has an abolished cyclic diurnal IL-1β expression pattern and enhanced *E. coli*–elicited IL-1β expression in the dark phase.

4. Discussion

Here, we reveal that rhythms in clock and inflammatory genes observed in microglia isolated from the hippocampus of young rats are disrupted in aged hippocampal microglia. Furthermore, we demonstrate that the abnormally high expression of cytokines during the dark phase in hippocampal microglia isolated from aged rats correlates with an exaggerated *E. coli*–induced inflammatory response. Specifically, there were several key findings reported here. First, diurnal rhythms in clock gene expression are disrupted in microglia isolated from the hippocampus of aged rats. In agreement with previous findings (Fonken et al., 2015), hippocampal microglia isolated from young rats rhythmically expressed core circadian clock genes. Microglia from aged rats displayed comparable rhythms in several clock genes including *BMAL1* and *Rev-erbα*; however, rhythmic expression of *Per1* was ablated and the *Per2* rhythm was shifted. Second, unstimulated microglia from young rats exhibited robust rhythms of *TNFα* and *IL-1β* mRNA, with peak cytokine expression occurring in microglia isolated during the middle of the light phase. In contrast, these proinflammatory cytokines were not regulated by time-of-day in microglia isolated from aged rats. Instead, *TNFα* and *IL-1β* mRNA were expressed at tonically high levels in unstimulated microglia isolated from the hippocampus of aged rats. Third, hippocampal microglia isolated from aged rats did not exhibit diurnal differences in responsiveness to ex vivo stimulation with LPS. In particular, aged microglia showed an exaggerated inflammatory response when they were isolated during the rat's dark (active) phase, when microglia isolated from young rats are typically less responsive. Fourth, similar patterns were observed in vivo: markers of microglia activation (*CD68* and *Iba1*) were diurnally regulated in the hippocampus of young rats, whereas *CD68* and *Iba1* were tonically elevated in the aged hippocampus with no time-of-day differences. Finally, diurnal variations in response to a peripheral *E. coli* challenge were only present in young rats, as aged rats displayed robust *E. coli*–induced hippocampal IL-1β increases in both the light and dark phases. Overall, these results indicate that intrinsic microglial rhythms in clock and inflammatory genes are dysregulated with aging, which could have implications for sickness susceptibility and/or duration and other neuroinflammatory conditions.

4.1. *Potential mechanisms underlying age-related dampening of circadian rhythms*

Aging is associated with decreased amplitude and precision of behavioral, metabolic, and other endocrine rhythms (Bonaconsa et al., 2014; Nakamura et al., 2015; Smith et al., 2005; Valentinuzzi et al., 1997) as well as slower re-entrainment of behavioral and extra-SCN oscillators (Davidson et al., 2008; Sellix et al., 2012). Here, we demonstrate that diminished rhythms with aging also apply to circadian regulation of the brains innate immune system. Dampening of extra-SCN rhythms in the aged brain may occur for several reasons. First, there may be age-associated decreases in input to the SCN. For example, aged animals exhibit a reduced response to light (Lupi et al., 2012). Second, aging is associated with diminished strength of SCN outputs (Farajnia et al., 2012; Kallo et al., 2004; Nakamura et al., 2011). Finally, global disruptions in epigenetic pathways that control the circadian clock may become dysregulated with aging (Orozco-Solis and Sassone-Corsi, 2014).

Central clocks may synchronize extra-SCN clocks by rhythmic expression of entraining factors, such as corticosterone and melatonin. Corticosterone is a synchronizing factor for several extra-SCN clocks (Balsalobre et al., 2000; Sujino et al., 2012) and induces expression of the core clock gene *Per1*, but not other circadian clock genes, in microglia (Fonken et al., 2015). Importantly, *Per1* mRNA expression was specifically dysregulated in hippocampal microglia isolated from aged rats. Indeed, *BMAL1* and *Rev-Erbα* expression were comparable across the day in hippocampal microglia isolated from aged and young rats and while the pattern of *Per2* mRNA expression was altered in aged

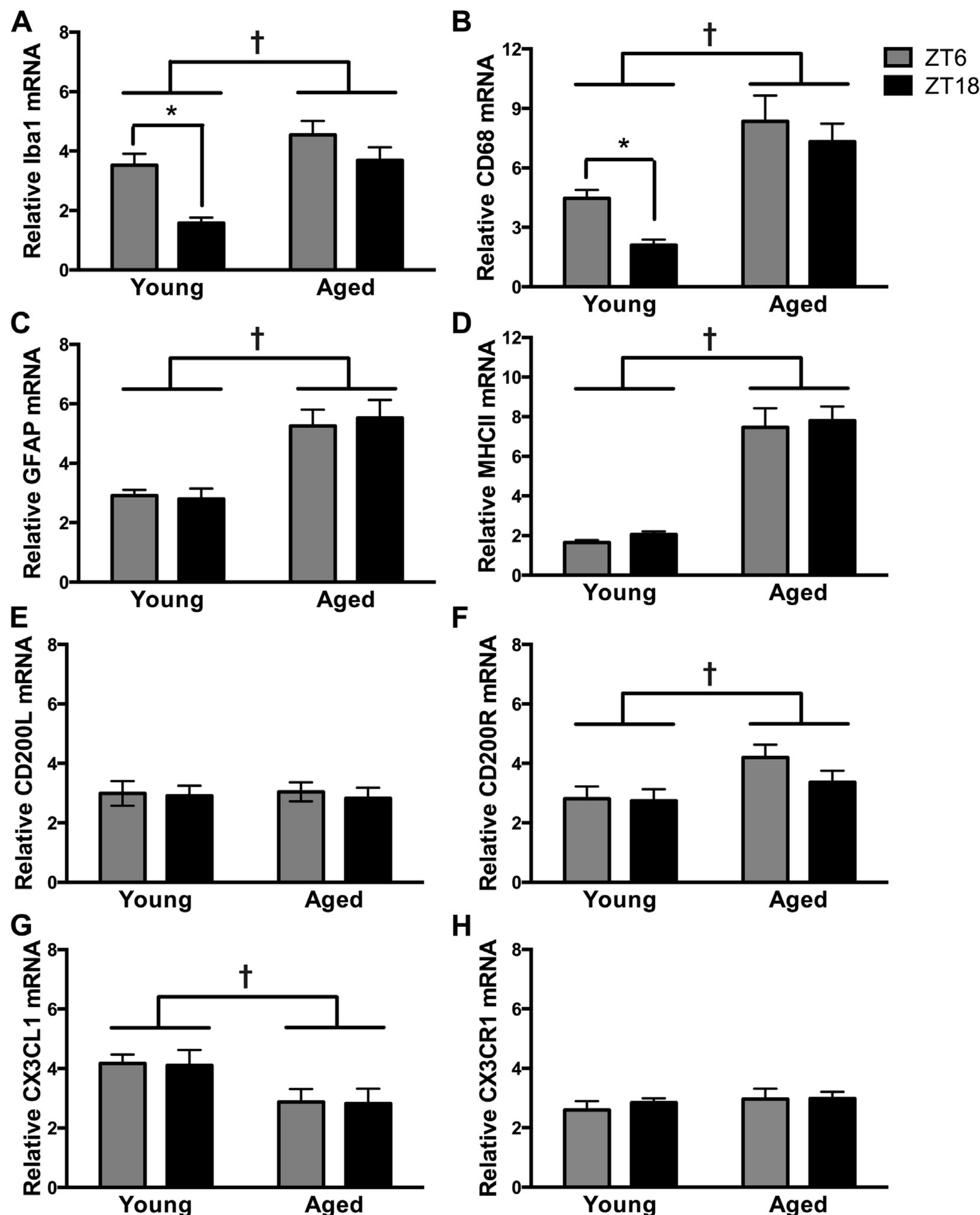

Fig. 4. The expression of some microglia-activation markers were increased in the light as compared to the dark phase in the hippocampus of young but not aged rats. Whole hippocampus was collected from young (4 months) and aged (25 months) male F334xBN rats during the middle of the light (Zeitgeber time 6; ZT 6) or dark (ZT 18) phase (n = 8–12 per group). Gene expression for (A) Iba1, (B) CD68, (C) GFAP, (D) MHCII, (E) CD200L, (F) CD200R, (G) CX3CL1, and (H) CX3CR1 were evaluated and analyzed using a 2×2 ANOVA with age and time as the between-subjects factors. Iba1, CD68, GFAP, MHCII, and CD200R were increased in aged hippocampus, while CX3CL1 was reduced in aged hippocampus. Both Iba1 and CD68, which are expressed in activated microglia, were diurnally regulated in young hippocampus but tonically elevated in aged hippocampus. All data are expressed relative to *β-actin* and presented as mean ± SEM. *Main effect of time within an age group, †main effect of age, in all cases $p < 0.05$. Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean; ZT, Zeitgeber time.

microglia, there was still a detectable rhythm. In contrast, rhythmic expression of *Per1* mRNA was completely ablated in hippocampal microglia isolated from aged rats. This pattern led us to examine whether there were differences in *Per1* induction in response to corticosterone stimulation in hippocampal microglia isolated from young and aged rats. Here, we found that corticosterone-elicited *Per1* induction was comparable in hippocampal microglia isolated from young and aged rats. However, previous work indicates that the amplitude of the corticosterone rhythm is diminished in the hippocampus of aged rats, with unusually elevated corticosterone concentrations occurring during the inactive phase in aged rats (Barrientos et al., 2015). This suggests that even though isolated microglia from aged rats respond comparably to corticosterone, they may receive disrupted rhythm in the corticosterone entrainment signal in vivo.

There are several alternative mechanisms through which circadian rhythms in microglia may become disrupted with age. First, melatonin signaling, which is another proposed entraining

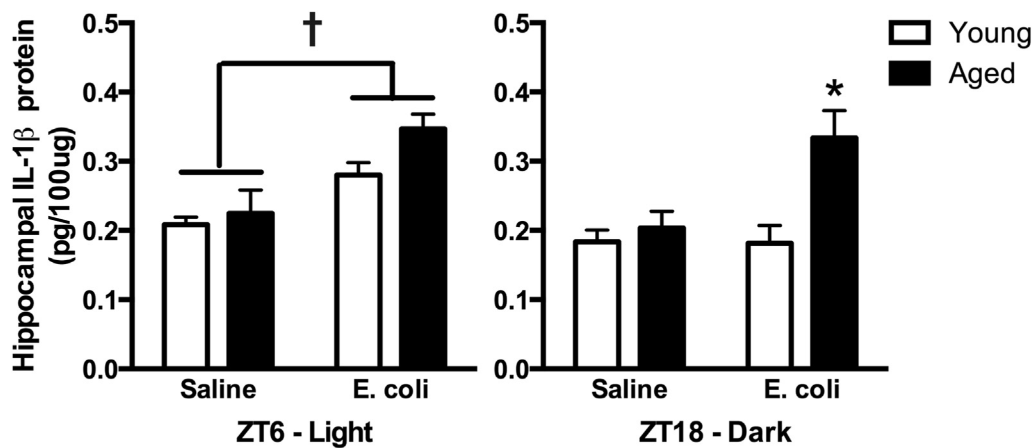

Fig. 5. *E. coli*-induced hippocampal IL-1β protein was amplified in aged rats, particularly during the dark phase. Rats received a single intraperitoneal injection of *E. coli* (2.5 × 10^9 colony forming units) during the light (ZT 6) or dark (ZT 18) phase (n = 6–7 per age and time). Hippocampal tissue was collected 3 hours later to assess IL-1β protein concentrations. For rats receiving saline and/or *E. coli* during the light phase, IL-1β was increased in *E. coli*–treated rats (young and old; main effect). For rats receiving saline and/or *E. coli* during the dark phase, IL-1β was increased specifically in the hippocampus of *E. coli*–treated aged rats. Data are expressed as mean ± SEM. †Main effect of *E. coli*, *differs from all other groups, in all cases $p < 0.05$. Abbreviations: SEM, standard error of the mean; ZT, Zeitgeber time.

signal for extra-SCN clocks, is also disrupted in the aged brain (Hardeland et al., 2015). Second, changes in *Per1* rhythmicity may reflect a disruption in CLOCK and/or BMAL1 protein as CLOCK and BMAL1 form the positive arm of the circadian clock and activate transcription of the *Per* and *Cry* genes (Partch et al., 2014). Of note, it is not unusual for aspects of the circadian feedback loop to remain intact when a single clock gene (such as *Per1*) is disrupted. This likely occurs because there is redundancy in the system—Per and Cry proteins both contribute to the negative arm of the circadian clock (Kume et al., 1999).

Changes in circadian clock genes and/or proteins in aged animals have previously been implicated in age-related pathologies. For example, the amplitude of *Rev-Erbα* mRNA rhythmic expression is diminished in peritoneal macrophages isolated from aged animals (Sato et al., 2014). Rev-erbα modulates the inflammatory function of macrophages through direct regulation of the chemokine CCL2. However, the present findings suggest that age does not affect *Rev-Erbα* expression in microglia. Disrupted BMAL1 expression is also associated with premature aging (Kondratov et al., 2006). BMAL1 deletion causes synaptic terminal degeneration, impaired cortical connectivity, as well as neuronal oxidative damage and impaired expression of several redox defense genes (Musiek et al., 2013). In aged microglia, Rev-erbα and BMAL1 expression were not altered; rather, *Per1* and *Per2* rhythmicity were blunted and correlated with exaggerated inflammatory responses. This could represent another age-related circadian disruption that contributes to neuroimmune priming.

4.2. Aged hippocampal microglia are hyperreactive during the dark phase

Diurnal changes in the microglial activation signature were observed in young but not aged rats. Hippocampal tissue from young rats displayed diurnal differences in microglia activation potential both in vivo (Fig. 4) and ex vivo (Figs. 2 and 3). In contrast, hippocampus from aged rats showed constitutively elevated microglia activation. Changes in microglia activation also paralleled intensity of neuroinflammatory responses elicited by an *E. coli* challenge. Indeed, IL-1β protein increases were not observed in the hippocampus of young rats challenged with *E. coli* during the middle of the dark phase (increased only in the light phase), whereas IL-1β protein was increased by an *E. coli* challenge during the light or the dark phase in aged rats. These results parallel the time at which the reduction in microglia activation was observed in the hippocampus of young rats. Although these findings implicate circadian differences in microglia in mediating time-of-day differences in the IL-1β protein response, there are also several alternative sources. For example, astrocytes also demonstrate circadian rhythms in activity and produce IL-1β (Prolo et al., 2005), although no time-of-day differences in GFAP expression were observed in the present study (Fig. 4). Alternatively, there could be time-of-day differences in immune cell migration into the CNS. Of note, the present experiments only evaluated 2 aging extremes (4 months and 25 months). Future studies should include "middle aged" groups to establish the age at which circadian rhythms in the immune response begin to erode.

The circadian system may rhythmically switch the immune system between 2 states: (1) a state of enhanced immunity, which would permit maximal immune surveillance and reactivity to pathogens and/or damage; and (2) a state of reduced immune responsivity, which could enable tissue repair and regeneration (Curtis et al., 2014). The healthy, young adult immune system appears to fluctuate between these 2 states over the course of the day, creating a diurnal rhythm in immune activation. Our results suggest that this natural, potentially beneficial diurnal fluctuation in inflammatory response may erode with age. The lack of diurnal regulation of immune responses may contribute to the protracted neuroinflammatory responses that are observed in aged animals (Barrientos et al., 2006, 2009a, 2009b). Unlike hippocampal microglia isolated from young rats, hippocampal microglia from aged rats do not appear to enter the state of decreased activation that putatively allows for immune system repair and regeneration (Curtis et al., 2014). Consistent with observations in macrophages (Keller et al., 2009), our results indicate that circadian clock genes continue to oscillate in microglia following the isolation procedure. This should be taken into consideration when interpreting results of the ex vivo immune stimulation experiments. For example, microglia isolated at ZT 6 were not stimulated with LPS until ∼ ZT 9 (as the isolation procedure takes several hours) and were then maintained in culture for 3 hours. Thus, RNA was not collected until almost a full 6 hours after the isolation procedure (placing the microglia at ZT 12 in this example). This suggests that while peak immune activation occurred in microglia isolated from young rats during the middle of the light and/or inactive phase (ZT 6), peak

immune activation in vivo may occur several hours later (between ZT 9 and 12).

4.3. "Inflammaging" and potential strategies to strengthen diurnal rhythms

Chronic low-grade inflammation that occurs with healthy aging (termed "inflammaging") may feed back on the circadian system to blunt daily rhythms (Hardeland et al., 2015; Popa-Wagner et al., 2015). Indeed, there is a higher density of astrocytes and microglia in the SCN of older rodents (Deng et al., 2010), and expression of cytokines and their receptors are altered in the aged SCN (Beynon and Coogan, 2010). Proinflammatory mediators can alter SCN neuronal function and circadian phase (Duhart et al., 2013; Sadki et al., 2007), suggesting "inflammaging" may affect circadian rhythms. Furthermore, aspects of circadian dysregulation that occur following sepsis mirror those reported in aged animals (O'Callaghan et al., 2012).

Taken together, these results indicate that strengthening circadian entrainment may be an effective strategy for resetting neuroinflammatory responses in the aged brain. In support of this idea, exercise is an intervention that entrains circadian rhythms (Droste et al., 2009; Edgar and Dement, 1991; Leise et al., 2013) and can prevent the exaggerated neuroinflammatory responses and microglia sensitization in the hippocampus of aged rats (Barrientos et al., 2011). Importantly, the effects of exercise on circadian clock entrainment depend on glucocorticoids (Hajisoltani et al., 2011).

5. Conclusions

These experiments reveal several key findings related to circadian rhythms in neuroinflammatory responses in aged rats. (1) Although microglia from aged rats display comparable rhythms in the clock genes *BMAL1* and *Rev-erbα*, *Per1* and *Per2* rhythms are suppressed; (2) Microglia from aged and young rats show comparable *Per1* induction in response to corticosterone, suggesting that suppressed glucocorticoid rhythms in aged hippocampus may mediate age-related dampening of microglial diurnal rhythms; (3) Typical diurnal rhythms in cytokine expression are abolished in microglia from aged animals; and (4) There is diurnal variation in hippocampal IL-1β in response to a peripheral immune challenge in the hippocampus of young but not aged rats. Together, these results highlight how aging can disrupt circadian rhythms in inflammatory cells, resulting in an overall heightened inflammatory response across the course of the day. The lack of diurnal variation in microglial responsivity in aged mammals could result in pathological tonic immune hyperreactivity and damaging, exaggerated responses to immune challenge. Thus, circadian rhythms should be considered when planning surgeries, chemotherapy, or other procedures that elicit an inflammatory response. Our study highlights that the circadian system is an underappreciated regulator of many homeostatic mechanisms in mammals. Future studies should investigate whether modulating circadian rhythms in inflammatory cells can diminish heightened and prolonged inflammatory responses that occur in the aged brain.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neurobiolaging.2016.07.019.

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