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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 CNS – 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 mos. F344XBN) rhythmically expressed circadian clock genes, whereas microglia isolated from the hippocampus of aged rats (25 mos.) had aberrant *Per1* and *Per2* rhythms. Unstimulated microglia from young rats exhibited robust rhythms of *TNFA* and *IL-1 β* 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-SCN 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.

Keywords

aging; neuroinflammation; glia; priming; IL-1 β

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 auto-regulatory feedback loop composed of key transcriptional activators (e.g. CLOCK, BMAL1,

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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 ((Straub and Cutolo, 2007, Marpegan et al., 2009, Spengler et al., 2012, Fonken et al., 2015), although see (Rahman et al., 2015)). Diurnal variations in immune responses are regulated in part by circadian rhythms in inflammatory cells (Boivin et al., 2003, Arjona and Sarkar, 2006, Hayashi et al., 2007, Keller et al., 2009, Bollinger et al., 2011, Silver et al., 2012, Fonken et al., 2015). 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 lifespan, with aged animals exhibiting diminished molecular and behavioral circadian rhythms. In the SCN of aged animals, several functional properties are altered including neuronal spiking (Nakamura et al., 2011, Farajnia et al., 2012), 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 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 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 (Godbout et al., 2005, Barrientos et al., 2009a, Fonken and Nelson, 2013). 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 mos.) and aged (24 mos.) male Fischer-Brown Norway crosses (F344xBN F1) were received from 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 mos) (Barrientos et al., 2006, Barrientos et al., 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 h:12 h light/dark cycle with lights on either at 0700 or 2100 h. Rats were given one month to acclimate to colony conditions before experimentation began (experimental manipulations occurred at ~4 and 25 mos. 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 min 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 below.

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 min, brains were removed, and the hippocampi were dissected out on ice. Each hippocampus was then homogenized in 3 mL of 0.2% glucose in 1X DPBS. The homogenate was passed through a 40µm 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 min at 22 °C and then the supernatant was removed. A Percoll gradient was created by re-suspending the pellet in 2 mL of 70% isotonic Percoll (GE Healthcare; isotonic Percoll is 10:1 Percoll with 10X PBS;

100% isotonic Percoll is then diluted with 1X 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 min 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 min at 22 °C. In total, this isolation procedure takes < 3 h. After isolation, microglia were re-suspended in media (filtered sterile high glucose DMEM (Gibco, 11960-044) +10%FBS [Atlanta biological, S11050]) and the concentration of viable microglia was determined by trypan blue dye exclusion. Microglia were plated at a density of 6,000 cells/100 μ L in a 96-well v-bottom plate. To assess microglia cytokine responsiveness, cells were challenged *ex vivo* with 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% CO₂ for 3 h (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, Frank et al., 2010). In a separate experiment, microglia were treated with corticosterone (Sigma-C2505) at concentrations of 1, 10, or 100 nM for 2 h (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 min at 4 °C to pellet the cells and then the supernatants were removed. Cells were washed with 0.1M 4 °C DPBS, centrifuged at 1000 *g* for 10 min at 4 °C, and then RNA was isolated (described below). 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, qPCR, and immunohistochemistry (Frank et al., 2006b, Frank et al., 2010).

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

2.2.4. Quantitative real-time PCR (qPCR)—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 μ g of RNA was reversed transcribed to cDNA using Superscript II (Life Technologies) according to the manufacturer's instructions. PCR amplification of cDNA was performed in duplicate using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA) with a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA).

Primers were previously designed using Genbank at the National Center for Biotechnology Information (NCBI), the Operon Oligo Analysis Tool, and the Basic Local Alignment Search Tool at NCBI 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: AAAATGCAAGGGAGGCCCCAC and R: TCTAACTTCCGGGACATCGC), *CD200* (F: CTCTCTATGTACAGCCCATAG and R: GGGAGTGAAGTCTCAGTACTAT), *CD200R* (F: TAGAGGGGGTGACCAATTAT and R: TACATTTTCTGCAGCCACTG), *CD68* (F: CAAGCAGCACAGTGGACATTC and R: CAAGAGAAGCATGGCCCGAA), *CLOCK* (F: CTGCTGACAAAAGCCAAGAT and R: GACTTTCTTGAGCTTCTGGA), *CX3CL1* (F: ATCATCCTGGAGACGAGACAGC and R: CCACACGCTTCTCAAACCTTGCC), *CX3CR1* (F: TCAGGACCTCACCATGCCT and R: CGAACGTGAAGACAAGGG), *GFAP* (F: AGATCCGAGAAACCAGCCTG and R: CCTTAATGACCTCGCCATCC), *Iba1* (F: GGCAATGGAGATATCGATAT and R: AGAATCATTCTCAAGATGGC), *IL-1 β* (F: CCTTGTGCAAGTGTCTGAAG and R: GGGCTTGGAAGCAATCCTTA), *MHCII* (F: AGCACTGGGAGTTTGAAGAG and R: AAGCCATCACCTCCTGGTAT), *Per1* (F: GTGCAGGCTAACCAGGAATA and R: GCGGAGAGTGTATTTCAGATG), *Per2* (F: ACAAGCGGCTGCAGTAGTGA and R: TTCAAGGTTGCCAGCGTGCT) and *TNFA* (F: CAAGGAGGAGAAGTTCCCA and R: TTGGTGGTTTGCTACGACG). There were no group difference in β -actin in experiments described in 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 h incubation as compared to the 3 h incubation; this likely reflects fewer surviving cells over time in culture.

2.2.5. Statistical analyses—All data are presented as mean $\pm$ standard error of the mean (SEM). Data were analyzed with Prism 5 (GraphPad Software) using two-way analysis of variance (ANOVA) with age and Zeitgeber time serving as the between subjects factors. F values are reported for each ANOVA and serve as the criteria for post hoc analysis (Tukey's HSD). Rhythm data were further tested for a fit to a 24 h 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 h intervals. Following isolations, microglia were plated in 100 μ L of media alone or, in some cases, with 10 or 100 ng/mL of lipopolysaccharide (LPS; *E. coli* serotype 0111:B4). These doses were selected based on previous work in our laboratory and reflect physiologically relevant concentrations (Frank et al., 2010, Fonken et al., 2015). After 3 h, RNA was extracted and the expression of clock genes and inflammatory cytokines was quantified using qPCR (as detailed above).

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) (see Barrientos et al 2006 and 2009). (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. (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 h 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 h, and then microglia were cultured for 3 h prior to RNA isolation. Although we expect 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 in order to establish whether the cells are one interval (6 h) behind the point of tissue collection. Unfortunately, microglia isolated from aged F344xBN rats are not viable for an entire 24 h 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 h (as described in our previous experiment) or for 15 h (12 h longer than the 3 h 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 (ZT6) or dark phase (ZT18) 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-1 β induction was examined. Young and aged rats received a 250 μ L IP injection of *E. coli* (2.5×10^9 CFU; ATCC#15746) or saline in the middle of the light (ZT6) or middle of the

dark (ZT18) phase (n = 6–8 per group) (as described in (Barrientos et al., 2006)). Hippocampal tissue was collected 3 h following the injection to evaluate IL-1 β 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, Barrientos et al., 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 h intervals (ZT0/24, ZT6, ZT12, and ZT18), and the expression of several circadian clock genes (*Per1*, *Per2*, *BMAL1*, *Rev-Erba*) was assessed. Both *BMAL1* and *Rev-Erba* 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 h 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-Erba* mRNA expression (main effect of time: $F_{3,30} = 4.9$, $p < 0.01$, Fig 1B) and a significant 24 h rhythm in *Rev-Erba* 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-Erba* (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 $\times$ time: $F_{3,30} = 4.6$, $p < 0.01$, Fig 1C; *Per2* - interaction of age $\times$ 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 h 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 h 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/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 h. 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 (ZT6) and then plated for either 3 or 15 h. 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 h 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 h, 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-1 β* 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 h rhythm with an acrophase of ZT 7.3 ($F_{3,16} = 12.3$, $p < 0.001$). In contrast, there was not a significant 24 h rhythm or diurnal differences in *IL-1 β* 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-1 β* mRNA expression collapsed across the course of the day ($F_{1,30} = 5.6$, $p < 0.05$, Fig 2B). *TNF α* was similarly regulated (interaction between age $\times$ time: $F_{3,30} = 3.0$, $p < 0.05$), with 24 h 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 *TNF α* 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-1 β* and *TNF α* expression (lower during the dark phase), microglia from aged rats expressed these pro-inflammatory cytokines at high levels throughout the day.

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 pro-inflammatory cytokines *IL-1 β* and *TNF α* throughout the day, suggesting that these cells may display an exaggerated inflammatory response – particularly during the dark phase (e.g., ZT18). To determine whether microglia are more sensitive to an inflammatory challenge at different times of day, microglia were isolated from the hippocampus of aged and young rats at 6 h intervals and treated *ex vivo* with LPS (Fig 3). In microglia isolated from the hippocampus of young rats, LPS-induced *IL-1 β* expression differed over the course of the day (interaction between ZT x 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-1 β* response during the light phase (ZT6) (compared to microglia isolated from young rats during the dark phase (ZT18); 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-1 β* mRNA increases at all four 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-1 β* response at ZT18 (versus young rat microglia at ZT18; interaction of age x LPS at ZT18: $F_{2,24} = 18.8$, $p < 0.05$).

Similar diurnal time- and age-dependent patterns of LPS-induced *TNF α* expression were observed. Microglia isolated from the hippocampus of young rats exhibited differences in LPS-induced *TNF α* mRNA at different times of day (interaction between ZT x LPS in young microglia: $F_{6,46} = 5.5$, $p < 0.001$). Hippocampal microglia isolated from young rats during the light phase (ZT6) had heightened LPS-induced *TNF α* mRNA expression (compared to microglia isolated from young rats during the dark phase (ZT18); $F_{3,46} = 26.8$, $p < 0.0001$; Fig 3B). There were no time-of-day differences in *TNF α* 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 *TNF α* response at ZT0 and ZT18 (versus young rat microglia at these times; interaction of age x LPS at ZT0 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 (ZT6) or middle of the dark (ZT18) 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) ($F_{1,38} = 25.1$ and $p < 0.0001$; 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 (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/astrocyte inflammatory potential (increased *Iba1*, *CD68*, *MHCII*, *GFAP*). Further, microglial activation markers (*Iba1*, *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-1 β response is abrogated in young but not aged rats during the dark phase

In order 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 h later. Hippocampal IL-1 β protein expression was increased 3 h 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-1 β 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-1 β expression during the middle of the dark phase ($F_{1,23} = 6.3$, $p < 0.05$): *E. coli* caused a significant elevation in IL-1 β 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-1 β expression pattern and enhanced *E. coli*-elicited IL-1 β 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-erba*; however, rhythmic expression of *Per1* was ablated and the *Per2* rhythm was shifted. Second, unstimulated microglia from young rats exhibited robust rhythms of *TNFA* and *IL-1 β* mRNA, with peak cytokine expression occurring in microglia isolated during the middle of the light phase. In contrast, these pro-inflammatory cytokines were not regulated by time-of-day in microglia isolated from aged rats. Instead, *TNFA* and *IL-1 β* 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-1 β 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/duration and other neuroinflammatory conditions.

Potential mechanisms underlying age-related dampening of circadian rhythms

Aging is associated with decreased amplitude and precision of behavioral, metabolic, and other endocrine rhythms (Valentinuzzi et al., 1997, Smith et al., 2005, Bonaconsa et al., 2014, Nakamura et al., 2015) 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 brain's 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 (Kallo et al., 2004, Nakamura et al., 2011, Farajnia et al., 2012). 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-Erba* 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 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 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/proteins in aged animals have previously been implicated in age-related pathologies. For example, the amplitude of *Rev-Erba* mRNA rhythmic expression is diminished in peritoneal macrophages isolated from aged animals (Sato et al., 2014). *Rev-erba* modulates the inflammatory function of macrophages through direct regulation of the chemokine CCL2. However, the present findings suggest that age does not affect *Rev-Erba* expression in microglia. Disrupted BMAL1 expression is also associated with pre-mature 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-erba* 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.

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* (Fig 2&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-1 β 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-1 β 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-1 β protein response, there are also several alternative sources. For example, astrocytes also demonstrate circadian rhythms in activity and produce IL-1 β (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 two aging extremes (4 mos and 25 mos.). Future studies should include “middle aged” groups in order 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 two states: (1) a state of enhanced immunity, which would permit maximal immune surveillance and reactivity to pathogens/damage; and (2) a state of reduced immune responsiveness, which could enable tissue repair and regeneration (Curtis et al., 2014). The healthy, young adult immune system appears to fluctuate between these two 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, Barrientos et al., 2009a, Barrientos et al., 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 ZT6 were not stimulated with LPS until ~ ZT9 (as the isolation procedure takes several hours) and were then maintained in culture for 3 h. Thus, RNA was not collected until almost a full 6 h after the isolation procedure (placing the microglia at ZT12 in this example). This suggests that while peak immune activation occurred in microglia isolated from young rats during the middle of the light/inactive phase (ZT6), peak immune activation *in vivo* may occur several hours later (between ZT9 and 12).

“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). Pro-inflammatory mediators can alter SCN neuronal function and circadian phase (Sadki et al., 2007, Duhart et al., 2013), 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 (Edgar and Dement,

1991, Droste et al., 2009, 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-erba*, *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. (4) There is diurnal variation in hippocampal IL-1 β 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Arjona A, Sarkar DK. Evidence supporting a circadian control of natural killer cell function. *Brain Behav Immun.* 2006; 20:469–476. [PubMed: 16309885]
- Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler U. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science.* 2000; 289:2344–2347. [PubMed: 11009419]
- Barrientos RM, Frank MG, Crysdale NY, Chapman TR, Ahrendsen JT, Day HE, Campeau S, Watkins LR, Patterson SL, Maier SF. Little exercise, big effects: reversing aging and infection-induced memory deficits, and underlying processes. *J Neurosci.* 2011; 31:11578–11586. [PubMed: 21832188]

- Barrientos RM, Frank MG, Hein AM, Higgins EA, Watkins LR, Rudy JW, Maier SF. Time course of hippocampal IL-1 beta and memory consolidation impairments in aging rats following peripheral infection. *Brain Behav Immun*. 2009a; 23:46–54. [PubMed: 18664380]
- Barrientos RM, Higgins EA, Biedenkapp JC, Sprunger DB, Wright-Hardesty KJ, Watkins LR, Rudy JW, Maier SF. Peripheral infection and aging interact to impair hippocampal memory consolidation. *Neurobiol Aging*. 2006; 27:723–732. [PubMed: 15893410]
- Barrientos RM, Thompson VM, Kitt MM, Amat J, Hale MW, Frank MG, Crysdale NY, Stamper CE, Hennessey PA, Watkins LR, Spencer RL, Lowry CA, Maier SF. Greater glucocorticoid receptor activation in hippocampus of aged rats sensitizes microglia. *Neurobiol Aging*. 2015; 36:1483–1495. [PubMed: 25559333]
- Barrientos RM, Watkins LR, Rudy JW, Maier SF. Characterization of the sickness response in young and aging rats following *E. coli* infection. *Brain Behav Immun*. 2009b; 23:450–454. [PubMed: 19486645]
- Beynon AL, Coogan AN. Diurnal, age, and immune regulation of interleukin-1beta and interleukin-1 type 1 receptor in the mouse suprachiasmatic nucleus. *Chronobiol Int*. 2010; 27:1546–1563. [PubMed: 20854134]
- Boivin DB, James FO, Wu A, Cho-Park PF, Xiong H, Sun ZS. Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood*. 2003; 102:4143–4145. [PubMed: 12893774]
- Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, Benedict C, Lange T, Westermann J, Oster H, Solbach W. Circadian clocks in mouse and human CD4+ T cells. *PLoS One*. 2011; 6:e29801. [PubMed: 22216357]
- Bonaconsa M, Malpeli G, Montaruli A, Carandente F, Grassi-Zucconi G, Bentivoglio M. Differential modulation of clock gene expression in the suprachiasmatic nucleus, liver and heart of aged mice. *Experimental gerontology*. 2014; 55:70–79. [PubMed: 24674978]
- Caldeira C, Oliveira AF, Cunha C, Vaz AR, Falcao AS, Fernandes A, Brites D. Microglia change from a reactive to an age-like phenotype with the time in culture. *Front Cell Neurosci*. 2014; 8:152. [PubMed: 24917789]
- Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M, Gewirtz AT, Davidson AJ. Dysregulation of inflammatory responses by chronic circadian disruption. *J Immunol*. 2010; 185:5796–5805. [PubMed: 20944004]
- Cleal JK, Shepherd JN, Shearer JL, Bruce KD, Cagampang FR. Sensitivity of housekeeping genes in the suprachiasmatic nucleus of the mouse brain to diet and the daily light-dark cycle. *Brain Res*. 2014; 1575:72–77. [PubMed: 24881883]
- Curtis AM, Bellet MM, Sassone-Corsi P, O'Neill LA. Circadian clock proteins and immunity. *Immunity*. 2014; 40:178–186. [PubMed: 24560196]
- Davidson AJ, Sellix MT, Daniel J, Yamazaki S, Menaker M, Block GD. Chronic jet-lag increases mortality in aged mice. *Current biology : CB*. 2006; 16:R914–916. [PubMed: 17084685]
- Davidson AJ, Yamazaki S, Arble DM, Menaker M, Block GD. Resetting of central and peripheral circadian oscillators in aged rats. *Neurobiol Aging*. 2008; 29:471–477. [PubMed: 17129640]
- Deng XH, Bertini G, Palomba M, Xu YZ, Bonaconsa M, Nygard M, Bentivoglio M. Glial transcripts and immune-challenged glia in the suprachiasmatic nucleus of young and aged mice. *Chronobiol Int*. 2010; 27:742–767. [PubMed: 20560709]
- Droste SK, Collins A, Lightman SL, Linthorst AC, Reul JM. Distinct, time-dependent effects of voluntary exercise on circadian and ultradian rhythms and stress responses of free corticosterone in the rat hippocampus. *Endocrinology*. 2009; 150:4170–4179. [PubMed: 19477935]
- Duhart JM, Leone MJ, Paladino N, Evans JA, Castanon-Cervantes O, Davidson AJ, Golombek DA. Suprachiasmatic astrocytes modulate the circadian clock in response to TNF-alpha. *J Immunol*. 2013; 191:4656–4664. [PubMed: 24062487]
- Edgar DM, Dement WC. Regularly scheduled voluntary exercise synchronizes the mouse circadian clock. *Am J Physiol*. 1991; 261:R928–933. [PubMed: 1928438]
- Farajnia S, Michel S, Deboer T, vanderLeest HT, Houben T, Rohling JH, Ramkisoensing A, Yassenkov R, Meijer JH. Evidence for neuronal desynchrony in the aged suprachiasmatic nucleus clock. *J Neurosci*. 2012; 32:5891–5899. [PubMed: 22539850]

- Fonken LK, Frank MG, Kitt MM, Barrientos RM, Watkins LR, Maier SF. Microglia inflammatory responses are controlled by an intrinsic circadian clock. *Brain Behav Immun*. 2015; 45:171–179. [PubMed: 25433170]
- Fonken LK, Nelson RJ. Mice exposed to dim light at night exaggerate inflammatory responses to lipopolysaccharide. *Brain Behav Immun*. 2013; 34:159–163. [PubMed: 24012645]
- Fonken LK, Nelson RJ. The effects of light at night on circadian clocks and metabolism. *Endocrine reviews*. 2014; 35:648–670. [PubMed: 24673196]
- Fonken LK, Weber MD, Daut RA, Kitt MM, Frank MG, Watkins LR, Maier SF. Stress-induced neuroinflammatory priming is time of day dependent. *Psychoneuroendocrinology*. 2016; 66:82–90. [PubMed: 26799851]
- Frank MG, Barrientos RM, Biedenkapp JC, Rudy JW, Watkins LR, Maier SF. mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiol Aging*. 2006a; 27:717–722. [PubMed: 15890435]
- Frank MG, Barrientos RM, Watkins LR, Maier SF. Aging sensitizes rapidly isolated hippocampal microglia to LPS ex vivo. *Journal of neuroimmunology*. 2010; 226:181–184. [PubMed: 20537730]
- Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. *Journal of neuroscience methods*. 2006b; 151:121–130. [PubMed: 16125247]
- Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *FASEB J*. 2005; 19:1329–1331. [PubMed: 15919760]
- Grabert K, Michael T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, Freeman TC, Summers KM, McColl BW. Microglial brain region-dependent diversity and selective regional sensitivities to aging. *Nature neuroscience*. 2016; 19:504–516. [PubMed: 26780511]
- Hajisoltani R, Rashidy-Pour A, Vafaei AA, Ghaderdoost B, Bandegi AR, Motamedi F. The glucocorticoid system is required for the voluntary exercise-induced enhancement of learning and memory in rats. *Behav Brain Res*. 2011; 219:75–81. [PubMed: 21147170]
- Hardeland R, Cardinali DP, Brown GM, Pandi-Perumal SR. Melatonin and brain inflammaging. *Prog Neurobiol*. 2015; 127–128:46–63.
- Hayashi M, Shimba S, Tezuka M. Characterization of the molecular clock in mouse peritoneal macrophages. *Biol Pharm Bull*. 2007; 30:621–626. [PubMed: 17409491]
- Kallo I, Kalamatianos T, Piggins HD, Coen CW. Ageing and the diurnal expression of mRNAs for vasoactive intestinal peptide and for the VPAC2 and PAC1 receptors in the suprachiasmatic nucleus of male rats. *Journal of neuroendocrinology*. 2004; 16:758–766. [PubMed: 15344914]
- Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B. A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci U S A*. 2009; 106:21407–21412. [PubMed: 19955445]
- Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes & development*. 2006; 20:1868–1873. [PubMed: 16847346]
- Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell*. 1999; 98:193–205. [PubMed: 10428031]
- Leise TL, Harrington ME, Molyneux PC, Song I, Queenan H, Zimmerman E, Lall GS, Biello SM. Voluntary exercise can strengthen the circadian system in aged mice. *Age (Dordr)*. 2013; 35:2137–2152. [PubMed: 23340916]
- Lupi D, Semo M, Foster RG. Impact of age and retinal degeneration on the light input to circadian brain structures. *Neurobiol Aging*. 2012; 33:383–392. [PubMed: 20409612]
- Marpegan L, Leone MJ, Katz ME, Sobrero PM, Bekinstein TA, Golombek DA. Diurnal variation in endotoxin-induced mortality in mice: correlation with proinflammatory factors. *Chronobiol Int*. 2009; 26:1430–1442. [PubMed: 19916840]
- Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci*. 2012; 35:445–462. [PubMed: 22483041]

- Musiek ES, Lim MM, Yang G, Bauer AQ, Qi L, Lee Y, Roh JH, Ortiz-Gonzalez X, Dearborn JT, Culver JP, Herzog ED, Hogenesch JB, Wozniak DF, Dikranian K, Giasson BI, Weaver DR, Holtzman DM, Fitzgerald GA. Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *The Journal of clinical investigation*. 2013; 123:5389–5400. [PubMed: 24270424]
- Nakamura TJ, Nakamura W, Tokuda IT, Ishikawa T, Kudo T, Colwell CS, Block GD. Age-Related Changes in the Circadian System Unmasked by Constant Conditions(1,2,3). *eNeuro*. 2015:2.
- Nakamura TJ, Nakamura W, Yamazaki S, Kudo T, Cutler T, Colwell CS, Block GD. Age-related decline in circadian output. *J Neurosci*. 2011; 31:10201–10205. [PubMed: 21752996]
- Nguyen KD, Fentress SJ, Qiu Y, Yun K, Cox JS, Chawla A. Circadian gene *Bmal1* regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes. *Science*. 2013; 341:1483–1488. [PubMed: 23970558]
- O’Callaghan EK, Anderson ST, Moynagh PN, Coogan AN. Long-lasting effects of sepsis on circadian rhythms in the mouse. *PLoS One*. 2012; 7:e47087. [PubMed: 23071720]
- Orozco-Solis R, Sassone-Corsi P. Circadian clock: linking epigenetics to aging. *Curr Opin Genet Dev*. 2014; 26:66–72. [PubMed: 25033025]
- Palomba M, Nygard M, Florenzano F, Bertini G, Kristensson K, Bentivoglio M. Decline of the presynaptic network, including GABAergic terminals, in the aging suprachiasmatic nucleus of the mouse. *J Biol Rhythms*. 2008; 23:220–231. [PubMed: 18487414]
- Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends in Cell Biology*. 2014; 24:90–99. [PubMed: 23916625]
- Popa-Wagner A, Buga AM, Dumitrascu DI, Uzoni A, Thome J, Coogan AN. How does healthy aging impact on the circadian clock? *J Neural Transm (Vienna)*. 2015
- Prolo LM, Takahashi JS, Herzog ED. Circadian rhythm generation and entrainment in astrocytes. *J Neurosci*. 2005; 25:404–408. [PubMed: 15647483]
- Rahman SA, Castanon-Cervantes O, Scheer FA, Shea SA, Czeisler CA, Davidson AJ, Lockley SW. Endogenous circadian regulation of pro-inflammatory cytokines and chemokines in the presence of bacterial lipopolysaccharide in humans. *Brain Behav Immun*. 2015; 47:4–13. [PubMed: 25452149]
- Sadki A, Bentivoglio M, Kristensson K, Nygard M. Suppressors, receptors and effects of cytokines on the aging mouse biological clock. *Neurobiol Aging*. 2007; 28:296–305. [PubMed: 16426706]
- Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. *Nature reviews Cancer*. 2009; 9:886–896. [PubMed: 19935677]
- Sato S, Sakurai T, Ogasawara J, Takahashi M, Izawa T, Imaizumi K, Taniguchi N, Ohno H, Kizaki T. A circadian clock gene, *Rev-erbalpha*, modulates the inflammatory function of macrophages through the negative regulation of *Ccl2* expression. *J Immunol*. 2014; 192:407–417. [PubMed: 24307731]
- Sellix MT, Evans JA, Leise TL, Castanon-Cervantes O, Hill DD, DeLisser P, Block GD, Menaker M, Davidson AJ. Aging differentially affects the re-entrainment response of central and peripheral circadian oscillators. *J Neurosci*. 2012; 32:16193–16202. [PubMed: 23152603]
- Silver AC, Arjona A, Hughes ME, Nitabach MN, Fikrig E. Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain Behav Immun*. 2012; 26:407–413. [PubMed: 22019350]
- Smith RG, Betancourt L, Sun Y. Molecular endocrinology and physiology of the aging central nervous system. *Endocrine reviews*. 2005; 26:203–250. [PubMed: 15561803]
- Spengler ML, Kuropatwinski KK, Comas M, Gasparian AV, Fedtsova N, Gleiberman AS, Gitlin II, Artemicheva NM, Deluca KA, Gudkov AV, Antoch MP. Core circadian protein *CLOCK* is a positive regulator of NF-kappaB-mediated transcription. *Proc Natl Acad Sci U S A*. 2012; 109:E2457–2465. [PubMed: 22895791]
- Straub RH, Cutolo M. Circadian rhythms in rheumatoid arthritis: implications for pathophysiology and therapeutic management. *Arthritis Rheum*. 2007; 56:399–408. [PubMed: 17265475]
- Sujino M, Furukawa K, Koinuma S, Fujioka A, Nagano M, Iigo M, Shigeyoshi Y. Differential entrainment of peripheral clocks in the rat by glucocorticoid and feeding. *Endocrinology*. 2012; 153:2277–2286. [PubMed: 22434077]

- Valentinuzzi VS, Scarbrough K, Takahashi JS, Turek FW. Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. *Am J Physiol.* 1997; 273:R1957–1964. [PubMed: 9435649]
- von Gall C, Weaver DR. Loss of responsiveness to melatonin in the aging mouse suprachiasmatic nucleus. *Neurobiol Aging.* 2008; 29:464–470. [PubMed: 17123666]

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Research highlights

- Diurnal rhythms in *Per1* and *Per2* are suppressed in hippocampal microglia isolated from aged rats.
- Cytokine rhythms are apparent in hippocampal microglia from young but not aged rats.
- Diurnal differences in hippocampal immune activation are only apparent in young rats.
- Corticosterone comparably induces *Per1* in aged and young hippocampal microglia.

Figure 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 h intervals across the day ($n = 5$ per age and time). Gene expression for (A) *BMAL1*, (B) *Rev-Erba*, (C) *Per1* and (D) *Per2* were evaluated in unstimulated microglia. There was a 24 h rhythm in *BMAL1* and *Rev-erba* 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 h 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 h cosinor curve. (E) In a separate experiment, hippocampal microglia were isolated from young and aged rats at ZT6 and plated for 2 h with corticosterone; then, *Per1* mRNA expression was evaluated in order 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 $\pm$ SEM. ~significant 24 h rhythm, *interaction between time and age; in all cases, $p < 0.05$.

Figure 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 h 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 h cosinor curve. Data are expressed relative to β -actin and presented as mean $\pm$ SEM. ~significant 24 h rhythm, *interaction between time and age, †main effect of age; in all cases $p < 0.05$.

Figure 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 h intervals across the day ($n = 5$ per age and time). To assess microglial inflammatory potential, microglia were stimulated for 3 h *ex vivo* with media alone (0 ng/mL LPS control), 10 ng/ml of LPS, or 100 ng/ml of LPS. (A) *IL-1 β* and (B) *TNF α* mRNA expression were evaluated and are expressed relative to β -actin. During the dark phase (ZT18), 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 $\pm$ SEM. *main effect of age, $p < 0.05$.

Figure 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 mos.) and aged (25 mos.) male F334xBN rats during the middle of the light (Zeitgeber time 6; ZT6) or dark (ZT18) 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 $\pm$ SEM. *main effect of time within an age group, †main effect of age, in all cases $p < 0.05$.

Figure 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 CFUs) during the light (ZT6) or dark (ZT18) phase ($n = 6-7$ per age and time). Hippocampal tissue was collected 3 h later to assess IL-1β protein concentrations. For rats receiving saline/*E. coli* during the light phase, IL-1β was increased in *E. coli*-treated rats (young and old; main effect). For rats receiving saline/*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 $\pm$ SEM. †main effect of *E. coli*, *differs from all other groups, in all cases $p < 0.05$