Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration

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Introduction

Circadian rhythms are controlled on a molecular level by cell-autonomous core clock machinery that is present in most cells in the body (1, 2). Circadian output from the suprachiasmatic nucleus (SCN) in the hypothalamus synchronizes tissue-specific cellular clocks to the light-dark cycle. The core circadian clock consists of a set of interacting transcriptional activators and repressors. The activators, or “positive limb” components BMAL1 and its binding partners CLOCK or NPAS2 heterodimerize, bind E-box motifs, and regulate the transcription of a wide variety of genes (3, 4). These positive limb proteins drive the transcription of circadian repressors, or “negative limb” components, including period (PER1-3) and cryptochrome (CRY1 and 2), which in turn inhibit the transcriptional activity of the BMAL1:CLOCK/NPAS2 heterodimers. This cell-autonomous clock machinery serves to synchronize intracellular gene expression to external cues such as light and to align physiologic oscillations in cells and tissues throughout the body. Furthermore, each core clock gene performs unique cellular functions that are distinct from its role in maintaining circadian oscillation, implying that clock genes might control key cellular processes via circadian or noncircadian mechanisms (5).

Brain aging is associated with diminished circadian clock output and decreased expression of the core clock proteins, which regulate many aspects of cellular biochemistry and metabolism. The genes encoding clock proteins are expressed throughout the brain, though it is unknown whether these proteins modulate brain homeostasis. We observed that deletion of circadian clock transcriptional activators aryl hydrocarbon receptor nuclear translocator–like (Bmal1) alone, or circadian locomotor output cycles kaput (Clock) in combination with neuronal PAS domain protein 2 (Npas2), induced severe age-dependent astrogliosis in the cortex and hippocampus. Mice lacking the clock gene repressors period circadian clock 1 (Per1) and period circadian clock 2 (Per2) had no observed astrogliosis. Bmal1 deletion caused the degeneration of synaptic terminals and impaired cortical functional connectivity, as well as neuronal oxidative damage and impaired expression of several redox defense genes. Targeted deletion of Bmal1 in neurons and glia caused similar neuropathology, despite the retention of intact circadian behavioral and sleep-wake rhythms. Reduction of Bmal1 expression promoted neuronal death in primary cultures and in mice treated with a chemical inducer of oxidative injury and striatal neurodegeneration. Our findings indicate that BMAL1 in a complex with CLOCK or NPAS2 regulates cerebral redox homeostasis and connects impaired clock gene function to neurodegeneration.

In peripheral tissues, clock genes serve as critical regulators of cellular metabolism and redox homeostasis and have been implicated in the aging process (6–9). Mice with targeted deletion of Bmal1 display loss of behavioral and physiologic circadian rhythms and develop increased systemic oxidative stress and signs of accelerated aging (9, 10). Conversely, aging is associated with diminished expression of positive-limb clock genes in mouse brain, and impaired circadian oscillation and oxidative injury are associated with brain aging and age-related neurodegenerative conditions in humans, suggesting a possible link between circadian clock dysfunction, oxidative stress, and age-related neurodegeneration (11–15). However, it is unknown whether core clock genes play any role in maintaining neuronal health or if these genes influence neurodegeneration.

Core clock genes are expressed throughout the brain (11, 16), though their function and importance in brain regions other than the SCN are poorly understood. BMAL1 has been implicated in hippocampal and astrocytic function (17–20). In Drosophila, deletion of the Period gene exacerbates brain pathology in neurodegeneration-prone mutants (21). In mice, Bmal1 deletion is associated with impairments in learning and memory as well as subtle increases in brain ROS (22), though no connection between clock genes and neurodegeneration has been clearly established in vertebrates. Thus, we hypothesized that core circadian clock function might regulate redox homeostasis in the mouse brain and that...
genetic disruption of circadian function might facilitate neuronal injury and neurodegeneration.

**Results**

**Oscillation of circadian clock genes is controlled by Bmal1 in cerebral cortex.** As circadian clock genes expressed in non-SCN brain regions might influence neuronal homeostasis, we examined the expression of selected core clock genes in cerebral cortex samples from young WT mice. Bmal1 and its transcriptional targets Per2 and Dbp all demonstrated circadian oscillation with phases that were similar to those observed in putitious tissue from a previous experiment (23), as well as to those described in rat cortex (ref. 17 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70317DS1). Expression of Dqb, a PAR bZIP transcription factor that is directly regulated by BMAL1/CLOCK-mediated transcription and serves as a marker of BMAL1/CLOCK transcriptional output (24), decreased by 86% in Bmal1 KO cortex, while expression of RevErba (Nrr1d1), another BMAL1 target gene, decreased by 83% (Supplemental Figure 1C). Notably, Per2 mRNA increased by an average of 46%, perhaps due to loss of transcriptional repression of Per2 by RevErba, as previously described (25). Thus, core clock genes are expressed and oscillate with appropriate phase in cerebral cortex, and deletion of Bmal1 elicits transcriptional changes in non-SCN regions similar to those seen in peripheral tissues.

**Bmal1 deletion causes age-dependent neuropathology and synaptic degeneration.** Global Bmal1 KO mice lack circadian rhythmicity in gene transcription and behavior and develop a variety of pathologies reminiscent of accelerated aging (9). Thus, we examined brain pathology in Bmal1 KO mice at 4 to 6 months of age, before peripheral pathologies become severe (9). Brains from 6-month-old Bmal1 KO mice appeared structurally normal with no developmental malformations, and cresyl violet staining demonstrated appropriate neural architecture with no significant thinning of hippocampal cell layers (Supplemental Figure 2). However, immunostaining for glial fibrillary acidic protein (GFAP) revealed striking astrocyte activation, a general marker of brain and neuronal injury that was most severe in cortical regions and hippocampus and less evident in thalamus and brainstem (Figure 1, A, B, and E). Astrocytosis was age dependent, as we did not observe it in 2-week-old Bmal1 KO mice, but it was clearly evident by 2.5 months of age and progressed by 6 months, as quantified by both Gfap mRNA levels and GFAP immunoreactivity (Figure 1, C and D). Astrocyte activation is often associated with increased expression of proinflammatory cytokines, including cyclooxygenase-2 (COX2, Ptghs2) and TNF-α (Tnfa), which exacerbate neurodegeneration (26, 27). We found that Ptghs2 and Tnfa mRNAs and COX2 protein were significantly increased in Bmal1 KO cortex, consistent with chronic neuroinflammation (Figure 1, F–H). Microglial activation was generally subtle in 6-month-old Bmal1 KO brain, though we observed substantial activation in isolated mice that lived until 8 months of age (Supplemental Figure 3). Fluoro-Jade C staining of Bmal1 KO cortex labeled numerous cellular processes in the dentate gyrus of the hippocampus and septal nuclei, which presumably represent injured neuronal processes (Supplemental Figure 2E). We performed ultrastructural analysis of the Bmal1 KO retrosplenial cortex with transmission electron microscopy, which showed many activated astrocytes throughout the tissue (Figure 2, A–D). Neuronal cell bodies and subcellular organelles appeared normal, as did dendritic structures. However, we noted frequent abnormal axonal synaptic terminals that were not seen in the control tissue. These presynaptic terminals were swollen and devoid of normal synaptic vesicles and cytoskeletal architecture, suggesting degeneration (Figure 2, B and C). Interestingly, the synaptic cleft itself appeared intact, as did the postsynaptic terminal. The axons themselves were structurally normal with intact myelination. Thus, Bmal1 deletion leads to widespread astrocytosis as well as discrete degeneration of presynaptic axonal terminals.

**Functional connectivity is impaired in Bmal1 KO brain.** We next sought to characterize the functional consequences of the neuropathological changes using optical intrinsic signal functional connectivity imaging (fOIS) (28). This approach visualizes alterations in regional cortical blood flow in anesthetized mice and generates maps of resting-state functional connectivity between contralateral brain regions. fOIS is highly sensitive to neuritic pathology in the absence of overt neuronal loss in mouse models of brain aging and Alzheimer disease (AD) (29). As shown in Figure 2, E and F, 5- to 6-month-old Bmal1 KO mice exhibited diminished functional connectivity throughout the cortex, most significantly in the retrosplenial cortex, a region that is severely affected in mouse models of brain aging and AD (29) and that showed severe astrogliosis in Bmal1 KO mice (see Figure 1E). Thus, the neuropathology seen in Bmal1 KO mice coincides in space and time with impaired neuronal network functional connectivity.

**Brain-specific deletion of Bmal1 replicates neuropathology despite intact behavioral rhythms and sleep-wake cycle.** Mice with global deletion of Bmal1 have a variety of peripheral pathologies and a lack of circadian oscillation in the sleep-wake cycle, which could potentially contribute to brain pathology (9, 10, 30). To address this, we generated NestinCre;Bmal1^floxed/lox^ mice (referred to herein as NestinCre; Bmal1^loxflo^) in which Bmal1 is deleted in most neurons, astrocytes, and oligodendrocytes, with residual Bmal1 expression in microglia (31). These mice do not display gross peripheral pathologies. Similar mice were previously reported to have partially intact Bmal1 expression in the SCN, rescuing the circadian regulation of locomotor activity and sleep (32). Locomotor circadian rhythms were largely retained in our NestinCre;Bmal1^loxflo^ mice as compared with mice lacking Cre, with no genotype difference in the free-running period, although there was a trend toward a slightly shorter free-running period, similar to that reported previously (ref. 32, Figure 3, A–C, and Supplemental Figure 4A). Using EEG, we saw that grossly normal sleep-wake oscillation was retained in NestinCre; Bmal1^loxflo^ mice in 12-hour light/12-hour dark housing conditions (Supplemental Figure 4, B and C). Despite relatively normal activity and sleep rhythms, the expression and oscillation of clock genes were disrupted in cortex tissue from NestinCre;Bmal1^loxflo^ mice. As shown in Figure 3D, Bmal1^loxflo^ control mice entrained to a 12-hour light/12-hour dark schedule that were then placed in constant darkness for 24 hours showed circadian oscillation of Bmal1, Dqb, and RevErba in cerebral cortex, similar to that seen in WT mice and in phase with rhythms observed in liver in previous experiments (see Supplemental Figure 1). However, expression of Dqb and RevErba, both BMAL1 target genes, declined by approximately 90% and became arrhythmic in NestinCre;Bmal1^loxflo^ littermates (Figure 3D).

Despite intact behavioral circadian rhythms and rhythmic sleep-wake, NestinCre;Bmal1^loxflo^ mice showed the same severe age-dependent astrogliosis we saw in global Bmal1 KO mice, as well as widespread microglial activation, which was more severe than that observed in global Bmal1 KO mice (Figure 4, A–G). We conclude that the brain phenotype seen in global Bmal1 KO mice is due...
to local loss of BMAL1 function within neurons/glia, and not to peripheral pathologies, changes in the sleep-wake cycle, or loss of peripheral circadian rhythms.

Since global Bmal1 KO mice exhibit behavioral abnormalities, including novelty-induced hyperactivity (22), we examined the performance of NestinCre;Bmal1−/− mice in a 1-hour locomotor behavior test. Like Bmal1 global KOs, NestinCre;Bmal1−/− mice displayed significant abnormalities in their response to a novel environment in 1-hour locomotor testing, as a repeated measures ANOVA of the total ambulation data yielded a significant genotype effect for total ambulations (F[1,12] = 6.60, P = 0.025) and vertical rearing (F[1,12] = 6.75, P = 0.023), with NestinCre;Bmal1−/− mice showing increased total activity (block 1 P = 0.029, block 2 P = 0.048) and number of rearings (block 1 P = 0.028) compared with WT controls. We found that all mice showed evidence of habituation on day 1 (P = 0.0004 and P < 0.00005, respectively), although only the control mice showed habituation on day 2 (P = 0.022). However, for rearing frequencies, only the NestinCre;
Bmal1f/f mice showed habituation on day 1 (P = 0.012), while neither group showed habituation to rearing on day 2. Thus, unlike the previous report with global Bmal1 KO mice, it was not clear that NestinCre+;Bmal1f/f mice showed impaired habituation. Ultimately, these data are consistent with the hypothesis that NestinCre+; Bmal1f/f mice respond abnormally to novelty. This may represent a behavioral consequence of the observed neuropathology.

Bmal1 regulates redox gene expression and oxidative stress in the brain. Circadian clock genes have been implicated in the regulation of oxidative stress in several organs (9). Using mass spectrometry, we observed that cortical F4-neuroprostanes (F4-NPs), markers of neuronal membrane lipid peroxidation (33), were increased 3-fold in 6-month-old Bmal1 KO mice, reflective of neuronal oxidative damage (Figure 5A). A similar, albeit nonsignificant, trend was evident for F2-isoprostanes (F2-IPs) (Supplemental Figure 6A), general markers of lipid peroxidation in all cell types. Marked increases in 4-hydroxynonenal Michael adducts, markers of lipid peroxidation, were also observed by immunohistochemistry throughout the Bmal1 KO brain (Supplemental Figure 6, B and C). To investigate the regulation of oxidative stress by the circadian clock, we examined the circadian oscillation of 89 candidate genes (see Supplemental Table 1) known to be involved in the regulation

Figure 2
Synaptic degeneration and impaired functional connectivity in Bmal1 KO cortex. (A–C). Electron micrographs showing presynaptic terminals (Sy) in 6-month-old WT (A) and Bmal1 KO (B and C) retrosplenial cortex. Note that in the Bmal1 KO cortex, the synaptic terminals are swollen and relatively devoid of synaptic vesicles, while the presynaptic and postsynaptic membranes, synaptic cleft, and dendritic spine (D) have normal morphology. Bmal1 KO mice showed both normal and abnormal terminals. (D) An activated astrocyte with a prominent Golgi complex (*) and islands of rough ER (**) around the nucleus (N). This cell is recognized by its abundance of intermediate filaments and cytoplasm with a lucent matrix. Activated astrocytes and numerous organelle-rich astrocytic processes were seen throughout the Bmal1 KO cortical tissue. Scale bars: 500 nm. (E) Composite functional connectivity maps from all mice generated using fcOIS. Shown are the seed locations (black circle) and the map of connectivity with that region (red indicates a positive correlation; blue indicates a negative correlation). (F) Connectivity (correlation coefficient z score) between corresponding contralateral cortical regions (n = 5 mice/genotype, all 6 months of age). Cing, cingulate; Sens, sensory; Rs, retrosplenial; Vis, visual. *P < 0.05 by 2-way ANOVA with Bonferroni’s post test.
of oxidative stress in several tissues using the CircaDB Circadian Expression Profiles database (http://bioinf.itmat.upenn.edu/circa), which queries microarray data from several published circadian microarray experiments in non-brain tissues. Circadian oscillation was detected using the JTK_CYCLE nonparametric algorithm (34), with a P value cutoff of 0.05. We also examined the expression level of this same panel of genes in WT and Bmal1 KO pituitary tissue using previously published microarray data (ref. 35; GEO accession number GSE29664). From this, we identified a set of candidate redox genes, each of which have been previously implicated in neurodegeneration, including Ucp2, Sod2, Prdx6, Aldh2, Nf2l2 (NRF2), and the NRF2 target genes Nqo1 and Hmox1. We examined the expression of these genes in WT and Bmal1 KO cortex at a single time point (Zeitgeber time [ZT] 6, Figure 5B and Supplemental Figure 7A) and in NestinCre;Bmal1 KO brain tissue at ZT 0, 6, and 12 (Figure 5B and Supplemental Figure 7A and B). Of these, the expression of Nqo1 and Aldh2 was significantly reduced in both Bmal1 KO and NestinCre;Bmal1 KO cortex as compared with controls at ZT 6. We thus examined the regulation of Nqo1 and Aldh2 in more detail.

ALDH2 scavenges reactive aldehydes generated during mitochondrial respiration and protects neurons against oxidative stress–induced neurodegeneration (36, 37). In our Bmal1 KO cortex samples, Aldh2 mRNA levels were significantly decreased on average by 58% and protein by 37% at ZT 6 (Figure 5, B–D). Nqo1 encodes NADPH dehydrogenase (quinone 1), a critical redox defense enzyme that reduces toxic quinones, suppresses oxidative damage, and may prevent neurodegeneration (38). Nqo1 mRNA and protein levels were also significantly decreased in Bmal1 KO brain, while mRNA was diminished by approximately 50% in NestinCre;Bmal1 KO cortex (Figure 5, B–D). We performed ChIP assays from WT mouse cortex to determine whether BMAL1 directly regulates the transcription of these genes. BMAL1 binds to noncanonical E-box motifs in the Nqo1 promoter (Figure SE). ChIP also showed that BMAL1 binds to a canonical E-box in the Aldh2 promoter, but does not bind a canonical E-box in the Nrf2 promoter.
promoter. Accordingly, the NRF2 target genes heme oxygenase 1 (Hmox1) and glutamate-cysteine ligase catalytic subunit (Gclc) showed nonsignificant trends toward increased expression at all time points in both Bmal1 KO and NestinCre+:Bmal1f/f cortex (Figure 5B and Supplemental Figure 7B), suggesting a normal NRF2-mediated response to increased oxidative stress, and showing that suppressed Nqo1 and Aldh2 expression in Bmal1 KO cortex is not due to general inhibition of NRF2 signaling. We examined the circadian expression of these redox-related transcripts in cortex also showed an opposite transcriptional pattern from that seen in Bmal1 KO mice, with increased levels of Dhp (demonstrating derepression of Bmal1 transcriptional activity) and Nqo1

Figure 3 for comparison), while Aldh2 and Nrf2 showed minimal oscillation (Figure 5F). Nqo1 and Aldh2 mRNA levels decreased at all time points and were arrhythmic in NestinCre+:Bmal1f/f cortex, further demonstrating transcriptional regulation by BMAL1. Thus, neuronal oxidative damage is evident in Bmal1 KO brain, and numerous redox-related transcripts exhibit circadian oscillation. Moreover, BMAL1 regulates the transcription of key redox response genes, including Nqo1 and Aldh2.

Dual deletion of Clock and Npas2 phenocopies Bmal1 deletion, while dual Per1 and Per2 mutation does not. Next, we sought to determine whether the neuropathology was specific to deletion of Bmal1 or common to other core clock genes. BMAL1 can heterodimerize with either CLOCK or NPAS2 to drive gene transcription. Because CLOCK and NPAS2 serve redundant roles in core clock function in the SCN (39) and are both expressed in the brain, we examined brains from Npas2 KO, Clock KO, and Npas2/Clock double-KO (DKO) mice for neuropathology. While Npas2 and Clock single-gene KOs resembled WT mice, Npas2/Clock DKO mice recapitulated the Bmal1 KO phenotype, showing marked astroglial activation throughout the brain, but was most severe in cortex (Figure 6A). Thus, disruption of the heterodimeric positive-limb transcriptional complex, either via
and a trend toward increased Aldh2 (Figure 6E). Thus, deletion of negative limb repressors (Per1/2) enhances BMAL1-mediated transcription of target genes such as Dbp and expression of Npq1/Aldh2. Taken together, these findings show that transcriptional regulation specifically by positive-limb BMAL1:Npas2/Clock heterodimers is required to prevent neuropathology in the brain.

**Diminished Bmal1 expression exacerbates neurodegeneration in vitro and in vivo.** In primary mouse neuron–enriched cultures, infection with a lentiviral shRNA (LV-shBMAL1) achieved an approximately 50% decrease in Bmal1 and an approximately 60% decrease in Dbp mRNA when compared with sister cultures treated with an identical lentivirus expressing a scrambled shRNA (LV-shSCR) (Supplemental Figure 8). By day 5 after lentiviral expression, LV-shBMAL1 cultures exhibited spontaneous neurite degeneration and cell death, while LV-shSCR–treated cells continued to appear healthy (Figure 7, A and B). Treatment with a low concentration of hydrogen peroxide (H2O2) exacerbated cell death even further in LV-shBMAL1 cultures. Knockdown of Bmal1 in Neuro2a neuroblastoma cells using siRNA targeted at a distinct sequence from the lentiviral shRNA did not induce spontaneous cell death, but did increase cell death caused by the mitochondrial toxin rotenone (Supplemental Figure 9, B and C), suggesting that the toxic effects of Bmal1 knockdown in primary neurons are not due to off-target effects of the shRNA. Conversely, siRNA-mediated knockdown of Bmal1 (~85% decrease) in primary astrocyte cultures had no effect on cell viability (Supplemental Figure 9A), did not induce significant astrocyte activation (as assessed by Gfap mRNA upregulation), did not suppress Aldh2 or Nqo1 expression, and did not induce inflammatory gene expression (Tnfα, Ptg2b, Il6), as compared with scrambled siRNA-treated cells (Figure 7C). This suggests that loss of Bmal1 expression in neurons, not astrocytes, is the primary driver of pathology. To address the hypothesis that gene dosage of Bmal1 might modulate oxidative neurodegeneration in vivo, we used the mitochondrial complex III inhibitor 3-nitropropionic acid (3-NP), which induces oxidative injury and striatal neurodegeneration (42). We treated WT and Bmal1 hemizygous mice (Bmal1+/–), which show no spontaneous neurite degeneration (Supplemental Figure 9A), did not induce significant astrocyte activation (as assessed by Gfap mRNA upregulation), did not suppress Aldh2 or Nqo1 expression, and did not induce inflammatory gene expression (Tnfα, Ptg2b, Il6), as compared with scrambled siRNA-treated cells (Figure 7C). This suggests that loss of Bmal1 expression in neurons, not astrocytes, is the primary driver of pathology. To address the hypothesis that gene dosage of Bmal1 might modulate oxidative neurodegeneration in vivo, we used the mitochondrial complex III inhibitor 3-nitropropionic acid (3-NP), which induces oxidative injury and striatal neurodegeneration (42). We treated WT and Bmal1 hemizygous mice (Bmal1+/–), which show no spontaneous neuropathologic phenotype in the age range we examined, with intrastriatal stereotactic injection of 3-NP, and 3 days later measured striatal lesion size by cresyl violet and Fluoro-Jade C staining (see Supplemental Figure 10). As shown in Figure 7, D–F,
Bmal1+/– mice had significantly larger lesions than WT mice, supporting the notion that Bmal1 expression levels play a critical role in neuronal redox homeostasis and neurodegeneration.

**Discussion**

It is now evident that core clock genes regulate critical aspects of cellular biology in many organs and that disruption of normal core clock function may precipitate disease pathology. Indeed, impaired clock gene expression in mice can cause a variety of pathologies, including diabetes, vascular disease, obesity, and accelerated aging (6, 8, 9, 43). Despite these findings, relatively little is known about the role of clock genes in other brain regions. Our results show that disruption of the positive limb of the circadian clock in the brain, either via deletion of Bmal1 (either globally or in a brain-specific manner) or Npas2 and Clock, induces oxidative stress, widespread astrocyte activation, axonal terminal degeneration, and disrupted resting-state functional connectivity. This neuropathology is not due to a disruption in peripheral physiology, sleep disruption, or systemic circadian mechanisms and is not recapitated by mutation of the negative-limb genes Per1 and Per2. The degree of astroglisosis and oxidative stress do not perfectly correlate, as thalamus shows minimal astrogliosis but significant increases in 4-HNE, suggesting that the regionality of brain pathology in Bmal1 KO mice is driven both by the degree of oxidative damage and the inherent susceptibility of a given region. The expression levels of Bmal1 appear to be critical, as Bmal1+/– mice, though not exhibiting overt pathology or circadian phenotype, are more sensitive than WT mice to oxidative neurodegeneration caused by the mitochondrial inhibitor 3-NP, while even partial knockdown of Bmal1 in primary neurons induces spontaneous neurodegeneration. These data reveal a critical role for the circadian clock positive-limb transcriptional complex (BMAL1: CLOCK/NPAS2) in neuronal redox homeostasis and protection from neurodegeneration.

BMAL1:NPAS2/CLOCK heterodimers regulate the transcription of many circadian and noncircadian genes, and both sets of transcripts are likely relevant to neuronal function (5). In our studies, the neuropathology in Bmal1 KO and Clock/Npas2 DK0 mice does not appear to be due to loss of systemic circadian rhythms or sleep-wake disturbance, since (a) NestinCre;Bmal1f/f mice develop neuropathology despite normal behavioral rhythms and sleep-wake oscillation; (b) Per1+/Per2+ mice, which have impaired circadian rhythms, do not develop pathology; and (c) Bmal1+/– mice, which exhibit normal circadian oscillation, have enhanced sensitivity to 3-NP-induced neurodegeneration. It thus appears that the levels of Bmal1 and/or Clock and Npas2 are the important factors, rather than the circadian oscillation of transcripts. However, the levels and activity of Bmal1, Npas2, and Clock are intimately tied to circadian oscillation, and conditions that disrupt oscillation can suppress Bmal1 expression or transcriptional activity. These include cellular senescence, sleep deprivation, and pulsed-light exposure (44–46). Therefore, these circadian “stressors” may predispose individuals to age-related neurodegeneration by suppressing positive-limb clock gene expression and activity. Circadian dysfunction
is observed in several neurodegenerative diseases, including AD and Parkinson disease (12, 13, 47), suggesting that disease-related loss of clock gene function might impact pathogenesis and disease progression. Accordingly, epidemiologic data show that diminished circadian function in humans imparts an increased risk of developing future dementia (48). Our findings suggest that further study of the regulation of circadian clock genes in non-SCN brain regions in aging and neurodegenerative diseases is warranted and that therapies targeted at bolstering positive-limb clock gene expression in the brain might have neuroprotective effects.

Aging is a major risk factor for neurodegenerative diseases, and the core clock is intimately intertwined with the aging process, as aging impairs the expression of Bmal1 and Clock (11), while disruption of Bmal1 recapitulates many aspects of age-related pathology (9). In vascular smooth muscle cells, senescence is associated with a marked decline in Bmal1 expression (44), while the expression of Bmal1 and Clock in cerebral cortex is substantially diminished in aged mice (11). Our results predict that aging-related declines in Bmal1 expression in the brain could impair redox defense gene expression, exacerbate oxidative stress, and facilitate neurodegeneration. Indeed, aged brain shares many characteristics with Bmal1 KO brain, including increased oxidative damage, diminished redox defense gene expression, increased Pysb2 expression, and impaired retinopulmonary functional connectivity. Thus, declining positive-limb core clock activity in non-SCN brain may be part of a feed-forward cycle with aging, which may exacerbate specific age-related pathogenic events that contribute to neurodegeneration (49–52).

Our findings, as well as previous studies, suggest that the core clock plays an important role in redox homeostasis. Bmal1 KO mice exhibit increased ROS levels in spleen, kidney, and brain, and treatment with the glutathione precursor N-acetyl cysteine extends lifespan in these mice (9, 22, 53). The cellular redox state shows circadian oscillation, which is dependent on Bmal1 expression both in cultured fibroblasts and the mouse SCN (54, 55). Furthermore, the acetylation of multiple critical mitochondrial proteins shows circadian oscillation, suggesting clock-mediated control of the mitochondrial redox state (56). Conversely, clock function is modulated by the redox state of the cell (7, 57, 58). In the brain, our observation of impaired expression of Aldh2 and Nqo1, despite ongoing oxidative stress, suggests that the core clock is required for appropriate protective responses to oxidant injury, illustrating a new aspect of this relationship between the clock...
and ROS. Aldh2 and Nqo1 are directly regulated by BMAL1, and their expression is diminished when Bmal1 is deleted. However, the expression of both genes increases in parallel with Dhp (a marker of positive-limb transcriptional activity) in Per1ldc/Per2ldc DKO brain, suggesting that alleviation of Per1/2-mediated repression of BMAL1 transcriptional activity enhances Aldh2 and Nqo1 expression. Both NQO1 and ALDH2 are critical mediators of the cellular antioxidant response and are closely linked to neurodegeneration. ALDH2 scavenges reactive electrophiles within mitochondria, and deletion of Aldh2 in mice causes oxidative damage and neuronal death, while impaired ALDH2 activity has been implicated as a cause of dopaminergic neurodegeneration in Parkinson disease (36, 37). NQO1 catalyzes the reduction of reactive quinones and oxidized proteins, prevents ROS-mediated cytotoxicity, and is upregulated in vulnerable brain regions in AD (38, 59). Thus, impaired Aldh2 and Nqo1 expression may contribute to neuronal pathologies in Bmal1 KO brain.

In summary, our findings draw a novel link between the core circadian clock, brain oxidative stress, and neurodegeneration. This relationship has many potential implications for age-related neurodegenerative diseases and suggests that further study of the regulation and function of core clock genes in non-SCN brain regions in health and disease is warranted.

**Methods**

**Reagents.** The following antibodies were used: ALDH2 and NQO1 monoclonals (Epitomics); BMAL1 polyclonal (Bethyl Laboratories, Inc.); COX2 polyclonal (Cayman Chemical); ERK (Cell Signaling Technology); GFAP (Dako); IBAI (Wako); and 4-HNE Michael Adduct (EMD Millipore). Cell culture media and reagents (neurobasal media, B27 supplement) and TaqMan quantitative PCR (qPCR) primer sets were obtained from Invitrogen. 3-Nitropropionic acid was purchased from Sigma-Aldrich. The following antibodies were used: ALDH2 and NQO1 monoclonals (Epitomics); BMAL1 polyclonal (Bethyl Laboratories, Inc.); COX2 polyclonal (Cayman Chemical); ERK (Cell Signaling Technology); GFAP (Dako); IBAI (Wako); and 4-HNE Michael Adduct (EMD Millipore). Cell culture media and reagents (neurobasal media, B27 supplement) and TaqMan quantitative PCR (qPCR) primer sets were obtained from Invitrogen. 3-Nitropropionic acid was purchased from Sigma-Aldrich.

**Mice.** Bmal1<sup>+/–</sup>, NestinCre<sup>+</sup>, and Bmal1<sup>+/+</sup> mice were obtained from The Jackson Laboratory and were bred at Washington University. A second strain of NestinCre<sup>+</sup>Bmal1<sup>f/f</sup> mice was bred at the University of Pennsylvania and was used in some experiments. A second strain of Bmal1<sup>+/–</sup> mice was originally obtained from C. Bradford (University of Wisconsin, Madison, Wisconsin, USA) and bred at the University of Pennsylvania. Npas2 KO (originally provided by S. McKeown, UT Southwestern Medical School, Dallas, Texas, USA), Clock KO, and Npas2/Clock DKO mice were bred and housed at the University of Massachusetts Medical School. Per1<sup>+/−</sup>/Per2<sup>+/−</sup> (referred to herein as Per1<sup>−/−</sup>/Per2<sup>−/−</sup>) were originally provided by S. Reppert (University of Massachusetts, Worcester, Massachusetts, USA) and were bred at Washington University. All mice were maintained on a C57Bl6 background. Mice were housed under a 12-hour light/12-hour dark conditions.

**Optical imaging of functional connectivity.** OIS imaging of resting-state functional connectivity in mice was performed as previously described (28, 29). Briefly, mice were anesthetized with i.p. pentobarbital, then perfused for 3 minutes with ice-cold Dulbecco’s modified PBS (DPBS) containing 3 g/l heparin. One hemisphere was fixed in 4% paraformaldehyde for 24 hours (4°C), then cryoprotected with 30% sucrose. Twenty-four hours later, the cells were harvested for RNA extraction, or cell viability was determined by MTT assay. For astrocyte cultures, P1 mice were dissected as described above. Cells were cultured in DMEM with F-12 supplement (Invitrogen) and 15% FBS and 10 ng/ml epidermal growth factor (Sigma-Aldrich).

**Immunohistochemistry.** Immunostaining was performed as previously described (29). Briefly, mice were anesthetized with i.p. pentobarbital, then perfused for 3 minutes with ice-cold Dulbecco’s modified PBS (DPBS) containing 3 g/l heparin. One hemisphere was fixed in 4% paraformaldehyde for 24 hours (4°C), then cryoprotected with 30% sucrose in PBS (4°C) for 48 hours, and 50-micron serial coronal sections were cut on a freezing sliding microtome. Sections were incubated in 0.3% hydrogen peroxide for 10 minutes, blocked for 30 minutes in TBS containing 3% serum and 0.25% Triton X-100, then incubated overnight in TBS plus 0.25% Triton X-100 (Sigma-Aldrich) with primary antibody and 1% serum at 4°C. Sections were incubated for 1 hour with biotinylated secondary antibody, then washed and incubated with 1:400 dilution of streptavidin-conjugated HRP (VECTASTAIN ABC Elite; Vector Laboratories), then with diaminobenzidine substrate with hydrogen peroxide and nickel chloride.

**Quantification of immunoreactivity.** Images from a given brain region from three sections per mouse that were 150 microns apart were converted to grayscale, thresholded such that all GFAP immunoreactivity was included, then the percentage of area was calculated using ImageJ software. Sections from all mice included in a given analysis were stained in a single batch, and threshold values were held constant for all mice in that batch. The
mean percentage of the area for each region from each mouse was compiled for statistical analysis.

**LV preparation.** shRNA sequences targeting Bmal1 (Arntl, clone NM_007489.1-2418s1c1, CCATGGATAAAGTCAATCTA) or a scrambled sequence were cloned into a lentiviral vector containing a U6 promoter (ubiquitous expression) upstream of the shRNA as well as a phosphoglycerate kinase (Pgk1) promoter driving a GFP tag.

**3-NP treatment.** Sterile 3-NP (100 mmol/ml) in PBS (pH 7.4) was injected with a 30-gauge Hamilton syringe needle at the following coordinates relative to the bregma: +0.98 mm anterior, +1.5 mm lateral, and depth of 2.6 mm. The needle was left in place for 5 minutes, then 0.5 μl (total of 50 nmol) of 3-NP was injected over a 5-minute period. Three days later, the mice were perfused as described above.

**siRNA experiments.** siRNAs (scrambled or Bmal1 targeted) were obtained from Thermof Scientific (Dharmacon SMARTpool siRNAs, which consists of a pool of 5 siRNA targeting Bmal1). Neuro2a neuroblastoma cells (ATCC) were cultured in DMEM plus 10% FBS and were plated at a density of 3 x 10^4 cells/ml (0.5 ml per well in a 24-well plate). One microliter of 20 μM siRNA was added to each well. Neuro2a cells were transfected with Lipofectamine 2000 reagent, while astrocytes were transfected with RNAiMax reagent (both from Invitrogen), according to the manufacturer’s instructions.

Additional methods, including behavioral testing, electron microscopy, ChIP, mass spectrometry, and Fluoro-Jade staining, are available in the Supplemental Methods.

**Statistics.** A 2-tailed Student’s t test was used when a single variable was compared between two genotypes, and 2-way ANOVA with Bonferroni’s post test was used when multiple variables were compared between genotypes. The cutoff for significance was P < 0.05. In all figures, the graphs depict the mean ± SEM.

**Study approval.** All animal studies were performed in accordance with protocols approved by the Animal Studies Committees of Washington University and the University of Pennsylvania.

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45. Mongrain V, La Spada F, Curie T, Franken P. Sleep loss reduces the DNA-binding of BMAL1, CLOCK, and NPAS2 to specific clock genes in the mouse central nervous system. Proc Natl Acad Sci U S A. 2013;110(9):3339–3344.