Circadian clock protein Rev-erbα regulates neuroinflammation

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Circadian dysfunction is a common attribute of many neurodegenerative diseases, most of which are associated with neuroinflammation. Circadian rhythm dysfunction has been associated with inflammation in the periphery, but the role of the core clock in neuroinflammation remains poorly understood. Here we demonstrate that Rev-erbα, a nuclear receptor and circadian clock component, is a mediator of microglial activation and neuroinflammation. We observed time-of-day oscillation in microglial immunoreactivity in the hippocampus, which was disrupted in Rev-erbα$^{-/-}$ mice. Rev-erbα deletion caused spontaneous microglial activation in the hippocampus and increased expression of proinflammatory transcripts, as well as secondary astrogliosis. Transcriptomic analysis of hippocampus from Rev-erbα$^{-/-}$ mice revealed a predominant inflammatory phenotype and suggested dysregulated NF-κB signaling. Primary Rev-erbα$^{-/-}$ microglia exhibited proinflammatory phenotypes and increased basal NF-κB activation. Chromatin immunoprecipitation revealed that Rev-erbα physically interacts with the promoter regions of several NF-κB–related genes in primary microglia. Loss of Rev-erbα in primary astrocytes had no effect on basal activation but did potentiate the inflammatory response to lipopolysaccharide (LPS). In vivo, Rev-erbα$^{-/-}$ mice exhibited enhanced hippocampal neuroinflammatory responses to peripheral LPS injection, while pharmacologic activation of Rev-erbs with the small molecule agonist SR9009 suppressed LPS-induced hippocampal neuroinflammation. Rev-erbα deletion influenced neuronal health, as conditioned media from Rev-erbα–deficient primary glial cultures exacerbated oxidative damage in cultured neurons. Rev-erbα$^{-/-}$ mice also exhibited significantly altered cortical resting-state functional connectivity, similar to that observed in neurodegenerative models. Our results reveal Rev-erbα as a pharmacologically accessible link between the circadian clock and neuroinflammation.

Rev-erbα | circadian | microglia | neuroinflammation

Circadian clocks allow organisms to precisely synchronize internal physiological processes with their external environment. A conserved transcriptional–translational feedback loop known as the core circadian clock controls cycles of protein expression that produce transcriptional and physiologic rhythms. This core circadian clock consists of the transcriptional activators BMAL1 and CLOCK, which drive transcription of their own transcriptional repressors, including CRYPTOCHROME (CRY), PERIOD (PER), and REV-ERB proteins (1). The circadian system regulates a variety of critical cellular processes, including aspects of metabolism, inflammation, and redox homeostasis (2). Disruptions of the clock or its associated proteins have been implicated in pathological conditions ranging from cancer to neurodegenerative diseases (2–4). However, the roles of cellular circadian clocks in brain health and neuroinflammation are still poorly understood.

Aberrant glial cell activation and neuroinflammation are hallmarks of many neurodegenerative conditions such as Alzheimer disease (AD) and Parkinson disease (PD) (5, 6). Circadian disruption is also a common symptom of these diseases (4). The circadian clock has been implicated in the regulation of peripheral inflammation (7–9), though its role in neuroinflammation is less clear. We have previously shown that deletion of *Bmal1*, which disrupts core clock transcriptional function, causes widespread glial activation, inflammation, and oxidative stress in the brain (10), though the downstream mechanisms have not been fully identified.

One potential mechanism by which BMAL1 could regulate neuroinflammation is via transcriptional regulation of Rev-erbα (gene name *Nr1d1*). Rev-erbα is a heme-responsive nuclear receptor protein as well as a circadian clock component which is directly regulated by BMAL1 (11–13). Rev-erbα is also a transcriptional repressor that inhibits *Bmal1* expression and other targets at specific sites within the genome in a tissue-specific manner (13–16). Rev-erbα has been implicated in control of diverse processes, including metabolism, macrophage function, and inflammation (17, 18). Because it is a nuclear receptor, Rev-erbα is an attractive therapeutic target which can be modulated with available small-molecule agonists and antagonists (19, 20). While the function of Rev-erbα in neuroinflammation and neurodegeneration has not been directly examined, Rev-erb agonist drugs appear to prevent inflammation and cell death when infused into the brain (21). Thus, we have examined the role for Rev-erbα in the control of neuroinflammation, using genetic and pharmacological manipulations both in vivo and in vitro. Our results demonstrate a key role for Rev-erbα in the regulation of glial activation and neuroinflammation.

Significance

Disruptions in the circadian clock and its component proteins have been shown to be associated with disease states ranging from cancer to neurodegeneration. Herein, we identify the circadian clock protein Rev-erbα as a critical regulator of neuroinflammation. Rev-erbα deletion causes spontaneous microglial and astrocyte activation, increased microglial NF-κB signaling, and neuronal injury. Accordingly, pharmacological activation of Rev-erbα suppressed brain inflammation. Our results establish Rev-erbα as a link between the circadian clock, glial activation, and neuroinflammation.

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Results

Rev-erbα Deletion Induces Spontaneous Hippocampal Microgliosis. Since Rev-erbα has been shown to regulate macrophage function in the periphery (18), we first investigated the effect of Rev-erbα deletion on the tissue resident macrophages of the CNS: microglia. We placed 6- to 8-mo-old wild-type (WT) and Rev-erbα$^{-/-}$ mice (referred to as RKO throughout) in constant darkness, then performed microglial staining with Iba1 at CT4 and CT16, timepoints at which REV-ERB–regulated gene *Fabp7* shows peak and trough expression, respectively (22). We observed that in the WT hippocampus, microglial volume varied by time of day, with increased Iba1 volume observed at CT16 (trough expression for Rev-erbα–regulated targets) (Fig. 1 *A* and *B*). This time-of-day effect was abrogated in the RKO mouse hippocampus, as microglial volume was similar to the WT CT16 timepoint at both CT4 and CT16 (Fig. 1 *A* and *B*). This finding suggested that oscillations in Rev-erbα may regulate genes which control basal microglial activation state. Accordingly, we observed markedly increased spontaneous microglial Iba1 staining in the hippocampus of 5-mo RKO mice (Fig. 1*C*), as well as increases in microglial-related inflammatory transcripts in the RKO hippocampus, including *Il1β* and *Trem2*, consistent with a proinflammatory response (Fig. 1*D*). Microglial activation can be characterized by morphologic changes, as well as increases in the phagocytic marker CD68 (23). We found a significant increase in CD68^{+} puncta within Iba1^{+} microglia by confocal imaging with 3D surface reconstruction in the hippocampus of RKO mice at 5–6 mo of age, indicating increased phagocytic activation [Fig. 1*E* and Movie S1 (WT cell) and Movie S2 (KO cell)]. We also observed decreased microglial branching morphologic changes in RKO microglia, consistent with activation (Fig. 1 *F* and *G*) (24). We also observed an increase in total number of Iba1^{+} microglia in the hippocampus of RKO mice (*SI Appendix*, Fig. S1*A*), as is observed in many neuroinflammatory conditions. However, this did not change with time of day and does not explain the microglial morphologic changes. Young (2–3 mo) RKO mice already had increased Iba1/Cd68 colocalization (*SI Appendix*, Fig. S1*B*), as well as increased *Aif1* and *Trem2* expression, though *Il1b* expression was age dependent (*SI Appendix*, Fig. S1*C*), suggesting that only some component of the phenotype is age dependent. Taken together, Rev-erbα appears to regulate microglial activation.

Enhanced NF-κB Signaling Is Associated with Immune Activation in RKO Microglia. To further characterize the neuroinflammatory phenotype of RKO mice, we performed microarray-based transcriptomic analysis of hippocampal tissue from 5-mo WT or RKO mice. Raw data are deposited in the ArrayExpress database (25, 26). We observed significant increases in proinflammatory chemokines (*Ccl2*, *Ccl5*, *Ccl8*, and *Cxcl5*), astrocyte activation markers (*Gfap*, *Timp1*, *S100a4*, and *S100A6*), and microglial activation transcripts (*Aif1*, *Cd68*, *Tyrobp*, *Trem2*, and *Csf1r*) (Fig. 2*A* and Dataset S1). We confirmed several of these transcriptional changes by qPCR in a separate cohort of 5-mo mice, including *Timp1*, *S100a6*, and *Cxcl5* (*SI Appendix*, Fig. S2*A*). Because Rev-erbα is a transcriptional repressor, we performed pathway analysis of transcripts which were significantly up-regulated (uncorrected P value <0.05, fold change >2) using DAVID software (27), which showed profound up-regulation of biological processes related to innate immune activation and inflammation (Fig. 2*B*), emphasizing the neuroinflammatory phenotype.

The NF-κB pathway is a critical regulator of innate immune activation which interacts with Rev-erbα (21, 28). We observed up-regulation of several genes involved in the positive regulation of NF-κB signaling, such as *Nfkb2*, *Tlr4*, *Stat3*, and *Traf2*, as well as down-regulation of two genes involved in inhibiting NF-κB signaling (*Nfkbib* and *Usp31*) (Fig. 2*A*). Of note, *Nfkbib* encodes the NF-κB pathway inhibitor IκBβ (29). We next examined basal and lipopolysaccharide (LPS)-induced p65 nuclear translocation in primary WT and RKO microglia. In WT microglia, nuclear p65 was minimal at baseline, increased markedly 1 h after LPS stimulation, then decreased by 3 h after LPS (Fig. 2*C*). However, RKO microglia showed significantly increased p65 nuclear translocation at baseline which remained elevated throughout the stimulation (Fig. 2*C*). Accordingly, primary RKO microglia showed increased basal cytokine production (Fig. 2*D*), though their response to LPS was similar to WT cells (Fig. 3*C*). We next performed chromatin immunoprecipitation (ChIP) assays in primary microglia using Rev-erbα antibody or IgG control, to identify direct targets of Rev-erbα. Using existing ChIP-seq databases for liver, adipose, and midbrain tissue (15), we identified Rev-erbα binding peaks located in the promoter regions of several genes which were altered in our transcriptomic data, including *Traf2*, *Nfkb2*, and *Nfkbib*. qPCR from WT microglial ChIP DNA revealed strong Rev-erbα binding to the promoters of *Bmal1* (a known Rev-erb target), *Traf2*, and *Nfkbib*, with lesser but still significant signal for *Nfkb2* (Fig. 2*E*). *S100a4*, an astrocyte gene included as a negative control, showed no signal, while DNA from RKO cells also showed no signal. These data suggest that loss of Rev-erbα directly regulates expression of multiple NF-κB–related genes to enhance basal NF-κB signaling in microglia and promote proinflammatory microglial activation.

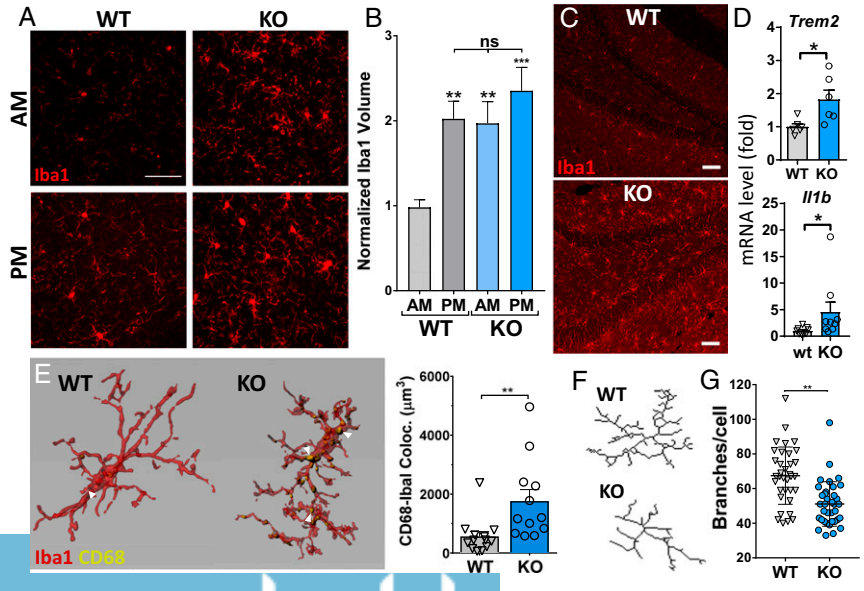

Fig. 1. Rev-erbα deletion induces hippocampal microglial activation. (*A*) Images (60×) of Iba1 staining in 6- to 8-mo WT (*Left*) and RKO (*Right*) mouse hippocampus, with Iba1 volume quantification (*B*). Mice were killed at CT4 (AM, *Top*) and CT16 (PM, *Bottom*). n = 4–5 mice per genotype, three images per mouse. (*C*) Iba1 staining in 5-mo RKO hippocampus (*Right*) and WT littermates (*Left*). (*D*) qPCR analysis for microglial inflammatory genes from the hippocampus of WT and RKO mice. n = 6 mice per group. (*E*) Representative 100× 3D surface rendering showing CD68^{+} puncta (yellow) within Iba1^{+} microglia (red), in RKO hippocampus compared with WT, with quantification. n = 4 mice per genotype with three 40× fields of view each. (*F*) Representative skeletonized microglial reconstructions and (*G*) quantification of branches per cell. n = 3 mice, n = 35 microglia. ns, not significant; *P < 0.05, **P < 0.01, or ***P < 0.001 by two-tailed t test with Welch's correction. (Scale bars, 50 μm for *A*, 120 μm for *C*.)

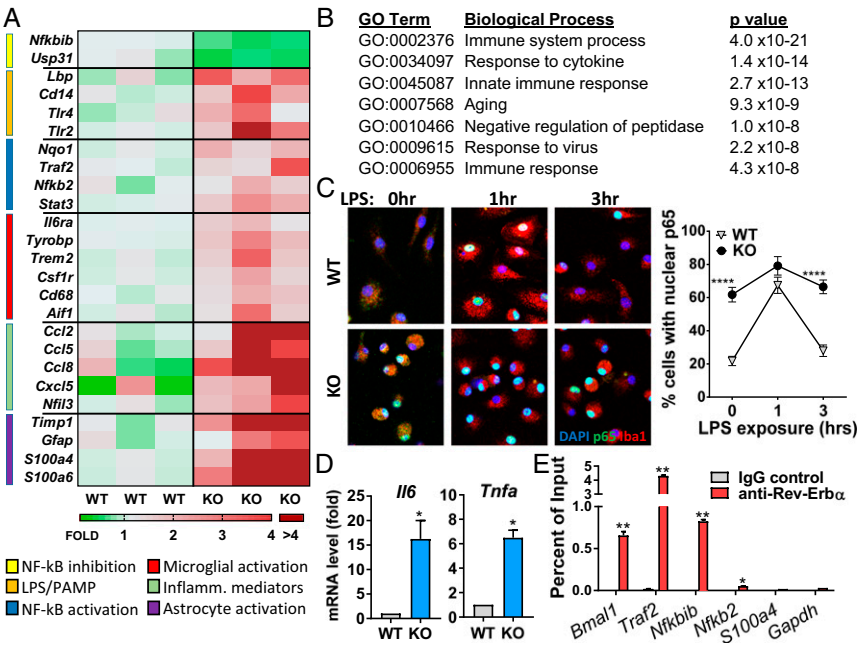

Fig. 2. Rev-erbα–mediated neuroinflammation is associated with dysregulated microglial NF-κB signaling. (*A*) Relative expression of transcripts related to different aspects of neuroinflammation taken from microarray analysis performed on 5-mo RKO and WT mice ($n = 3$ per genotype). Colored bars on *Left* indicate hand-curated functional groupings. (*B*) Gene Ontology (GO) term analysis of microarray data for biological processes up-regulated in RKO hippocampus compared with WT ($P > 0.05$, fold change >2). (*C*) Representative images showing p65 nuclear translocation in RKO microglia compared with WT at baseline, 1 h and 3 h, following LPS stimulation. Quantification of the percent microglia with nuclear p65 staining (DAPI/p65 overlap) is shown (*Right*). $n = 3$ separate experiments. (*D*) qPCR showing mRNA expression for the proinflammatory mediators *Il6* and *Tnfa* in primary WT and RKO microglia. (*E*) ChIP analysis of WT microglia for the Rev-erbα target *Bmal1*, NF-κB signaling components *Traf2* as well as *Nfkbib*, a negative control *S100a4* and a normalization control *Gapdh*. $*P < 0.05$ or $**P < 0.01$ by two-tailed *t* test with Welch's correction. $****P < 0.001$ by two-way ANOVA with Tukey's multiple comparisons test.

Rev-erbα Deletion Exacerbates LPS-Induced Neuroinflammation in the Hippocampus. Based on our in vitro findings, we next investigated the neuroinflammatory response of RKO mice to LPS. Notably, we observed an extremely high fatality rate specifically in RKO mice following intracerebroventricular LPS injection, and thus employed intraperitoneal (i.p.) LPS injections instead. i.p. LPS caused striking increases of inflammatory transcripts within the hippocampus of WT mice 6 h after injection (Fig. 3*A*). Mice were perfused before tissue harvest to minimize RNA contribution from inflammatory cells in blood. Rev-erbα deletion augmented LPS-induced expression of proinflammatory mediators in the hippocampus, including *Il6* and *Ccl2*, the astrocyte activation marker *Gfap*, and the oxidative stress-responsive transcript *Hmox1* (Fig. 3*A*). These data suggest that Rev-erbα deletion exacerbates the LPS-induced neuroinflammation in vivo.

Pharmacologic Activation of Rev-erbα Suppresses LPS-Induced Neuroinflammation. We next sought to determine if activating Rev-erbα could mitigate LPS-induced neuroinflammation. Rev-erbα is an orphan nuclear receptor and thus can be activated using brain permeant small-molecule agonists such as SR9009 (19). To test the effect of Rev-erbα activation on inflammation, cells were stimulated with LPS with or without SR9009 for 8 h to activate NF-κB, with ATP added during the final 30 min to facilitate secretion of IL-1β into the media. In BV-2 immortalized microglial cells, SR9009 exhibited dose-dependent suppression of LPS+ATP-induced IL-1β protein secretion (*SI Appendix*, Fig. S4*A*). In primary WT microglia, SR9009 again inhibited LPS+ATP-induced IL-1β secretion (Fig. 3*B*), as well as *Il6* mRNA expression (Fig. 3*C*). SR9009 did not significantly suppress cytokine expression in RKO microglia, suggesting some specificity of the drug for Rev-erbα (Fig. 3 *B* and *C*). SR9009 also significantly suppressed LPS-induced *Il6*, *Tnfa*, and *Il1b* expression in primary astrocyte-enriched cultures (Fig. 3*D* and *SI Appendix*, Fig. S4*B*), suggesting that this agent can mitigate inflammation across cell types in the brain. We next investigated the effects of SR9009 on LPS-mediated neuroinflammation in vivo in the

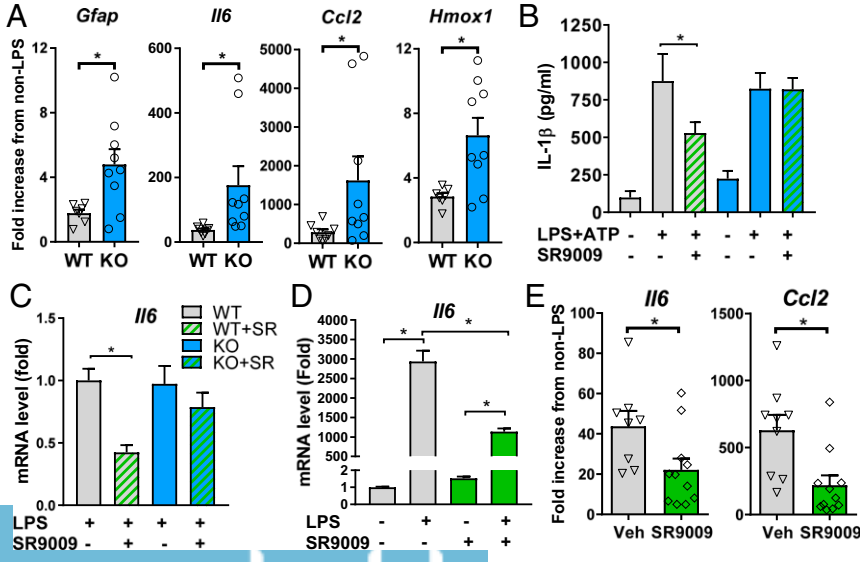

Fig. 3. Rev-erbs modulate LPS-induced neuroinflammation in vivo. (*A*) qPCR analysis of the hippocampal tissue from 5-mo RKO mice or WT littermates treated with i.p. LPS for 6 h. (*B*) ELISA for IL-1β levels in media from WT or RKO primary microglia treated with LPS alone or in combination with SR9009 as well as ATP for inflammasome maturation. (*C*) qPCR of WT or RKO primary microglia treated with LPS alone or in combination with SR9009 for *Il6* mRNA expression. Gene expression is normalized to LPS-treated WT cells. (*D*) mRNA expression of *Il6* in WT astrocytes treated with LPS and/or SR9009. (*E*) qPCR analysis of hippocampus from WT mice pretreated with SR9009 or vehicle (Veh) and then stimulated with i.p. LPS for 6 h. In both *A* and *E*, data are shown as fold increase from vehicle-treated WT mice (no LPS). $*P < 0.05$ by two-tailed *t* test.

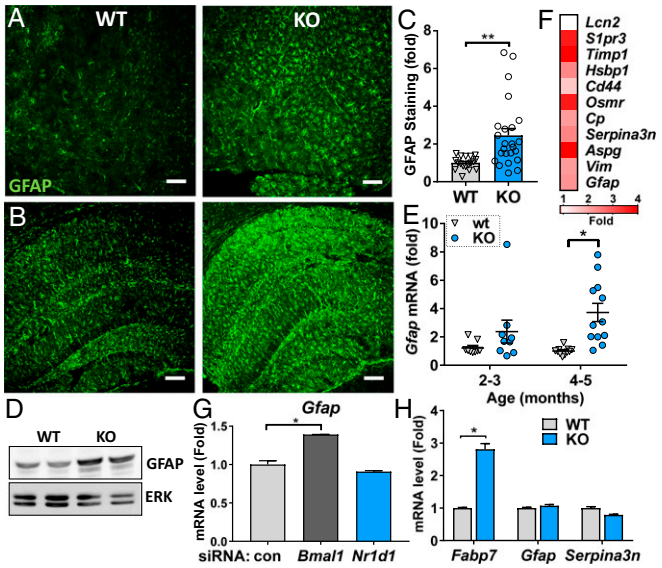

Fig. 4. Astrocyte activation in Rev-erbα$^{-/-}$ brain is cell nonautonomous. (*A* and *B*) GFAP staining in the piriform cortex (*A*) and hippocampus (*B*) of 5-mo RKO mice (KO, *Right*) compared with WT (*Left*). (*C*) Quantification of the percent area GFAP immunoreactivity in the cortex of RKO (KO) and WT littermates at 2–5 mo. n = 10–11 mice per genotype. (*D*) Western blot analysis for relative GFAP protein levels in WT and RKO mouse hippocampi as well as the associated ERK loading control. (*E*) qPCR analysis of *Gfap* mRNA expression in hippocampus from 2- to 3-mo and 4- to 5-mo RKO (KO) and WT mice. n = 10–12 mice per group. (*F*) Average expression fold changes for pan-reactive astrocyte activation markers in the hippocampus of 5-mo Rev-erbα$^{-/-}$, compared with WT littermates, n = 3 mice per genotype. (*G*) qPCR for *Gfap* mRNA expression in primary WT astrocytes treated with control siRNA as well as siRNA targeting *Bmal1* and *Nr1d1*. (*H*) Relative mRNA expression levels for the Rev-erbα target *Fabp7*, and astrocyte activation markers *Gfap* as well as *Serpina3n* in primary WT and RKO astrocytes. *P < 0.05 or **P < 0.01 by two-tailed t test with Holm–Sidak correction for multiple comparisons.

hippocampus. WT mice were pretreated with twice-daily injections of SR9009 (100 mg/kg) or vehicle, at 7 AM and 7 PM for 2 d, then injected with LPS (2 mg/kg i.p.). WT mice pretreated with SR9009 showed a diminished response to LPS stimulation as evidenced by reduced levels of proinflammatory transcripts, including *Il6* and *Ccl2*, in the hippocampus 6 h after LPS injection (Fig. 3*E*). Together, these data demonstrate that deletion of Rev-erbα exacerbates LPS-induced hippocampal inflammation, while Rev-erbα activation with SR9009 mitigates it.

Rev-erbα Deletion Induces Cell Nonautonomous Astrocyte Activation. Since astrogliosis is associated with microglial activation and occurs in *Bmal1*$^{-/-}$ mice (10), we next examined astrocyte activation in RKO brain. We observed diffuse astrocyte activation, as assessed by increases in GFAP immunoreactivity (Fig. 4 *A*–*C*), protein (Fig. 4*D*), and mRNA (Fig. 4*E*) expression, throughout the cortex (Fig. 4*A* and *SI Appendix*, Fig. S3*A*) and hippocampus (Fig. 4*B*). Increases in mRNA expression is evident in 2- to 3-mo and 4- to 5-mo RKO mice (Fig. 4*E*). The degree of astrogliosis was variable between mice, though it was consistently observed beginning in the outer cortical layers and hippocampus by 3 mo (*SI Appendix*, Fig. S2*A*). We examined expression of pan-reactive astrocyte activation transcripts, as described previously (30), and found the majority of these to be significantly up-regulated in RKO hippocampus (Fig. 4*F*). We have recently demonstrated that BMAL1 can regulate astrocyte activation in a cell-autonomous manner (31). However, we observed no increase in *Gfap* expression in WT astrocytes following siRNA-mediated *Nr1d1* knockdown (Fig. 4*G*) or in primary astrocyte cultures from RKO mice (Fig. 4*H*). Notably, RKO astrocyte cultures did show increased *Il6* and *Il1b* expression in response to LPS (*SI Appendix*, Fig. S3*D*), suggesting that Rev-erbα may regulate NF-κB in multiple glial cell types. Thus, our results suggest that Rev-erbα deletion induces astrocyte activation indirectly, perhaps as a result of microglial activation, neuronal injury, or other factors.

Glial Rev-erbα Deficiency Promotes Neuronal Death in a Coculture Model. Aberrant glial activation can result in neuronal damage in the context of disease. Therefore, we explored the consequences of Rev-erbα deficiency on neuronal injury using a primary neuron-mixed glia coculture model. We prepared mixed glial cultures containing microglia and astrocytes from postnatal WT mice, then treated cultures with siRNA targeting *Nr1d1* (siNr1d1) to knock down Rev-erbα, or with a nontargeting siRNA (siSCR) (Fig. 5 *A* and *B*). We removed the siRNA, then prepared conditioned media (CM) from these mixed cultures. We then grew WT primary cortical neurons in CM for 7 d, then treated neurons with hydrogen peroxide (H_2O_2) for 24 h to induce neuronal oxidative stress and death. WT neurons grown in siNr1d1 CM showed increased cell death at similar H_2O_2 concentrations compared with neurons grown in siSCR CM (Fig. 5 *C*–*E*). In a separate experiment, we depleted microglia by shaking (leaving 1–2% microglia) (31) from WT or RKO mixed glial cultures, leaving astrocyte-enriched cultures. WT neurons were grown on the astrocyte-enriched cultures and then stressed with H_2O_2. Here, we observed no significant differences between WT neurons grown on WT and RKO astrocytes (*SI Appendix*, Fig. S5). This illustrates the necessity of microglial Rev-erbα deficiency in promoting neuronal injury in vitro.

Altered Resting-State Functional Connectivity in RKO Mice. Given the effects on neuronal injury in vitro, we next asked if genetic deletion of Rev-erbα leads to impairment of systems-level brain function by examining cortical resting-state functional connectivity (FC) using optical intrinsic signal imaging (OISI) (32). Using OISI, changes in FC between different cortical regions can be observed in healthy mice (33, 34) or in models of neurodegeneration (10, 35). We performed OISI in anesthetized 5-mo WT and RKO (KO) mice, using a region-of-interest approach (Fig. 6*A*). Compared with WT mice, mice lacking Rev-erbα exhibited significantly reduced FC within the retrosplenial cortex (Fig. 6 *B* and *C*), a region which is selectively affected in other neurodegenerative models (35, 36). Interestingly, we also observed significantly increased FC within the somatosensory region of KO mice compared with WT mice (*Bottom* row, Fig. 6B and boxed blue region, Fig. 6*C*).We next examined quantitative, global FC differences between WT and KO mice, using spatial principal component analysis (PCA) of the group-averaged whole-cortex correlation difference matrix. The first two principal components, which explain 55% of the total variance between the groups, overlap remarkably with the retrosplenial (PC1, 39% variance) and somatosensory (PC2, 16% variance) regions examined above (Fig. 6*D*). Thus, Rev-erbα deletion causes in vivo changes in

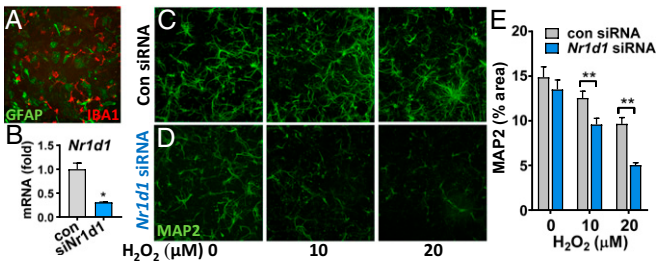

Fig. 5. Loss of Rev-erbα in glia impacts neuronal health in vitro. (*A*) GFAP/Iba1 costain of mixed glial culture used to generate the CM. (*B*) *Nr1d1* mRNA levels in mixed glia following treatment with control or *Nr1d1* siRNA. (*C* and *D*) Representative 10× images of MAP2 staining of neurons grown in CM from mixed glia treated with control (*C*) or *Nr1d1* siRNA (*D*). After 7 d in CM, neurons were treated with H_2O_2 for 24 h. (*E*) Quantification of MAP2 staining in *C* and *D*. n = 51–60 wells per group from at least three separate experiments. *P < 0.05 by two-tailed t test and **P < 0.05 by two-tailed t test with Holm–Sidak correction for multiple comparisons.

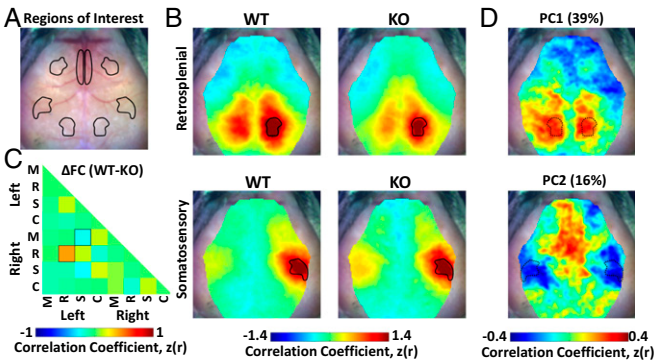

Fig. 6. Impaired cortical resting-state functional connectivity in Rev-erbα KO mice. (*A*) Functional parcellation of resting-stage networks using the average correlation structure across all mice studied. (*B*) Resting-state FC maps in WT and KO mice (n = 5–6 mice per genotype). *Top* row: Group-averaged retrosplenial FC. High correlation is indicated by reds; anticorrelation by blues. KO mice exhibit reduced interhemispheric FC as well as reduced anterior–posterior anticorrelations. *Bottom* row: Group-averaged somatosensory FC. WT mice exhibited lower interhemispheric FC compared with KO mice while both groups exhibited qualitatively similar ipsilateral FC. (*C*) Regional differences in correlation structure for each region-of-interest (ROI) pair. Maps of FC for each ROI examined in *A* were compressed into a difference matrix for quantitative evaluation of pairwise regional FC between WT and KO. WT mice exhibit significantly increased retrosplenial FC and significantly decreased FC in somatosensory regions compared with KO mice (black boxes). Cortical regions: C, cingulate; M, motor; R, retrosplenial; S, somatosensory. (*D*) Spatial PCA performed on the whole cortex correlation difference matrix assesses the topography of all pairwise correlation differences across groups. The first two PCs from this process explain 39% (PC1) and 16% (PC2) of the variance, respectively. This unbiased approach also reveals differences in KO mice involving retrosplenial (PC1) and somatosensory regions (PC2). Dotted black outlines of the ROIs are for reference.

regional FC which resemble those observed in other mouse models of neurodegenerative disease.

Discussion

Here, we show that the circadian clock protein Rev-erbα regulates microglial activation in vitro and in vivo, and that loss of Rev-erbα function promotes spontaneous neuroinflammation and neuronal dysfunction in vivo. We observed a time-of-day effect on microglial immunoreactivity in the hippocampus which was lost following Rev-erbα deletion, suggesting that Rev-erbα may control circadian oscillation of microglial activation, and that loss of Rev-erbα may lock brain microglia in a proinflammatory state. ChIP experiments revealed that Rev-erbα can interact with promoter regions of transcripts involved in NF-κB signaling, with loss of Rev-erbα driving proinflammatory NF-κB activation in cultured microglia and in the mouse hippocampus. While astrocyte activation was observed in the RKO brain, Rev-erbα deletion did not induce *Gfap* expression in cultured astrocytes, suggesting that, in contrast to *Bmal1* deletion (31), astrocyte activation in this model is noncell autonomous. Activation of Rev-erbs with the small-molecule agonist SR9009 suppressed inflammatory gene expression in cultured glia and in vivo. Taken together, these results implicate Rev-erbα as an important regulator of neuroinflammation.

A growing literature supports the link between the circadian clock and mammalian innate and adaptive immune function (37). Rev-erbα regulates various aspects of peripheral innate immune activation and cytokine release in various tissues. Rev-erbα has been implicated in the regulation of pulmonary inflammation (38) and in the transcriptional suppression of inflammatory mediators, including IL-6 and CCL-2 (18, 39). Rev-erbα also controls circadian oscillations in the NLRP3 inflammasome, with Rev-erbα deletion exacerbating IL-1β production and toxin-induced inflammatory hepatitis in mice (43). Despite the wealth of information regarding Rev-erbα in peripheral immune function, little is known about its role in innate immune responses in the brain. Microglia exhibit robust circadian clock function and inflammatory activation of microglia varies in a circadian manner (41, 42). Thus, loss of rhythmic Rev-erbα–mediated transcriptional repression could play a key role in the regulation of the microglial activation state, as demonstrated by our finding that microglial Iba1 volume changes with time of day in a Rev-erbα–dependent manner. Of note, RKO mice display nearly normal wheel-running activity due to compensation by Rev-erbβ (13, 17).Thus, the inflammatory phenotypes observed are not due to gross circadian rhythm disruption. Future studies are needed to understand the circadian and noncircadian transcriptional functions of Rev-erbs in various cell types in the brain.

We observed activation of the NF-κB pathway in both our primary microglia experiments and RKO brain. This is consistent with the previously established links between NF-κB signaling and the circadian clock (43, 44), and a recent study using a Rev-erbα–targeted drug (21). Notably, our experiments showed enhanced basal NF-κB p65 nuclear accumulation in RKO microglia. Our data suggest that loss of Rev-erbα could promote NF-κB signaling by directly regulating transcription of several NF-κB–associated transcripts, including *Traf2*, *Nfkbib*, and *Nfkb2*. *Traf2* conveys proinflammatory signals from the TNF receptor to NF-κB and can promote inflammation in glia (45). Rev-erbα deletion leads to loss of repression at the *Traf2* promoter and increased *Traf2* expression. Interestingly, Rev-erbα also binds to the *Nfkbib* promoter, but Rev-erbα deletion leads to decreased *Nfkbib* expression, suggesting that Rev-erbα binding may either act to somehow positively regulate *Nfkbib* transcription, or that the direct binding effects of Rev-erbα at the *Nfkbib* locus are minor and are counteracted by some indirect effect. Since *Nfkbib* acts as an inhibitor of NF-κB activation (29), loss of *Nfkbib* expression in Rev-erbα$^{-/-}$ cells could also promote inflammation. In macrophages, Rev-erbα/β integrate transcriptional signals from multiple damage-associated transcription factors, including NF-κB, Nrf2, and Smad3 (46), suggesting complex transcriptional regulation programs beyond canonical NF-κB signaling. Adding to the complexity, Rev-erbs can repress transcription by several distinct mechanisms, including DNA binding domain (DBD)-dependent binding to RORE sites (14), DBD-independent tethering by tissue-specific transcription factors (15), altering enhancer RNA expression (16), and by modulating chromatin looping (47). Notably, the Rev-erbα binding regions which we identified in *Traf2* and *Nfkbib* did not contain canonical RORE sites, suggesting possible DBD-independent regulation. Future ChIP-seq experiments will be needed to fully elucidate the cell type-specific targets of Rev-erbα.

We demonstrate that Rev-erbα KO mice have impaired functional connectivity in the retrosplenial cortex, similar to other mouse models of neurodegeneration, including models of amyloid-β or tau aggregation, or *Bmal1* deletion (10, 35, 36). It is unclear if this is due to damage related to neuroinflammation, or a direct effect of Rev-erbα in neurons. Rev-erbα is known to regulate dopaminergic neurotransmission via transcriptional control of tyrosine hydroxylase in the hippocampus and midbrain (48, 49), and Rev-erbα deletion alters cerebellar neuron development (50), suggesting that Rev-erbα could directly regulate cortical neuron function and/or health. Future studies using cell type-specific Rev-erbα deletion are needed to delineate the role of neuronal versus glial Rev-erbα in brain homeostasis.

Rev-erbα is a nuclear receptor that can be targeted pharmacologically with existing agonists (19). Previous studies show that Rev-erb agonists can suppress LPS-induced inflammatory cytokine production in C6 glioma cells and cultured microglia and in the murine brain following LPS infusion (21, 51). Our results are consistent with these previous studies, but greatly expand upon them by demonstrating the necessity of Rev-erbα for maintaining microglial homeostasis. Furthermore, we have demonstrated that the antiinflammatory effects of SR9009 are at least partially dependent on Rev-erbα, as we observed a blunted antiinflammatory response to SR9009 in Rev-erbα$^{-/-}$ microglia. Our studies use SR9009 as a proof-of-concept agent to demonstrate

that development of more potent Rev-erb agonists may be a viable strategy for treatment of neurodegenerative pathology.

In summary, our data establish a role for Rev-erbα in the control of microglial activation and neuroinflammation. This work solidifies Rev-erbα as a potential therapeutic target for treatment of neuroinflammation and illustrates the need for future study of the role of Rev-erbα and Rev-erb–targeted therapies in neuroinflammatory and neurodegenerative diseases.

Methods

For detailed methods, see *SI Appendix, SI Methods*.

Mice. All experiments were performed on RKO or WT littermate mice on a C57BL/6J background obtained from The Jackson Laboratory, and were approved by the Washington University Institutional Animal Care and Use Committee. Mice were housed on a 12/12 light/dark cycle unless noted. Drugs were administered by i.p. injection.

Cell Culture. Astrocytes and microglia were isolated from postnatal day 2 (P2) mice. Mixed cultures of astrocytes and microglia were grown in GM-CSF (5 ng/mL) containing media for 1 wk and then shaken at 225 rpm at 37 °C for 2 h to isolate microglia. Primary microglia were kept in culture for 9–12 d, then treated with LPS (50 ng/mL) for a specified time.

Optical Intrinsic Signal Imaging. OISI was performed in mice anesthetized with ketamine-xylazine using sequential illumination provided at four wavelengths by light emitting diodes, as previously described (32). See *SI Appendix, SI Methods* for details.

Data Analysis. Unless otherwise stated, values are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 7 or Matlab. A two-tailed Student's *t* test was used when a single variable was compared between genotypes (with Welch's correction when appropriate), and a two-way ANOVA with Tukey's posttest was used when multiple variables were compared between genotypes.

Confocal Imaging and 3D Reconstructions. Z stacks were obtained from 50-μm-thick sections using a Nikon A1Rsi confocal microscope. Colocalization analyses and 3D reconstructions were created using Imaris 9 software (Bitplane).

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