Presenilin 2 N141I mutation induces hyperactive immune response through the epigenetic repression of REV-ERBa

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Abstract

Hyperimmunity drives the development of Alzheimer disease (AD). The immune system is under the circadian control, and circadian abnormalities aggravate AD progress. Here, we investigated how an AD-linked mutation deregulates expression of circadian genes and induces cognitive decline using the knock-in (KI) mice heterozygous for presenilin 2 (*Psen2*) N141I mutation. This mutation causes overproduction of clock-controlled cytokines through the epigenetic repression of the clock protein REV-ERBa in innate immune cells, which show normal circadian period but lower amplitude. The KI/+ mice displayed normal circadian behaviour patterns, but were vulnerable to an otherwise innocuous, mild immune challenge. The antipsychotic chlorpromazine restored the REV-ERBa level and prevented the overexcitation of innate immune cells and cognitive decline in KI/+ mice. These results highlight a new pathogenic link between this AD mutation and immune cell-intrinsic circadian alteration through the epigenetic suppression of REV-ERBa.

Introduction

Alzheimer disease (AD) is the most common neurological disorder characterized by progressive loss of cognitive functions. The key dual pathological hallmarks of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles¹. With the increase in lifespan, AD poses a severe burden on global public health. Most cases of AD are sporadic, but a small percentage is inherited and is caused by the mutated versions of the genes including *APP*, *PSEN1*, and *PSEN2*, which encode amyloid precursor protein, presenilin 1, and presenilin 2, respectively². Identification of amyloid b (Ab) peptide in the senile plaques and widespread occurrence of amyloid deposition have led to the prevailing amyloid cascade hypothesis³. Genetic studies also suggested that familial AD (FAD) mutations in the above genes elevate amyloidogenic Ab₄₂ production⁴. However, intensive efforts over the last several decades aimed at clearing or preventing amyloid deposits have failed to prove the central role of Ab production and amyloid deposition in the pathogenesis of AD⁵.

Circadian rhythm is an endogenous biological rhythm with an approximately 24-h period that allows organisms to accommodate daily environmental changes and optimize their behaviour and physiology⁶. In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is the circadian pacemaker and serves as the master clock by synchronizing peripheral clocks found in various peripheral tissues and cell types throughout the body⁷. Rhythmic expression of clock genes is regulated by interlocked transcription–translation feedback loops⁸. REV-ERBa (encoded by the *NR1D1* gene), a representative clock protein, is a core inhibitory component of the circadian system; it functions as a transcriptional repressor in the auxiliary loop by suppressing the expression of BMAL1 (encoded by the *ARNTL1* gene)⁹. Molecular clock machineries are found not only in the SCN, but also in almost every peripheral organ¹⁰. In the brain, rhythmic expression of canonical clock genes is not restricted to neurons, but is also found in glia, suggesting the roles of glial cells in the control of the circadian rhythm and the importance of a functional molecular clockwork for proper glial function^{11,12}.

Recent studies have revealed an intimate link between circadian abnormalities and AD. Circadian disruptions, including sleep–wake cycle disturbance, sundowning, and altered daily rhythms of body activity and core temperature are highly prevalent among AD patients¹³ and in AD animal models^{14–16}. Circadian rhythm also affects Ab dynamics^{17,18}, and circadian alterations precede the onset of AD symptoms^{19,20}. As demonstrated in clock gene knockout animal models, circadian alterations can contribute to the pathogenesis of AD from early stages and exacerbate cognitive impairment,^{21,22}. Regardless of which is the cause and which is the effect, there is clear evidence of a positive feedback loop between circadian dysfunction and AD progress. However, clock gene deletion abolishes molecular rhythmicity and induces a wide range of pathological phenotypes, some of which may be irrelevant to AD. Therefore, model systems carrying clock gene deletions have limitations in representing the pathological changes in clock components or rhythmicity during neurodegeneration, and may not be well suited to study the AD-related circadian deficits²³. Transgenic AD mouse models also suffer from limitations, since these mice express mutated human genes, sometimes multiple genes, with the expression levels several to ten times those of endogenous mouse genes. Because of these limitations, molecular mechanisms underlying the bidirectional association between circadian dysregulation and AD pathogenesis remain largely unknown.

There is increasing recognition that innate immune response contributes to AD pathogenesis²⁴. Systemic inflammation markers are strongly associated with increased likelihood of dementia²⁵, and high levels of inflammatory mediators including interleukin-6 (IL-6) are found in the plasma of AD patients²⁶. Cognitive status in patients with AD is inversely correlated with microglial activation but not Ab load^{27,28}. Furthermore, genome-wide association studies have clearly implicated innate immunity and inflammation in the pathogenesis of AD^{29,30}. Therefore, it is now widely appreciated that inflammation is not only a central epiphenomenon in AD, but represents a major risk factor for the pathogenesis of AD²⁵.

Innate immunity is under the circadian control, and circadian proteins can directly control the expression of genes involved in innate immune system^{31,32}. Therefore, it is imperative to understand functional roles of the immune cell-intrinsic clock system in the progress of AD.

PSEN has been implicated in inflammation^{33,34}. However, how the *PSEN2* FAD mutations affect innate immune system remains largely unknown. Therefore, mouse models mimicking human *PSEN2* mutations would be desirable to delineate the molecular pathways underlying hyperimmunity-driven AD pathogenesis and cognitive impairment. The Asn residue at position 141 in *PSEN2* is conserved between human and mouse, and its substitution by Ile (N141I) is tightly linked to a form of early-onset FAD with strong penetrance³⁵.

Here, we generated knock-in (KI) mice expressing *Psen2* with the N141I mutation and provide evidence that mice heterozygous for *Psen2* N141I are vulnerable to an otherwise innocuous, mild inflammatory challenge and exhibit a hyperactive immune response and memory deficits because of overproduction of clock-controlled cytokines. This hyperimmunity is due to the DNA methylation-mediated epigenetic suppression of the clock protein REV-ERBa. Therefore, we identify epigenetic repression of REV-ERBa as a missing link between the *Psen2* N141I FAD mutation and hyper immune activity.

Results

Circadian clock-controlled cytokines respond with higher magnitude in *Psen2*^{N141I} KI/+ mice than in WT control. To investigate the pathogenic function of the *Psen2*^{N141I} mutation in vivo, we generated KI mice harbouring the *Psen2*^{N141I} allele (*Psen2*^{N141I/+} and *Psen2*^{N141I/N141I}) (Fig. 1a). The substitution of N to I (AAC to ATC) was confirmed by genomic sequencing (Fig. 1b). Human AD patients with FAD mutations are heterozygous³⁵. Therefore, to more accurately recapitulate human AD and to maintain the endogenous expression level, we used heterozygous *Psen2*^{N141I/+} (KI/+) mice for all experiments. The protein and mRNA levels of PSEN2 in KI/+ primary microglia and bone marrow-derived macrophages (BMDM) were comparable to those in WT cells (Supplementary Fig. 1a, b), indicating that the N141I mutation does not affect PSEN2 expression. Also, this mutation did not induce a compensatory change in PSEN1 expression (Supplementary Fig. 1a, b). We estimated γ -secretase activity on the basis of the cleavage of N-cadherin and mRNA expression level of *Hes5* as a downstream target of the Notch pathway³⁶, and found no difference between N141I KI/+ and WT genotypes in microglia and BMDM (Supplementary Fig. 1c, d).

To explore the functional consequences of the *Psen2*^{N141I} mutation in innate immunity, we examined whether this mutation affects inflammatory functions of immune cells following activation of Toll-like receptors (TLRs)³⁷. We treated primary microglia from WT or KI/+ mice with N-palmitoyl-S-dipalmitoylglycerol Cys-Ser-(Lys)₄ for TLR1/2, heat-killed *Listeria monocytogenes* for TLR2, polyinosinic-polycytidylic acid (high or low molecular weight) for TLR3, lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 for TLR4, flagellin from *Salmonella typhimurium* for TLR5, Pam₂CGDPPKHPKSF for TLR6, 20-mer single-strand RNA derived from HIV-1 long terminal repeat for TLR7, and oligonucleotides containing unmethylated CpG dinucleotides for TLR9. As a readout of the inflammatory response, we measured the release of tumour necrosis factor- α (TNF- α) and IL-6 to the medium. All but two TLR ligands induced a significantly higher secretion of IL-6 in KI/+ microglia than in WT (Fig. 1c). However, no such effect was observed for TNF- α release (Fig. 1d). Similarly, a more robust release of IL-6 but not TNF- α in KI/+ cells than in WT triggered by the TLR ligands was observed in BMDM (Fig. 1e, f). Therefore, markedly increased selective secretion of IL-6 caused by the *Psen2*^{N141I} mutation seems to be a common response in innate immune cells.

Next, to explore the spectrum of the cytokines affected by the *Psen2*^{N141I} mutation, we used enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array with WT and KI/+ microglia after LPS treatment. Among 40 targets, we detected 13 cytokines and observed increased secretion of C-X-C motif chemokine ligand 1 (CXCL1), C-C motif chemokine ligand 2 (CCL2), and CCL5 in addition to IL-6 in KI/+ microglia (Fig. 2a, b). Furthermore, their transcript levels were significantly up-regulated in KI/+ microglia and BMDM relative to WT (Fig. 2c, d). Interestingly, all 4 increased cytokines (IL-6, CXCL1, CCL2, and CCL5) are known to be regulated by the circadian rhythm³⁸. In contrast, secretion of TNF- α and its mRNA level were changed by LPS treatment to the same extent between WT and KI/+ genotypes in both microglia and BMDM (Fig. 2c, d). IL-1 β is another major cytokine and is released through the inflammasome complex, such as the NLRP3 inflammasome in innate immune cells³⁹. We activated the NLRP3 inflammasome by stimulating LPS-primed microglia with nigericin, as in our previous study³⁹, and found a similar amount of released IL-1 β and its transcript levels between WT and KI/+ microglia (Supplementary Fig. 2). Of note, the expression of both *Tnf* and *Il-1b* is circadian cycle-independent³⁸. As we saw a higher magnitude of LPS response in KI/+ microglia than in WT, we examined the expression level of the *Tlr4* gene and found no difference between the genotypes (data not shown). To sum up, the *Psen2*^{N141I} mutation induced hyperactive responses of clock-controlled cytokines in microglia and BMDM, innate immune cells representative in brain and periphery, respectively.

***Psen2*^{N141I} mutation alters the rhythmic expression pattern of clock genes and decreases robustness of circadian oscillation in innate immune cells.**

Because the *Psen2*^{N141I} mutation increased production of clock-controlled cytokines, we measured steady-state expression levels of representative clock genes in WT and KI/+ microglia and BMDM without cell synchronization to examine whether there is an alteration in their expression associated with the hyperactivation. The *Nr1d1*, *Clock*, *Cry1*, *Per1*, and *Per2* mRNA levels were significantly reduced and conversely the *Arntl* (encoding BMAL1) level was increased in KI/+ microglia and BMDM (Fig. 3a, b). Next, we examined the intrinsic oscillation of clock genes more in detail in microglia after synchronization by exposure to 100 nM dexamethasone (DEX) for 2 h⁴⁰ followed by culturing for 24 h in the absence of DEX with sampling every 4 h. The oscillation of *Nr1d1* was severely impaired and its amplitude was greatly decreased in KI/+ microglia (Fig. 3c). Oscillation of the other genes was also slightly shifted and amplitude of some genes was markedly decreased in KI/+ microglia (Supplementary Fig. 3a–i). These results suggest that the *Psen2*^{N141I} mutation impairs the microglia-intrinsic clock rhythm.

To compare the robustness of daily oscillations in microglia and BMDM between the WT and KI/+ genotypes, we monitored circadian clock oscillations in primary microglia and BMDM derived from *Per2::Luc* mice crossed with KI/+ (*Per2::Luc;Psen2*^{N141I/+}) or WT littermates (*Per2::Luc;Psen2*^{+/+}). *Per2::Luc* reporter mice, in which the luciferase (*Luc*) gene is fused in-frame to the 3' end of the endogenous mouse *Per2* gene, show robust circadian rhythms in both SCN and peripheral tissues⁴¹. Since cell-autonomous circadian clock dampens gradually over time if it is not synchronized with the master SCN clock through neuronal signalling⁴², this reporter line is a good system to compare and analyse the dynamics and robustness of cell-autonomous circadian oscillations by bioluminescence recording. WT microglia cultures exhibited robust luminescence oscillations for 3 days, whereas KI/+ microglia cultures displayed two robust circadian peaks followed by a barely recognizable third peak (Fig. 3d). Statistical analysis revealed that the amplitude was significantly reduced in KI/+ microglia compared to WT, while the circadian period was similar between the two groups (Fig. 3d). BMDM also exhibited similar alterations in circadian oscillation, comparable period but decreased amplitude in KI/+ cells relative to WT (Fig. 3e). Circadian rhythm disruption can manifest as changes in the circadian period, phase, or amplitude. Amplitude dampening is an especially good indicator of the reduced robustness of the circadian rhythm⁴³. Therefore, our results suggest that *Psen2*^{N141I/+} immune cells exhibit the same period of the molecular circadian rhythm, but its amplitude is lower and dampens faster than in WT cells. To examine whether this is also the case with the central clock, we obtained SCN tissue slices from *Per2::Luc;Psen2*^{N141I/+} and *Per2::Luc;Psen2*^{+/+} mice for explant study and found that the *Psen2* mutation did not alter central oscillation (Fig. 3f). These data suggest that immune cells derived from *Psen2*^{N141I/+} mice remain rhythmic, but have less robust autonomous circadian clocks.

Circadian locomotor activity and body temperature oscillation remain intact in *Psen2*^{N141I/+} mice. On the basis of the results on the SCN clock rhythm, we expected the circadian locomotor activity of *Psen2*^{N141I/+} mice to be normal. Actogram recording under 12-h light/12-h dark cycles for 14 days followed by constant darkness for another 14 days revealed no differences in the periods or amplitudes of the locomotor activity rhythm between WT and KI/+ mice (Fig. 4a, b). Likewise, body temperature fluctuations were the same in both genotypes (Fig. 4e, f). Periodograms showed no differences in the amplitudes of locomotor activity (Fig. 4c, d) or body temperature (Fig. 4g, h). These data indicate that *Psen2*^{N141I/+} mice preserve normal central clock and circadian behaviour, and suggest that circadian phenotypes caused by the *Psen2* N141I mutation are limited to certain cell types, including the components of the immune system.

***Psen2*^{N141I/+} mice display exacerbated inflammation and memory deficit in response to LPS.** The aggravated immune response of microglia and BMDM from *Psen2*^{N141I/+} mice suggests that these animals are predisposed to developing inflammation and cognitive decline. To test this idea, we compared the immune response between WT and KI/+ mice treated with a broad range of LPS concentrations. In mice, the immune response peaks in the hours around the beginning of the active phase¹². Therefore, we injected WT and KI/+ mice intraperitoneally (i.p.) with various doses of LPS at 6:00 PM (Zeitgeber time 11 with light-on at 7:00 AM) (Supplementary Fig. 4a) and monitored the inflammatory response after 20 h. Compared with WT mice, KI/+ mice exhibited higher circulating level of IL-6 at all LPS doses tested, and the relative difference between the genotypes was more pronounced at the lower doses (Supplementary Fig. 4b). On the other hand, the blood levels of TNF- α were the same in both genotypes at all doses (Supplementary Fig. 4c). The lowest dose (0.35 mg/kg body weight) we tested did not inflict inflammation in WT mice, but increased the blood levels of clock-controlled cytokines in KI/+ mice (Fig. 5a). Secretion of CCL3, CCL4, and CXCL2 was lower in KI/+ microglia than in WT upon LPS treatment (Fig. 2b), but these cytokines showed no difference in blood serum levels between WT and KI/+ mice following LPS injection (Supplementary Fig. 4d). Therefore, reduced secretion of this set of cytokines seems to be limited to in vitro microglia cultures and is subject to additional regulation in vivo.

Since microglia morphology is closely related to their function and microglial activation is characterized by cell shape change⁴⁴, we wondered whether the increased production of clock-controlled cytokines is associated with changes in morphology in KI/+ microglia. We examined microglia shapes in the hippocampus of KI/+ and WT mice by immunohistochemical analyses with an antibody against a microglia-specific marker, Iba-1. In the WT mice, microglia had a small cell body with highly ramified processes; consistent with no induction of cytokine release, a low dose of LPS did not change their morphology (Fig. 5b). On the other hand, hippocampal microglia in KI/+ mice, even in the absence of LPS challenge, already had a round enlarged soma with shorter processes, and these morphological features were furthered by LPS injection (Fig. 5b). Thus, we used the confocal images of microglia to reconstruct their 3D morphology and measured morphological parameters using IMARIS software (Fig. 5c). We examined total dendrite length and branching number, and performed Sholl analysis. Total dendrite length and the number of dendrite terminal points of each microglial cell were lower in KI/+ than in WT mice and further reduced by LPS injection (Fig. 5d). Sholl analysis measures the average number of dendrite intersections on concentric rings spaced at 1 μ m intervals from the centre of the cell body. Sholl analysis revealed that the hippocampal microglia of *Psen2*^{N141I/+} mice had fewer dendrite intersections than those of WT mice and exhibited dwindled morphology, and these characteristics became more distinct in response to LPS injection (Fig. 5d), consistent with microglial activation data. Therefore, on the basis of morphology, microglial activation was evident in KI/+ mice at baseline and was further induced by mild LPS challenge.

To determine whether the differential increase in the production of clock-controlled inflammatory cytokines and hippocampal microglial activation in KI/+ in response to i.p. injection of LPS is reflected by cognitive decline, we conducted a Y-maze test 20 h after LPS injection and examined spatial learning and memory. The arm alternation in the Y-maze was significantly decreased by LPS only in KI/+ mice (Fig. 5e), while the total number of arm entries was similar across all the groups, indicating normal motor function (Fig. 5f). To further examine learning and memory, we conducted a T-maze test with a food reward 20 h after LPS injection. Similar to the Y-maze test, the success rate for entering the correct arm was significantly lower in LPS-injected KI/+ mice than in the other groups (Fig. 5g, h). KI/+ mice developed no sickness behaviour nor did they differ from WT mice in spontaneous home-cage behaviours at this low dose of LPS, when locomotor activity and other behaviours were measured by LABORAS for 24 h (Supplementary Fig. 4e, f). Therefore, there was no difference with respect to basal activity patterns between the genotypes. In conclusion, a low dose of LPS induced a hyperactive immune response and caused memory deficit through the overproduction of clock-controlled cytokines including IL-6 in *Psen2*^{N141I/+} mice, while the same dose of LPS was innocuous to WT mice.

Down-regulation of REV-ERBa underlies exacerbated responses of clock-controlled cytokines to LPS in KI/+ microglia. Next, we focused on the REV-ERBa transcription factor, which showed the most striking difference between the genotypes (Fig. 3a, c). REV-ERBa is a transcriptional repressor of *Il-6* in macrophages⁴⁵ and links the clock in macrophages and microglia to immune processes such as cytokine release^{38,46}. Therefore, we examined the role of REV-ERBa in the hyperreactive immune response to LPS. Consistent with the lower *Nr1d1* mRNA level in KI/+ cells (Fig. 3a, b), protein levels of REV-ERBa was also much lower in KI/+ microglia and BMDM than in WT (Fig. 6a), suggesting that the *Psen2* N141I mutation decreases REV-ERBa levels in innate immune cell types. Consistent with a previous report⁴⁵, the chromatin immunoprecipitation (ChIP) assay showed that REV-ERBa bound directly to the proximal RORE (AGGTC A) promoter site of the *Il-6* but not *Tnf* gene (Fig. 6b). Promoters of *Amtl* and the non-specific TATA box-binding protein (*Tbp*) gene were used as positive and negative control, respectively⁴⁷. The REV-ERBa binding level was significantly lower in KI/+ microglia than in WT (Fig. 6b), suggesting that down-regulation of REV-ERBa may underlie the marked increase in IL-6 response to LPS. To confirm this notion, we knocked down the *Nr1d1* gene in WT microglia by transduction with lentivirus expressing *Nr1d1*-targeting shRNA (Fig. 6c). Similar to the *Psen2* N141I mutation, suppression of *Nr1d1* expression in WT microglia potentiated the inflammatory response, as shown by an increase in the secretion (Fig. 6d) and mRNA expression (Fig. 6e) of IL-6 in response to LPS. Thus, the knockdown of REV-ERBa was sufficient to induce a selective increase in IL-6 production. We next overexpressed REV-ERBa in KI/+ microglia using lentivirus (Fig. 6f). As expected, expression of human *NR1D1* in KI/+ microglia was sufficient to revert the response of IL-6 to LPS to the WT levels (Fig. 6g,

compared with Fig. 1c and Fig. 2c). In contrast, the secretion and transcript level of TNF- α were not impacted by modulating REV-ERBa expression in microglia (Fig. 6d, e, g, h). These data indicate that the *Psen2*^{N141I} mutation elevates clock-controlled cytokines, as represented by IL-6, in response to LPS through down-regulation of REV-ERBa in microglia.

***Nr1d1* promoter is hypermethylated in KI/+ innate immune cells.** The circadian rhythm is driven by the cyclic regulation of gene expression, including epigenetic regulation⁴⁸. Notably, the methylation of DNA, which occurs mostly on CpG dinucleotides (CpG islands) and results in transcriptional repression, is altered in AD brains⁴⁹ and is associated with circadian dysregulation⁵⁰. To examine whether promoter methylation is associated with REV-ERBa expression, we demethylated DNA in KI/+ microglia using 5-azacytidine (5-Aza) and determined *Nr1d1* transcript level by qRT-PCR. Treatment of KI/+ microglia with 5-Aza for 24 h rescued *Nr1d1* mRNA (Fig. 7a) and REV-ERBa protein levels (Fig. 7b), and subsequently decreased IL-6 expression (Fig. 7c) and secretion (Fig. 7d) to the levels similar to those in WT, without affecting TNF- α expression (Fig. 7c) or secretion (Fig. 7d). The same treatment with 5-Aza also restored the *Nr1d1* transcript and REV-ERBa protein levels, and reverted elevated expression and secretion profiles of IL-6 without affecting TNF- α in KI/+ BMDM (Fig. 7e–h). The same dosage of 5-Aza had no effects in WT cells (Fig. 7a–h).

We searched CpG islands in the *Nr1d1* promoter using the prediction algorithm at www.urogene.org/methprimer and performed whole-genome DNA methylation analysis of WT and KI/+ microglia to estimate methylation levels in the predicted CpG islands. Methylation sequencing and differential cytosine methylation analysis showed that many of the CpG islands in the *Nr1d1* promoter are hypermethylated in KI/+ microglia compared with WT (Fig. 7i). As a result, the overall methylation ratio of *Nr1d1* ~2 kbp upstream of the transcription start site was significantly higher in KI/+ microglia than in WT (Fig. 7j). Taken together, these data suggest that DNA hypermethylation of the promoter of the *Nr1d1* gene is critical for reducing its expression in KI/+ innate immune cells.

The antipsychotic chlorpromazine restores REV-ERBa expression and prevents hyperactive immune response and cognitive decline in *Psen2*^{N141I/+} mice.

Aiming at enforcing the circadian amplitude in immune cells including microglia by restoring the REV-ERBa level and thereby preventing hyperactive immune response, we screened a variety of CNS-targeting drugs that can cross the blood–brain barrier but have not been well studied in terms of their therapeutic efficacy in AD. Among the drugs tested, the antipsychotic chlorpromazine (CPZ, 0.5 mM for microglia and 1 mM for BMDM) efficiently reduced IL-6 secretion and mRNA level (Fig. 8a, b) without affecting those of TNF- α (Supplementary Fig. 5a, b) in KI/+ microglia and BMDM. Also, CPZ reversed LPS-induced increases in the mRNA levels of clock-controlled cytokines in KI/+ microglia and BMDM (Supplementary Fig. 5c–e). At these concentrations, CPZ was not toxic to the cells (cell death of $2.76\% \pm 1.78\%$ in WT and $2.39\% \pm 0.42\%$ in KI/+ microglia; $2.4\% \pm 0.69\%$ in WT and $2.26\% \pm 0.19\%$ in KI/+ BMDM). Furthermore, CPZ treatment of KI/+ microglia and BMDM for 12 h rescued *Nr1d1* mRNA (Fig. 8c) and protein levels (Fig. 8d). To examine whether CPZ can attenuate the overproduction of clock-controlled cytokines in vivo, we injected CPZ into WT and KI/+ mice 4 h before LPS injection (Fig. 8e). Administration of CPZ prior to LPS decreased the blood levels of clock-controlled cytokines in KI/+ mice without affecting TNF- α (Fig. 8f). To examine whether CPZ can rescue LPS-induced memory deficits in KI/+ mice, we conducted Y-maze test (Fig. 8g, h). The arm alternation was recovered in KI/+ mice pre-treated with CPZ (Fig. 8g). The total number of arm entries was the same among all groups, indicating that CPZ-injected mice had normal locomotor activity (Fig. 8h). To examine whether CPZ can normalize circadian amplitude, we carried out bioluminescence recording with KI/+ microglia with and without treatment of CPZ. Interestingly, despite the restoration of REV-ERBa protein level, amplitude was not rescued by CPZ (Supplementary Fig. 5f). This result suggests that REV-ERBa's immunomodulatory effects are critical to the KI/+ mice phenotypes and circadian alteration may be secondary. Taken together, these data reveal a novel action of the antipsychotic drug CPZ via the restoration of *Nr1d1* expression and thereby prevention of hyperactive inflammation and memory deficits caused by LPS in *Psen2*^{N141I/+} mice.

Discussion

Here, we show that *Psen2*^{N141I} mutation down-regulates REV-ERBa in innate immune cells and mutant mice fail to optimize the innate immune response to an otherwise innocuous immune challenge, leading to overproduction of clock-controlled cytokines and cognitive decline (Fig. 9). We revealed hypermethylation of the *Nr1d1* promoter as the underlying epigenetic repressive mechanism. Therefore, our findings highlight a novel mechanism linking the *Psen2*^{N141I} FAD mutation to altered expression of circadian genes, which renders mutant mice vulnerable to AD immunopathology.

In addition to *Nr1d1*, other clock genes including *Per1*, *Per2*, *Cry*, and *Clock* were also markedly down-regulated in KI/+ microglia and BMDM. However, the reduced circadian amplitude of KI/+ cells is not readily explained by their down-regulation. CLOCK forms a complex with the NF- κ B subunit p65 and increases the transcriptional activity of NF- κ B⁵¹. Therefore, *Clock*-deficient BMDM are less responsive to LPS⁵². PER2 can also promote inflammation, and *Per2*^{-/-} mice are protected from LPS-induced endotoxic shock and show decreased serum levels of several cytokines, but the IL-6 level remains normal⁵³. *Cry1*^{-/-} *Cry2*^{-/-} BMDM show increased inflammatory features, but without selectivity for IL-6 or clock-controlled cytokines, and the levels of both IL-6 and TNF- α are increased by the absence of CRY⁵⁴. Therefore, the selectively enhanced production of clock-controlled cytokines in *Psen2*^{N141I/+} mice is mostly likely due to the repression of REV-ERBa.

The mediation of circadian regulation by REV-ERBa in innate immune cells has been associated with inflammation in the periphery³⁸. However, the role of REV-ERBa in central immunity has not been well known. Recently, the link between REV-ERBa and microglia function has been studied using gene knockout approaches⁴⁶. Deletion of REV-ERBa activates NF- κ B signalling, and multiple proinflammatory genes, including TNF- α and circadian-regulated cytokine genes, are up-regulated, which is different from the selective elevation of expression of circadian-controlled cytokines observed in this study. In addition, unlike in REV-ERBa KO microglia, we did not observe the activation of NF- κ B signalling in *Psen2*^{N141I/+} microglia (data not shown). Also, the repression of REV-ERBa

due to the *Psen2*^{N141I} mutation did not phenocopy the mildly disturbed circadian locomotor behaviours, neurodegeneration, or mood dysregulation observed in REV-ERBa KO mice^{9,55}, suggesting that the residual expression of REV-ERBa is enough to maintain normal central clock and neural function. There has been great interest in understanding how circadian proteins go awry during the progress of neurodegeneration and the cause of their dysfunction. Our study uncovers the effects of the *Psen2*^{N141I} FAD mutation on the expression of REV-ERBa, and reveals a functionally important pathogenic pathway leading to hyperreactive innate immunity in AD.

Chlorpromazine was introduced into psychiatry for the treatment of schizophrenia and other psychoses in the 1950s⁵⁶. Its psychotropic effects were mainly attributed to blockade of dopamine receptors in neurons⁵⁷, and partially to inhibition of neuroinflammation⁵⁸. However, the action of CPZ on innate immune cells is largely unknown and the rescue of REV-ERBa expression reveals a previously unrecognized effect of CPZ, suggesting a potentially promising therapeutic strategy to suppress inflammation for the treatment of AD.

It is known that antipsychotic drugs such as CPZ, when taken for a long time at high doses, increase mortality in elderly patients with dementia-related psychotic symptoms⁵⁹. However, our study suggests that low doses of CPZ may be effective in preventing immunopathology and improving cognition, warranting further studies to optimize the CPZ regimen.

It remains to be answered how the *Psen2*^{N141I} mutation induces hypermethylation in the promoter region of *Nr1d1* and whether promoters of other clock-regulated cytokine genes are also hypermethylated. It also needs to be determined whether CPZ restores REV-ERBa level through demethylation of the *Nr1d1* promoter. Another interesting question is regarding the relative contributions of REV-ERBa's circadian or immunomodulatory effects to the phenotypes. CPZ restored REV-ERBa expression and efficiently blocked hyperactive immune response in KI/+ cells, but failed to rescue dampened circadian robustness. This suggests that REV-ERBa's immunomodulatory activity may be directly related with the phenotypes with reduced amplitude as an accompanying secondary effect, although further study will be required to confirm this notion. Our findings suggest that PSEN2 plays an important role in innate immunity by optimizing the immune response and limiting overactive inflammation. In the future, it will be necessary to analyse the role of the interaction between REV-ERBa and other down-regulated clock proteins in regulating the function of PSEN2 in the negative feedback of inflammation.

Overall, our findings show that the *Psen2*^{N141I} mutation leads to dysregulated innate immunity through epigenetic suppression of REV-ERBa. Even though the diminished circadian amplitude observed at the immune-cell level was not manifested at the central-clock or whole-animal level, mutant mice were highly vulnerable to immune challenge and predisposed to cognitive decline and AD immunopathology.

Methods

Animals

All procedures for the care and use of laboratory animals were approved by the Institutional Animal Care and Use Committee of DGIST. Animals were maintained in a specific pathogen-free environment under a standard 12-h light/12-h dark cycle at the DGIST animal facility. *Psen2*^{N141I/+} mice were generated using homologous recombination. Targeting vector included the I141 mutation in exon 4 and the *Neof-Loxp* sequence, and mutated sequence in the targeting vector was inserted into *Psen2* of the WT allele. *Psen2*^{N141I/N141I;loxP-Neof-Loxp} mice were crossed with Cre mice to generate knock-in mice carrying the *Psen2*^{N141I} mutation using the Cre-loxP system. *Per2::Luc* knock-in mice were a generous gift from Joseph Takahashi⁴¹. *Psen2*^{N141I/+} mice were crossed with *Per2::Luc* mice to monitor real-time circadian dynamics using bioluminescence recording.

Cell culture

Primary microglia were obtained from 1–3-day-old neonatal mice as described previously³⁷ and cultured in Dulbecco's modified Eagle's medium (DMEM, Corning) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Hyclone) and 1% penicillin–streptomycin (Hyclone). Microglia were isolated at days in vitro 12 by tapping. Purity of primary microglia and no contamination by astrocytes were confirmed by immunostaining with antibodies against Iba-1 and GFAP, specific microglia and astrocyte markers, respectively, as we previously reported³⁷. BMDM were obtained from femurs and tibias of 6–7-week-old mice, as we previously reported⁶⁰.

Reagents and antibodies

The following antibodies were used: horseradish peroxidase–conjugated β -Actin (sc47778) from Santa Cruz Biotechnology; PSEN 1 (5643) and REV-ERBa (13418) from Cell Signaling Technology; Iba-1 (019-19741) from Wako Chemicals; N-cadherin (610920) from BD Bioscience; PSEN 2 (ab51249) from Abcam; REV-ERBa (PA5-29865) from Thermo Fisher Scientific. The following reagents were used: LPS from *Escherichia coli* 0111:B4 (L4391), DEX (50-02-2), and chlorpromazine hydrochloride (C8138) were purchased from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS); mouse Toll-like receptor ligands kit (Tlr-kit1mw) and nigericin (Tlr-nig) from Invivogen; SR9009 (554726) from Merck Millipore; 5-azacytidine (ab142744) from Abcam; and D-luciferin (E1601) from Promega.

Quantitative RT-PCR

RNA was isolated using the ImProm-II Reverse Transcriptase kit (Promega) and cDNA was synthesized using oligo dT. PCR primers were commercially synthesized (Cosmo Genetech). qRT-PCR was performed on the reverse transcribed product using Taq Polymerase (Invitrogen) and primers (Table 1) specific for mouse cDNAs. TOPreal™ qPCR 2 × PreMIX (SYBR Green with low ROX) (Enzynomics) was used. Amplification (50-cycles) was performed in a CFX96 Real-Time System (Bio-Rad). *Actb* was used as the reference gene for normalization.

Enzyme-linked immunosorbent assay

ELISA kits for mouse IL-6, TNFα, IL-1β, CCL2, CXCL1, CCL3, CCL4, and CXCL2, and a Proteome Profiler Mouse Cytokine Array Panel A kit were purchased from R&D Systems and used to measure cytokines in culture media and blood serum according to the manufacturer's instructions.

Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaCl in 20 mM Tris-HCl, pH 7.4) with 1× protease and phosphatase inhibitors (Thermo Fisher Scientific) and 0.1 M dithiothreitol (Sigma-Aldrich). Cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated with the appropriate primary antibodies, and bound antibodies were detected by species-specific, horseradish peroxidase–conjugated secondary antibodies. Chemiluminescence detection was performed to analyze the protein bands of interest.

ChIP-qPCR analysis

Cells were fixed and proteins were cross-linked to DNA using 0.75% paraformaldehyde (PFA) in PBS. Glycine (125 mM) was added to quench cross-linking reaction, and cells were collected and lysed in radio-immunoprecipitation assay buffer (Sigma-Aldrich) containing 1 × protease and phosphatase inhibitor cocktail, and DNA was fragmented by sonication to 500–1000-bp size. Protein A-agarose/salmon sperm DNA beads (Millipore) were incubated with DNA and antibody against the protein of interest or IgG, and the samples were immunoprecipitated overnight with rotation at 4°C. DNA was eluted by adding elution buffer (10 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0). Input samples was incubated with RNase A (20 µg/mL) and Proteinase K (0.5 mg/mL) at 65°C overnight to reverse cross-linking. DNA was precipitated with isopropanol, and qRT-PCR was performed to assess the binding of the protein to the anticipated binding sites in DNA using the primers used listed in Table 1.

Y-maze assay

Y-maze was used to evaluate spatial working memory. The assay was conducted in white plastic arms of a Y-shaped maze. A mouse was placed into the centre and was allowed to freely explore the arms for 5 min. The experiment was recorded with EthoVision software (Noldus). The number of arm entries and the number of triads were analysed to calculate the percentage of alternation by dividing the number of three consecutive arm entries (triads) by the number of possible triads × 100 (total arm entries – 2).

T-maze assay

T-maze was used to evaluate spatial learning and memory with reward alternation. The assay was conducted in white plastic arms of a T-shaped maze. Mice were familiarized with the maze and food reward for 5 min before test. Then, in the test run, rewards were placed in both arms, and one arm was blocked. Mice were placed at the base and ran to open arms to eat the reward. At the next trial, the previously closed arm was opened. Mice were placed back again at the base and chose one arm. If mice chose the newly opened arm, they were able to eat the reward. If mice incorrectly chose the previously visited arm, they did not get any rewards. The number of trials in which the correct arm was visited was expressed as percentage of total arm entries.

LABORAS

Mice were placed in each single cage and recorded during 24 hours starting from night cycle at 7:00 PM. Activities of mice including locomotion, climbing, rearing, grooming and eating were recorded and automatically analyzed using the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris).

Microglial transduction

sh*Nr1d1* (22747) was purchased from Addgene. Human *NR1D1* cDNA was cloned into the PLJM1-EGFP vector to generate lentiviruses. Lentiviruses were produced by transfection of Lenti-X 293T cells with the transfer vector (PLKO.1-sh*Nr1d1* or PLJM1-*NR1D1*-EGFP), packaging vector (psPAX2, Addgene), and

VSV-G envelope-expressing vector (PMD2.G, Addgene). After 3 days, the supernatant was harvested and ultracentrifuged at 25,000 $\times g$ for 2 h (Optima XPN-100, Beckman Coulter) for virus concentration. Primary microglia were infected with the lentiviruses in medium supplemented with hexadimethrine bromide (8 $\mu\text{g/mL}$). After 24 h, medium was replaced with fresh culture medium. eGFP expression was monitored using fluorescence microscopy after 72 h to estimate the lentiviral transduction efficiency.

Immunohistochemistry

Mice were anesthetized by injection of a mixture of Zoletil (Virbac, 50 mg/kg) and Rompun (Bayer, 10 mg/kg). Then, the mice were perfused with PBS, followed by 4% PFA. Brains were collected, post-fixed in 4% PFA for 16 h, transferred to 30% sucrose until they sank to the bottom of the tube, and frozen with optimal cutting temperature compound. Embedded brains were cut into 50- μm -thick coronal sections and slices were floated in PBS. Samples were incubated in sodium citrate buffer (10 mM tri-sodium citrate dihydrate, 1 N HCl, 0.05% Tween-20 in water) in a 1.5-mL tube at 95°C for 5 min for antigen retrieval. Samples were washed with PBS three times and blocked with PBS containing 5% normal donkey serum and 0.01% Triton X-100. Slices were incubated with Iba-1 antibody in PBS containing 3% bovine serum albumin for 24 h at 4°C and then with appropriate secondary antibody for 2 h at room temperature. Images were acquired with an LSM 7 confocal laser scanning microscope (Carl Zeiss).

Microglia morphology analysis

Confocal images were obtained along the entire Z-axis of a randomly selected field. Then, 3D images were reconstructed from confocal images using IMARIS software (version 9.2.1, Bitplane AG). Dendrite length, dendrite branch points, and Sholl radius of microglia were quantified using FilamentTracer function following Iba-1 signals.

SCN slice culture

SCN explant cultures were prepared and monitored with minor modifications⁶¹. One-week-old WT or *Psen2*^{N141I/+} mice with the *Per2::Luc* knock-in allele⁴¹ were sacrificed and their brains were quickly removed, chilled and moistened in Gey's Balanced Salt Solution (GBSS) supplemented with 0.01 M HEPES and 36 mM D-glucose, and aerated with 5% CO₂ and 95% O₂. The brains were then cut coronally into 400- μm -thick slices using a Leica VT1000 S vibratome (Leica). The slices were maintained on a culture insert membrane (Millicell-CM, Millipore) and dipped into culture medium (50% minimum essential medium, 25% GBSS, 25% horse serum, 36 mM glucose, and 1 $\times$ antibiotic-antimycotic) at 37°C. The SCN slices were cultivated for >2 weeks before being used in experiments.

Bioluminescence recording

To monitor real-time circadian dynamics in cells and SCN slices derived from *Per2::Luc* reporter mice, luminescence was continuously measured at 36°C with a cell culture incubator–incorporated luminometer (Kronos Dio, Atto). Light emission was integrated for 1 min with 10-min intervals between measurements. For astrocytes, microglia, and BMDM, cells were synchronized with DEX (150 nM) in culture media. At 15 min after DEX addition, cells were washed and medium was changed to fresh recording medium (culture medium supplemented with 0.3 mM D-luciferin). SCN cultures were maintained in a 35-mm Petri dish with 1 mL culture medium containing 0.3 mM D-luciferin without DEX synchronization. Bioluminescence was analysed in real time by the cosinor procedure^{43,62}.

Behaviour recording

The locomotor activity and body temperature of the mice were measured using E-mitter, a radio transmitter–based telemetry system (Starr Life Science). E-mitter was implanted beneath the skin on the back using aseptic techniques under general anaesthesia induced by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) injection⁶³. After implantation, the mice recovered for at least one week and acclimatized to a regular 12-h light/dark cycle. Activity and temperature data detected by the implanted sensor were transmitted to a receiver (ER-4000 Energizer/Receiver). Data acquisition and digital transformation were performed using VitalView software (Starr Life Science) every 6 min. Chi-square periodogram analysis was performed using the xsp package⁶⁴ in R⁶⁵.

Methylation sequencing

Genomic DNA was isolated from 10 $\times$ 10⁶ microglial cells from WT and KI/+ mice using the MasterPure DNA purification kit (Epicentre) according to the manufacturer's instructions. The extracted genomic DNA was resuspended in TE buffer and quantified by fluorometry. Bisulphite conversion of genomic DNA was conducted using the EZ DNA Methylation Lightning kit (Zymo Research). In this method, non-methylated cytosine residues are converted into uracil by bisulphite treatment and read as thymine when sequenced. Methylated cytosines, protected from conversion, are still read as cytosines. Bisulphite-converted DNA was purified and used to prepare a sequencing library using the EpiGnome Methyl-Seq kit (Epicentre). In this procedure, to synthesize DNA containing a

specific sequence tag, DNA is randomly primed using a polymerase able to read uracil nucleotides. Only the complement to the original bisulphite-treated DNA is used as the sequencing template; thus, the resulting Read 1 will always be the same sequence as the original bisulphite-treated strand.

Statistical analysis

Data were acquired in at least 3 independent experiments and are presented as mean $\pm$ standard error of the mean (SEM) values. Statistical analysis was performed by using unpaired Student *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA and Bonferroni post-test. Statistical significance was analysed using GraphPad Prism (GraphPad Software).

Declarations

Acknowledgements

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Author contributions

S.-W.Y. designed the experiments, analysed and interpreted the data, and wrote the manuscript. H.N. designed and performed most of the experiments, collected and analysed the data, and wrote manuscript. Y.L., J.-W.L., Y.P., and B.K. performed additional experiments. B.K. and H.K.C. provided mice, analysed and interpreted the data. H.-K.A., K.M.C., and J.H. generated and provided *Psen2*^{N141I/+} mice. K.K., E.-K.K. and H.K.C. provided critical experimental materials and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

No potential conflict of interest was reported by the authors.

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Table

Table 1. Primers for qRT-PCR and CHIP-qPCR analyses

qRT-PCR		
Gene	Gene reference number	5'- primer sequence -3'
<i>Psen1</i>	NM_001362271.1	F CAG TCA CCT GGG GCC TCA TCG R GTT GTG TTC CAG TCT CCA CTG GC
<i>Psen2</i>	NM_011183.3	F GTA TGG GGC GAA GCA TGT GAT R ACG CAC AGA CTT GAT AGT GGC
<i>Il-6</i>	NM_031168.2	F CTG GAT ATA ATC AGG AAA TTT GC R AAA TCT TTT ACC TCT TGG TTG A
<i>Cxcl1</i>	NM_008176.3	F CAT GGC TGG GAT TCA CCT CA R TGA GGT GAA TCC CAG CCA TG
<i>Ccl2</i>	NM_011333.3	F GTC CCT GTC ATG CTT CTG GG R CCC CAA GAA GGA ATG GGT CC
<i>Ccl5</i>	NM_013653.3	F CCT CAC CAT ATG GCT CGG AC R TGC TCC AAT CTT GCA GTC GT
<i>Tnf</i>	NM_013693.3	F CAT CTT CTC AAA ATT CGA GTG ACA A R TGG GAG TAG ACA AGG TAC AAC CC
<i>Hes5</i>	NM_010419.4	F GGT ACA GTT CCT GAC CCT GC R GCA GGG TCA GGA ACT GTA CC
<i>Il-1b</i>	NM_008361.4	F AGG CCA CAG GTA TTT TGT R GCC CAT CCT CTG TGA CTC
<i>Nr1d1</i>	NM_145434.4	F TGC TGG CAT GTC CCA TGA AC R GAC TGT AGG TTG TGC GGC TC
<i>Nr1d2</i>	NM_011584.4	F AGC TTC CAG TCC TCG TCC TC R ATC CTG ATG CCA CAT CCC CA
<i>Arntl</i>	NM_007489.4	F GCT GTA CCG TCC CTG TCA AA R GGT GCC TTC GTG ACT CTG AA
<i>Clock</i>	AF000998.1	F TAG CAC TCC TCC CAG ACA GC R CTA TCA TCC GTG TCC GCT GC
<i>Cry</i>	AF351609.1	F CTC GCC TCG GTC CCT TCT AA R CGG AGC TTC TCC CTT GCT TG
<i>Per1</i>	NM_011065.5	F CAG TGG AGT CTG GAG GAG GG R GGA GCC AGG AGC TCT GAG AA
<i>Per2</i>	NM_011066.3	F GCA GAA ATG CAA AGC AGC CC R GGA TGT TGG CTG GGA ACT CG
<i>Dbp</i>	NM_016974.3	F GGA GCC TTC TGC AGG GAA AC R AAA GGC AAA GTG CGT TCC CA
<i>Rora</i>	NM_013646.2	F CGC TCG TGG CTT CAG GAA AA R GGA GTC GCA CAA TGT CTG GG
<i>Csnk1a1</i>	NM_146087.3	F AGT CGC CGA GAT GAC ATG G R GGG TCC TGA AAA GGA TGC GG
ChIP-qPCR		
Gene	Gene reference number (primer recognition site)	5'- primer sequence -3'
<i>Il-6</i>	NM_031168.2	F GGA GAG GGA GTG TGT GTC TT
	(Promoter region)	R GTG CTG GTT TAA ATA ACA TCA

<i>Arntl</i>	NM_007489.4 (Promoter region)	F	GGT TGG AAT TAC AGA CTA CGC C
		R	ACA TTT TAG GAA CAC AGA GTG
<i>Tbp</i>	NM_013684.3 (Promoter region)	F	CCA CAC CCG CCA CCA GTT CG
		R	TAC AGC CCG GGG AGC ATC GT
<i>Tnf</i>	NM_013693.3 (Promoter region)	F	ACA CTT CCC AAC TCT CAA GC
		R	TTG TAG AAA GAC CAT GCC TGT

Figures

Figure 1

Psен2 N141I mutation causes selective increase in the production of IL-6 in innate immune cells. **a** Strategy for targeted insertion of the N141I point mutation into the murine *Psен2* locus to generate a KI mouse model. **b** Sanger sequencing chromatograms of genomic DNA from wild type (WT), *Psен2*N141I/+ (KI/+), and *Psен2*N141I/N141I (KI/KI) mice. **c**, **d** ELISA of IL-6 (**c**) and TNF-α (**d**) in culture supernatants from WT and KI/+ microglia after treatment with the TLR ligands for 12 h (n = 4). Cells were stimulated with N-palmitoyl-S-dipalmitoylglycerol Cys-Ser-(Lys)4 (Pam3CSK4; 100 ng/mL), heat-killed *Listeria monocytogenes* (HKLM; 107 cells/ mL), polyinosinic-polycytidylic acid high molecular weight (poly (I:C) HMW; 10 μg/ mL) or low molecular weight (poly (I:C) LMW; 1 μg/ mL), lipopolysaccharide (LPS; 1 μg/ mL), flagellin from *Salmonella typhimurium* (FLA-ST; 1 μg/ mL), Pam2CDGPKHPKSF (FSL-1; 100 ng/ mL), single-strand (ss) RNA (1 μg/ mL), or ODN1826 (0.5 μM). **e**, **f** ELISA of IL-6 (**e**) and TNF-α (**f**) in culture supernatants from WT and KI/+ BMDM after treatment with the TLR ligands for 12 h (n = 4). BMDM were stimulated by TLR ligands as microglia. All data are mean ± SEM. #p < 0.05, ##p < 0.01, and ###p < 0.001 for the indicated comparisons.

Figure 2

Psen2 N141I mutation increases production of clock-controlled cytokines in innate immune cells. **a** Proteome ELISA of 40 cytokines in the culture media of WT and KI/+ microglia after LPS (1 μ g/mL) treatment for 12 h. Signals were normalized to pixel density of indicated reference (Ref) spots. **b** Quantification of the average signal (pixel density) as relative changes in cytokine levels between WT and KI/+ samples ($n = 4$). **c**, **d** Relative mRNA expression levels of the increased cytokines (*Il-6*, *Cxcl1*, *Ccl2*, and *Ccl5*) and *Tnf* after LPS (1 μ g/mL) treatment for 12 h in primary microglia (**c**) and BMDM (**d**). mRNA levels were normalized to *Actb* ($n = 3$). All data are mean $\pm$ SEM. n.s., not significant. * $p < 0.05$ and *** $p < 0.001$ vs. untreated WT control. ††† $p < 0.001$ vs. untreated KI/+ control. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ for the indicated comparisons.

Figure 3

Psen2 N141I mutation decreases robustness of circadian oscillations in innate immune cells. a, b Comparison of relative mRNA expression levels of clock genes between WT and KI/+ microglia (n = 4) (a) and BMDM (b) (n = 3). c Altered rhythmic expression pattern of the Nr1d1 gene at different circadian times (CT). Microglia were synchronized with dexamethasone (DEX, 100 nM) treatment for 2 h and harvested every 4 h for RNA extraction and qPCR (n = 4). mRNA levels were normalized to Actb. d, e Representative bioluminescence recording of rhythmic Per2::Luc expression in primary microglia (d) and BMDM (e) from Per2::Luc;Psen2+/+ or Per2::Luc;Psen2N141I/+ mice. Cells were cultured and exposed to DEX (100 nM) for 2 h, followed by measurement of luciferase bioluminescence (each measurement for 1 min with 10-min interval) for 5 days. Circadian period and amplitude were analysed in microglia (d) and BMDM (e) (n = 7). f Representative bioluminescence recordings of rhythmic expression in SCN slices derived from Per2::Luc;Psen2+/+ or Per2::Luc;Psen2N141I/+ mice. Circadian period and amplitude were analysed using representative recordings (n = 8). Data are mean ± SEM. #p < 0.05. ##p < 0.01, and ###p < 0.001 for the indicated comparisons.

Figure 4

Psen2 N141I mutation does not alter circadian behaviour in mice. a, b Representative actograms of locomotor activity of WT and KI/+ mice. Grey backgrounds indicate periods of constant darkness. Mice were subjected to 12-h light/dark cycles (LD, 14 days), as indicated by the empty and filled bars above the records, followed by constant darkness (DD, 14 days; grey background). c, d Analyses of the peaks in periodograms for each animal showing the average period (h) during constant darkness (23.9 ± 2.275 h in WT and 23.9 ± 0.212 h in KI/+; $n = 8$). Dark line, average of each animal; red line, significance at $p < 0.001$. e, f Representative actograms of body temperature in WT and KI/+ mice. g, h Analyses of the peaks in periodograms for each animal showing the average period (h) in body temperature change during constant darkness (23.9 ± 0.025 h in WT and 23.8 ± 0.160 h in KI/+; $n = 8$). Dark line, average of each animal; red line, significance at $p < 0.001$.

Figure 5

Psen2N141I/+ mice display exacerbated inflammation and memory deficit in response to a low dose (0.35 μg/kg) of LPS. **a** ELISA of the cytokines IL-6, CXCL1, CCL2, CCL5, and TNF-α in blood serum from WT and KI/+ mice 20 h after i.p. injection of LPS (n = 10). **b** Iba-1 staining in hippocampus of WT and KI/+ mice 20 h after i.p. injection of LPS. Insets, 2.5-fold enlarged images. Scale bar, 25 μm. **c** Representative 3D filament tracking images of Iba-1 signals made by IMARIS software. Scale bar, 20 μm. **d** Dendrite length, number of branching branch points, and Sholl radius analysis. Data extracted from FilamentTracker in IMARIS analysis (n > 30 per group). **e**, **f** Y-maze test with WT and KI/+ mice 20 h after LPS i.p. injection for assessment of spatial working memory (n = 13 or 14 per group). **g**, **h** T-maze test with WT and KI/+ mice 20 h after LPS i.p. injection for assessment of spatial learning and memory (n = 10 or 11 per group). Tracking images indicate mouse head (blue line), centre (red line), and tail (purple). Behaviour tests were analysed by two-way ANOVA and Bonferroni post-test. All data are mean ± SEM. n.s., not significant. †p < 0.05, ††p < 0.01 and †††p < 0.001 vs. untreated KI/+ control. #p < 0.05 and ###p < 0.001 for indicated comparisons.

Figure 6

Psen2 N141I mutation down-regulates REV-ERBa in innate immune cells. **a** Analyses of REV-ERBa protein levels by western blotting in microglia and BMDM ($n = 3$). **b** Primer target regions: proximal RORE of the *Il-6* promoter and κ B3 at a similar location in the *Tnf* promoter. Average fold enrichment of immunoprecipitated DNA fragments in microglia was analysed by qRT-PCR in ChIP assay. *Arntl* was used as a positive control; *Tnf* and *Tbp* were used as negative controls of promoter binding by REV-ERBa. Average fold enrichment was normalized to IgG of each gene ($n = 4$). **c** Relative REV-ERBa expression levels in microglia transduced with lentivirus expressing shCon or shNr1d1. **d, e** Secretion (**d**) and mRNA levels (**e**) of IL-6 and TNF- α in WT microglia transduced with shNr1d1 after LPS (1 μ g/mL) treatment for 12 h ($n = 3$). **f** Relative REV-ERBa expression levels in KI/+ microglia expressing human NR1D1. **g, h** Secretion (**g**) and mRNA levels (**h**) of IL-6 and TNF- α in NR1D1-overexpressing KI/+ microglia after LPS (1 μ g/mL) treatment for 12 h ($n = 3$). Signals on western blots were normalized to those of ACTB. The blots shown are representative of 3 experiments with similar results. mRNA levels were normalized to Actb. All data are mean $\pm$ SEM. n.s., not significant. * $p < 0.05$ vs. untreated WT control. † $p < 0.01$ vs. untreated KI/+ control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for the indicated comparisons.

Figure 7

DNA hypermethylation underlies the repression of REV-ERB α in *Psen2N141/+* microglia and BMDM. a-h Pharmacological modulation of DNA methylation rescues *Nr1d1* mRNA and protein levels. 5-Azacytidine (5-Aza, 10 μ M) treatment for 24 h restores *Nr1d1* transcript (a, e) ($n = 3$) and protein levels (b, f) ($n = 4$) in KI/+ microglia (a, b) and BMDM (e, f). c, d 5-Aza-induced demethylation reduces the mRNA level (c, g) and secretion (d, h) of IL-6 but not TNF- α ($n = 4$) in KI/+ microglia (c, d) and BMDM (g, h). Cells were pre-treated with 5-Aza for 12 h, and mRNA levels and secretion were analysed after co-treatment with LPS for 12 h. mRNA levels were normalized to Actb. i Methylation analysis of CpG islands in the *Nr1d1* promoter. Difference in the value of the sum of methylation rate (calculated as methylation depth / (methylation depth + non-methylation depth)) between KI/+ and WT at each methylated CpG site is shown as a graph with a blue bar for each position. j Comparison of overall DNA methylation ratios in the 2-kbp *Nr1d1* promoter region between KI/+ and WT microglia. All data are mean $\pm$ SEM. n.s., not significant. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ and $\dagger\dagger\dagger p < 0.001$ vs. untreated KI/+ control. $\# p < 0.05$, $\#\# p < 0.01$, and $\#\#\# p < 0.001$ for the indicated comparisons.

Figure 8

Chlorpromazine recovers REV-ERB α level and prevents hyper immune response and cognitive decline in *Psen2N141I/+* mice. a, b Chlorpromazine (CPZ, 0.5 μM in microglia and 1 μM in BMDM) treatment for 12 h reduces the secretion (a) and mRNA level (b) of IL-6. c, d CPZ treatment restores Nr1d1 transcript (n = 3) (c) and protein (n = 3) levels (d) in KI/+ microglia and BMDM. e Schematic illustration of the experimental schedule. f ELISA of IL-6, CXCL1, CCL2, CCL5, and TNF-α in blood serum from WT and KI/+ mice 20 h after i.p. injection of LPS (0.35 μg/kg). CPZ (0.25 mg/kg) was administered i.p. 4 h prior to LPS injection (n = 8). g, h Y-maze test with WT and KI/+ mice 20 h after LPS i.p. injection (0.35 μg/kg) for assessment of spatial working memory (n = 7–10 per group) with arm alternation (g) and number of arm entries (h). CPZ (0.25 mg/kg) was administered i.p. 4 h prior to LPS injection. Behaviour test results were analyzed by two-way ANOVA and Bonferroni post-test. All data are mean ± SEM. n.s., not significant. tp < 0.05, tp < 0.01 and tp < 0.001 vs. untreated KI/+ control. ##p < 0.01 and ###p < 0.001 for the indicated comparisons.

Figure 9

Schematic diagram suggesting hyperactive immune responses caused by the *Psen2* N141I mutation. The mutation induces excessive immune responses through the epigenetic suppression of REV-ERB α and reduction in the amplitude of the immune cell-intrinsic clock rhythm. An antipsychotic, CPZ, rescues REV-ERB α expression and prevents cognitive decline.

Supplementary Files

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