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The role of circadian rhythm proteins Rev-Erba and β in the development of neuronal injury after traumatic brain injury

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Background/aim: Circadian rhythm proteins (CRPs) play critical roles in both physiological and pathophysiological conditions, including neurodegenerative disorders. As members of CRPs, the nuclear receptors Rev-Erba/ β regulate circadian rhythm particularly by inhibiting Bmal1 protein and are involved in the neuroinflammation and cell death processes. However, their roles in the development of neuronal injury after traumatic brain injury (TBI) were largely unexplored, and so were investigated in the present study.

Materials and methods: For the induction of TBI, animals were subjected to the cryogenic model of TBI, which is a commonly used animal model and shares essential similarities with cerebral ischemia in terms of pathophysiological cascades. To assess the impact of Rev-Erb proteins on TBI, both Rev-Erba and Rev-Erb β proteins were activated or deactivated, and their expression profiles were determined by western blot analyses. Infarct volume and brain swelling were analyzed by cresyl violet staining. Blood–brain barrier (BBB) permeability was analyzed by immunoglobulin G extravasation. Neuronal survival was analyzed by NeuN immunohistochemistry.

Results: Our observations indicate that Rev-Erb β significantly reduced brain injury after TBI, which was reversed by inhibiting this protein. Not activation but the inhibition of both Rev-Erb proteins increased brain swelling significantly. In addition, both Rev-Erba and Rev-Erb β improved BBB permeability and neuronal survival significantly, which were reversed by their inhibitions.

Conclusion: Our results show that Rev-Erba and particularly Rev-Erb β play significant roles in the development of neuronal injury after TBI. Our findings suggest that Rev-Erb proteins would be a potential therapeutic target for the treatment of neurodegenerative diseases.

Key words: Circadian rhythm proteins, neuronal survival, Rev-Erb, traumatic brain injury

1. Introduction

Traumatic brain injury (TBI) is a significant global health problem, primarily affecting young adults and children and emerging as a leading cause of disability and death [1,2]. Worldwide, there are an estimated 939 cases of traumatic brain injury per 100,000 people, with around 5.48 million individuals experiencing severe TBI annually [3].

The pathophysiology of TBI is complex, starting with an immediate primary injury to the brain, followed by a series of secondary injuries that can persist for days [4]. The primary injury occurs at the moment of the initial trauma, causing irreversible tissue loss at the core of the lesion. It can be classified as either penetrating (open-head) or nonpenetrating (closed-head or blunt) based on whether the skull is ruptured. The secondary injury involves a cascade of biochemical, cellular, and molecular events

that contribute to further brain damage. Key mechanisms include neuroinflammation, blood–brain barrier (BBB) disruption, excitotoxicity, oxidative stress, cerebral edema, and apoptosis [5]. Understanding these processes is crucial for developing therapeutic interventions to reduce the impact of secondary injuries and improve patient outcomes.

Circadian rhythms, present in all life forms from microorganisms to humans, play a crucial role in aligning a variety of internal physiological processes with the daily cycles of the external environment. These intrinsic oscillations, observed in behaviors, physiological states, and metabolic activities, follow a roughly 24-h cycle, hence the term “circadian,” derived from the Latin “circa diem,” meaning “about a day.” These rhythms primarily regulate the human biological clock and influence various physiological functions [6–8].

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At the molecular level, circadian rhythm is regulated by a transcription–translation feedback loop involving several gene-encoded proteins. Core clock proteins are the brain and muscle Arnt-like protein 1 (ARNTL, also known as BMAL1) and circadian locomotor output cycles kaput (CLOCK) protein, which form a heterodimer that activates the transcription of period (PER) and cryptochrome circadian regulator (CRY) genes. The PER and CRY proteins accumulate in the cytoplasm and then translocate back into the nucleus to inhibit the activity of the BMAL1–CLOCK complex [9,10]. Rev-Erb plays a crucial role in the molecular clock mechanism by acting as a transcriptional repressor. It binds to specific DNA sequences called ROR elements (ROREs) to inhibit the expression of BMAL1 and CLOCK, thus contributing to the stability and precision of the circadian rhythm. By repressing these core clock genes, Rev-Erb helps fine-tune the feedback loops that drive the circadian cycles, integrating various physiological and metabolic processes [11,12].

Rev-Erba (Nr1d1) and Rev-Erb β (Nr1d2) are the main components of the nuclear receptor (NR) superfamily, playing crucial roles in regulating circadian rhythm, metabolic functions, and immune systems [13,14]. Pharmacological activation of Rev-Erb, for instance, has been shown to inhibit the NF- κ B signaling pathway, thereby reducing microglial activation and neuroinflammation [15]. Furthermore, Rev-Erb proteins have been implicated in regulating apoptosis and autophagy, as evidenced by their influence on Cyclin A2, ATG5, and BECN1 gene expression [16]. Notably, deficiency of the NR1D1 gene, encoding Rev-Erba, exacerbates oxidative stress by increasing reactive oxygen species (ROS) and reducing antioxidative genes, such as catalase (CAT), superoxide dismutase 1 (SOD1), and glutathione peroxidase 4 (GPX4) [17]. Nevertheless, within neurodegenerative diseases, elevated concentrations of Rev-Erb exhibit a protective role by diminishing mitochondrial dysfunction and excitotoxicity, which are characteristic manifestations of secondary damage in TBI [18].

In the literature, there are important studies showing the role of circadian rhythm and Rev-Erbs in neurodegenerative diseases [13,19–24]. However, there is a notable absence of investigations that specifically examine its function within the pathophysiology of TBI. Understanding the molecular mechanisms underlying the effects of Rev-Erb proteins on TBI pathophysiology may pave the way for novel therapeutic strategies, offering hope for improved outcomes in TBI patients. In the present study, we investigated the role of Rev-Erba and/or Rev-Erb β proteins either by inhibiting or activating their expression levels after TBI. For this aim, we constructed lentiviral vectors and treated animals with them. Three days after TBI, the expression profiles of Rev-Erba and

Rev-Erb β were identified by western blot analyses, brain injury and brain swelling were analyzed by cresyl violet staining, BBB permeability was evaluated by IgG extravasation, and neuronal survival was determined by neuronal nuclei (NeuN) staining.

2. Materials and methods

2.1. Ethics statement

The study was conducted in accordance with the ethics standards of the EU Guidelines on the Care and Use of Laboratory Animals (Directive 2010/63/EU) and in agreement with Türkiye's Animal Research Ethics Committee local guidelines and legislation. The study was approved by local government authorities (İstanbul Medipol University, Animal Research Ethics Committee; reference number: 03/02/2021-09). All animals were maintained under a constant 12-h light/dark cycle (lights on at 07:00 daily) with ad libitum access to food and water. The investigators remained unbiased throughout all experiments and data analysis stages, as they were blinded to the experimental groups.

2.2. Experimental groups

A total of 64 BALB/c mice (8–10 weeks old, weighing 20–25 g) were used. They were divided into 8 different groups to increase or decrease Rev-Erba/ β protein expression levels. The groups were as follows: (i) Lv-GFP (empty lentiviral vector), (ii) Lv-RevErba (for Rev-Erba overexpression), (iii) Lv-RevErb β (for Rev-Erb β overexpression), (iv) Lv-RevErba/ β (for both Rev-Erba and Rev-Erb β overexpression), scRNA (scrambled RNA), (vi) Sh-RevErba (for Rev-Erba inhibition), (vii) Sh-RevErb β (for Rev-Erb β inhibition), and (viii) Sh-RevErba/ β (for both Rev-Erba and Rev-Erb β inhibition). Since viral transfections require approximately 7–10 days to infect the target tissue [25], the mice were subjected to virus injection 10 days before the induction of TBI. Following the 10 days of virus injections, the cold-induced TBI model was performed on all mice. After TBI, the mice were monitored for 72 h. At the end of this period, they were deeply anesthetized and euthanized, and their brains were preserved by freezing with dry ice. Subsequently, the brains were sectioned into 18- μ m coronal slices using a cryostat (CM1850; Leica, Germany).

2.3. Cloning and preparation of lentivirus

A second-generation lentivirus packaging system was used for efficient vector production. High amounts of viral particles were produced using this system to increase or decrease the levels of Rev-Erba and Rev-Erb β . For increasing Rev-Erba/ β protein expression, a vector containing GFP protein expression (Lenti-Ef1-GFP-2A-Puro Vector ABM) was used. To inhibit the expression of Rev-Erba/ β commercially available plasmids from

Dharmacon (Nr1d1 mEF1a-TurboRFP shRNA glycerol set and Nr1d2 mEF1a-TurboRFP shRNA glycerol set) were used.

Briefly, the coding sequence of human Rev-Erba (NCBI Reference Sequence: NM_021724.5) and Rev-Erb β (NCBI Reference Sequence: NM_005126.5) were amplified using appropriate primers (Rev-Erba forward: 5'- AGT CAG TCG ACA TGA CGA CCC TGG ACT CCA AC -3', Rev-Erba reverse: 5'- AGT CAG GAT CCT CAC TGG GCG TCC ACC CG -3', Rev-Erb β forward: 5'- AGT CAG TCG ACA TGG AGG TGA ATG CAG GAG G -3', and Rev-Erb β reverse: 5'- AGT CAT CTA GAT TAA GGG TGA ACT TTA AAG GCC A -3'). The PCR products and the Lenti-Ef1-GFP-2A-Puro vector were treated with restriction enzymes for Nr1D1, SalI (FD0644 Thermo Fisher Scientific), BamHI (FD0054 Thermo Fisher Scientific), Nr1D2, SalI (FD0644 Thermo Fisher Scientific), and XbaI (FD0684 Thermo Fisher Scientific). Next, they were ligated using T4 DNA ligase (EL0014, Thermo Fisher Scientific). After that, the insert was confirmed by sequencing. pMD2.G and psPAX plasmids, which were kindly provided by Dr Didier Trono (Ecole Polytechnique Federale, Lausanne, Switzerland), were used as complementary vectors for packaging of the lentiviral system (12259; 12260, Addgene, UK). ScRNA and inhibition vectors for Rev-Erba and Rev-Erb β were purchased from Dharmacon.

Seven million HEK293T cells were seeded in 10-cm petri dishes for virus production. The next day, the cells were transfected with 7 μ g of expression plasmids (Lv-GFP, Lv-RevErba, Lv-RevErb β , Lv-RevErba/ β , scRNA, Sh-RevErba, Sh-RevErb β , or Sh-RevErba/ β), 7 μ g of psPAX, and 3.5 μ g of pMD2.G using Lipofectamine 3000 (L30000015, Thermo Fisher Scientific). Six hours posttransfection, the culture medium was replaced with fresh DMEM. The medium was harvested 24 and 52 h later, centrifuged at 2000 rpm at 4 °C for 10 min, and filtered through a 0.45- μ m filter. After ultracentrifugation at 100,000 $\times$ g for 2 h, virus pellets were resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS; P04-3650, Pan Biotech) without calcium and magnesium. The virus aliquots were stored at -80 °C. Virus titration was calculated as described before [25].

2.4. Administration of lentiviral vectors

The mice were anesthetized with 2% isoflurane (30% O₂, remainder N₂O) and placed in a stereotaxic frame (Stoelting, Illinois, USA). The animals' skulls were drilled and Lv-GFP, Lv-RevErba, Lv-RevErb β , Lv-RevErba/ β , scRNA, Sh-RevErba, Sh-RevErb β , or Sh-RevErba/ β vectors (10⁸ lentivirus particles in 2 μ L of 0.1 M PBS) were administered into the left hemisphere (ipsilesional) (injection coordinates: bregma 1.0 mm posterior, 1.5 mm lateral, and 0.5 mm deep) via a micro syringe pump (Micro 4; World Precision Instrument, Florida, USA).

2.5. Cryogenic model of TBI

To perform brain injury, the animals underwent cold-induced TBI, a commonly used model that mimics essential pathophysiological aspects of trauma [26]. In brief, the mice were anesthetized with 1.5% isoflurane (30% O₂, remainder N₂O) (AWN-340150-01, Adeka, Turkey), and their rectal temperature was maintained between 36.5 and 37 °C using a feedback-controlled heating system (K 023152, Harvard, UK). The mice were placed in a stereotaxic device (WPI Instruments, USA) with their heads secured using ear bars and a tooth bar. The skull landmarks were carefully aligned and leveled. After sterilizing the scalp with 70% ethanol, a small incision was made, and the connective tissue was removed. A copper probe cooled with liquid nitrogen (tip diameter 1.5 mm) was applied to the skull (1.0 mm posterior and 1.5 mm lateral to bregma). The copper probe was cooled for 3 min in liquid nitrogen before each application to ensure consistent injury. The probe was then placed on the skull for 60 s, after which it was carefully removed. The wounds were sutured after 1 min, and the mice were returned to their home cages. At 72 h post-TBI, the animals were sacrificed using high-dose anesthesia (4% isoflurane, 30% O₂, N₂O). Eighteen micrometer coronal cryosections were used to analyze brain injury, brain swelling, serum IgG extravasation, and neuronal survival.

2.6. Cresyl violet staining

Coronal brain sections were obtained at equidistant intervals of 1 mm. Then sections were stained with cresyl violet (C5042; Sigma Aldrich) according to the previously published protocol [20]. Within the sections, the border between injured (I) and noninjured (NI) tissues was delineated using image analysis software (ImageJ; National Institute of Health, Maryland, USA). The infarct area was quantified by subtracting the area of the ipsilesional hemisphere noninfarcted (I_{NI}) tissue from that of the contralesional hemisphere (C_H). Infarct volume was calculated by the (C_N - I_{NI}) $\times$ distance between injured areas. Brain swelling was determined as the volume difference between the ipsilesional hemisphere (I_H) and the C_H and expressed in cubic millimeters (mm³).

2.7. Blood-brain barrier permeability analysis

With gentle stirring, coronal brain sections were rinsed for 10 min at room temperature in 0.1 M phosphate-buffered saline (PBS) to remove intravascular IgG and were fixed in 4% paraformaldehyde (PFA) [27]. Following the blocking of endogenous peroxidase with methanol/0.3% H₂O₂ and immersion in 0.1 M PBS containing 5% bovine serum albumin (BSA) and normal swine serum (1:1000), sections were incubated for 1 h in biotinylated horse anti-mouse IgG (BA-1300-2.2, Vectastain Elite; Vector Labs, California, USA) and stained with an avidin peroxidase kit (PK-7800; Vectastain Elite; Vector Labs) and DAB

Substrate Kit (SK-4100; Vector Labs). For reasons of data comparability, all sections were processed in parallel. Sections were scanned, and the integrated density of the ipsilesional and contralesional hemispheres was quantified using the NIH ImageJ software. Afterward, density was compared between the ipsilesional tissue and the contralesional tissue to determine BBB leakage.

2.8. Neuronal survival analysis

For the evaluation of neuronal survival, coronal brain sections were fixed with 4% paraformaldehyde (PFA)/0.1 M PBS and incubated at room temperature for 1 h with 0.1 mol/L PBS including 0.3% Triton X-100 (PBS-T)/10% normal goat serum (NGS). Then sections were incubated overnight at 4 °C with Alexa Fluor 488-conjugated monoclonal mouse anti-NeuN (Mab377X; Chemicon). The next day, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and were analyzed using a confocal microscope (LSM800, Carl Zeiss, Jena, Germany) [28]. NeuN-positive cells were counted in 62,500 μm^2 cortical areas to determine the neuronal survival rate.

2.9. Western blot

Mice cortical tissue from TBI subjects was homogenized in RIPA buffer (R0278-50ML, Sigma Aldrich) supplemented with a 1:100 protease/phosphatase inhibitor cocktail (5782, Cell Signaling) and incubated on ice for 15 min. Lysates were centrifuged (14,000 rpm, 15 min, 4 °C), and the supernatant was collected for protein quantification. Protein concentration was determined via Qubit 3.0 Fluorometer (Q33216, Invitrogen) using the Qubit protein assay kit (Q33211, Invitrogen) with 1:50 sample dilution. For western blotting, 20 μg of protein lysate was mixed with 2X Laemmli buffer (1610737, Bio-Rad) and separated on Mini-PROTEAN TGX Precast gels (4569033, Bio-Rad) using 10X Tris/Glycine/SDS running buffer (1610732, Bio-Rad). Proteins were transferred to PVDF membranes (162-0174, Bio-Rad) using a Trans-Blot Turbo System (1704155 Bio-Rad). Membranes were blocked (5% skim milk in TBS-T, 1 h, RT) and incubated overnight with primary antibodies against Rev-Erba (13418, Cell Signaling Technology), Rev-Erb β (398252, Santa Cruz Biotechnology), and β -Actin (4970, Cell Signaling Technology). HRP-conjugated goat anti-rabbit (31460, Thermo Fisher Scientific) or goat anti-mouse (31430, Thermo Fisher Scientific) secondary antibodies were used before visualization with a WesternBright ECL kit (K-12045-D20, Advansta) and Chemidoc MP imaging (1708280, Bio-Rad). Protein expression was quantified relative to β -Actin abundance.

2.10. Statistics

Statistical analysis was performed using SPSS (version 15, SPSS Inc., Chicago, USA). Data for the western blot

experiments were evaluated using the independent samples t-test to compare means between two groups. Data for infarct volume, brain swelling, BBB leakage, and neuronal survival were evaluated using one-way ANOVA to compare means across multiple groups. One-way ANOVA was chosen because it is suitable for comparing the means of three or more independent groups, and our data met the assumptions of normality and homogeneity of variances, as confirmed by the Shapiro–Wilk and Levene's tests, respectively. Data are presented as mean $\pm$ SD values. Exact p-values and effect sizes (partial eta squared) are provided to enhance transparency and replicability. Throughout the study, p-values <0.05 were considered statistically significant.

3. Results

3.1. Expression profiles of Rev-Erba and β after lentiviral vector application

To investigate the contribution of Rev-Erba/ β to TBI outcomes, we developed specific lentiviral vectors aimed at modulating the expression of these proteins within the mouse cortex. Using western blot analysis, we quantified the expression of Rev-Erba and β and proteins in cortical tissue samples after intracerebral injection of lentiviral vectors. This method allowed us to confirm the vectors' capability to effectively upregulate or downregulate the target proteins, providing a foundation for their subsequent application in examining the roles of Rev-Erba/ β and in the context of TBI (Figure 1). As compared with Lv-GFP control, Lv-RevErba and Lv-RevErb β treatment increased the level of Rev-Erba and Rev-Erb β proteins significantly ($p < 0.01$; Figures 1A and 1B). In contrast, Sh-RevErba and Sh-RevErb β treatments decreased these proteins significantly compared to scRNA control ($p < 0.01$; Figures 1C and 1D).

3.2. Activation or inhibition of Rev-Erba and β proteins plays a significant role in brain protection after TBI

To investigate the potential protective effects of Rev-Erba and β overexpression or inhibition on brain injury, mice were exposed to the TBI and 72 h later brain injury, swelling/edema, and BBB permeability were evaluated. Following cold-induced TBI, lesion volumes were as follows: $55.53 \pm 16.74 \text{ mm}^3$ for the Lv-GFP group, $52.52 \pm 14.87 \text{ mm}^3$ for the Lv-RevErba group, $31.99 \pm 16.44 \text{ mm}^3$ for the Lv-RevErb β group, and $33.91 \pm 8.71 \text{ mm}^3$ for the Lv-RevErba/ β group (Figure 2A). The Lv-RevErb β group showed a significant reduction in lesion volume compared to the Lv-GFP group ($p < 0.01$). Similarly, the Lv-RevErba/ β group exhibited a significantly smaller lesion volume compared to the Lv-GFP group ($p < 0.05$). However, the Lv-RevErba group did not show a statistically significant difference in lesion volume compared to the Lv-GFP group. The eta squared (η^2) calculated for the groups

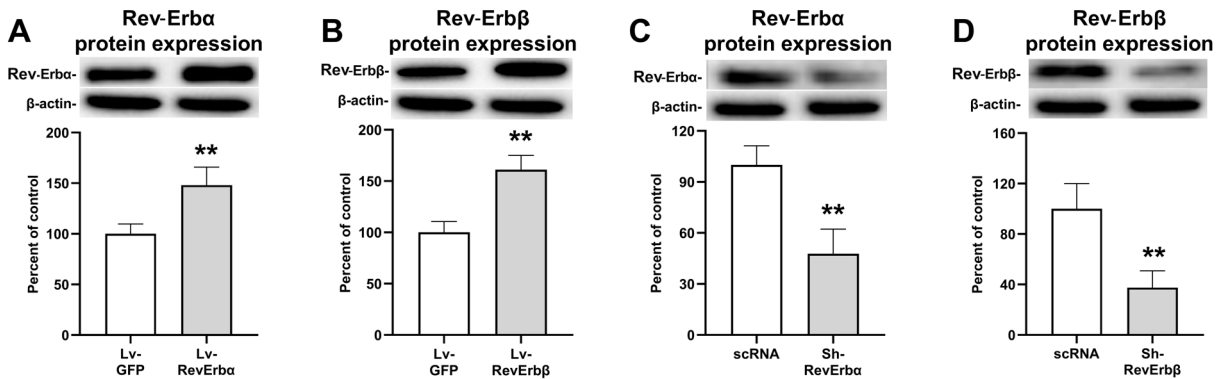


Figure 1. Lentiviral vectors increase or decrease of Rev-Erb proteins. Western blot analysis of overexpression of Rev-Erba (A) and Rev-Erb β (B) and inhibition of Rev-Erba (C) and Rev-Erb β (D). Representative images of western blot analysis from three independent experiments are given above their corresponding graphs. Data are represented as mean $\pm$ SD values of three independent experiments. ** $p < 0.01$ compared with the vehicle (Lv-GFP for overexpression, scRNA for inhibition groups) treated group.

was 0.347. The effect size (Cohen's f) was 0.729, suggesting a large effect size according to Cohen's conventions.

For the inhibition groups (scRNA, Sh-RevErba, Sh-RevErb β , and Sh-RevErba/ β) as shown in Figure 2B, the lesion volumes were as follows: $23.62 \pm 9.17 \text{ mm}^3$ for the scRNA group, $26.63 \pm 11.72 \text{ mm}^3$ for the Sh-RevErba group, $49.06 \pm 14.12 \text{ mm}^3$ for the Sh-RevErb β group, and $40.61 \pm 18.79 \text{ mm}^3$ for the Sh-RevErba/ β group. The Sh-RevErb β group showed a significant increase in lesion volume compared to the scRNA group ($p < 0.01$). Similarly, the Sh-RevErba/ β group exhibited a significantly larger lesion volume compared to the scRNA group ($p < 0.05$). However, the Sh-RevErba group did not show a statistically significant difference in lesion volume compared to the scRNA group. The η^2 calculated for the groups was 0.356. The Cohen's f was 0.744, suggesting a large effect size according to Cohen's conventions. These results demonstrate the significant impact of Rev-Erb β knockdown on increasing lesion volume following TBI.

The analysis of brain edema following TBI in the groups with protein overexpression (Lv-GFP, Lv-RevErba, Lv-RevErb β , and Lv-RevErba/ β) revealed the following mean $\pm$ standard deviation values: Lv-GFP $4.0 \pm 1.7 \text{ mm}^3$, Lv-RevErba $4.1 \pm 2.4 \text{ mm}^3$, Lv-RevErb β $2.5 \pm 0.8 \text{ mm}^3$, and Lv-RevErba/ β $3.4 \pm 2.7 \text{ mm}^3$ (Figure 2C). The η^2 calculated was 0.089. The Cohen's f was 0.313, suggesting a small to moderate effect size. These results demonstrate that the Lv-RevErb β group had a lower brain edema volume compared to the other groups, although the differences were not statistically significant. In the groups with protein knockdown (scRNA, Sh-RevErba, Sh-RevErb β , and Sh-RevErba/ β), the brain edema volumes were as follows: scRNA $1.8 \pm 1.7 \text{ mm}^3$, Sh-RevErba $1.3 \pm 1.2 \text{ mm}^3$, Sh-RevErb β $1.9 \pm 0.5 \text{ mm}^3$, and Sh-RevErba/ β

$3.7 \pm 1.8 \text{ mm}^3$ (Figure 2D). The η^2 for this analysis was 0.297, indicating that 29.7% of the variance in brain edema was due to group differences. The Cohen's f calculated was 0.650.

3.3. The role of Rev-Erba and β in BBB permeability after TBI

The analysis of BBB permeability in the groups with protein overexpression showed the following mean $\pm$ standard deviation values: Lv-GFP $31.65 \pm 9.99\%$, Lv-RevErba $15.91 \pm 6.17\%$, Lv-RevErb β $19.58 \pm 5.13\%$, and Lv-RevErba/ β $21.98 \pm 7.63\%$ (Figure 2E). The η^2 for BBB permeability was 0.379, suggesting that 37.9% of the variance in permeability can be attributed to the differences between groups. The Cohen's f was 0.781, indicating a large effect size. These results highlight significant reductions in BBB permeability in the Lv-RevErba and Lv-RevErb β groups compared to the Lv-GFP group, while the Lv-RevErba/ β group did not show a significant change. In the groups with protein knockdown, the BBB permeability values were as follows: scRNA $34.38 \pm 11.51\%$, Sh-RevErba $20.26 \pm 9.98\%$, Sh-RevErb β $40.26 \pm 9.98\%$, and Sh-RevErba/ β $52.98 \pm 11.89\%$ (Figure 2F). The η^2 for this analysis was 0.539, indicating that 53.9% of the variance in permeability was due to group differences. The Cohen's f was 1.081, suggesting a very large effect size. These findings demonstrate a significant increase in BBB permeability in the Sh-RevErb β and Sh-RevErba/ β groups compared to the scRNA group, with the Sh-RevErba group showing no significant change.

3.4. The role of Rev-Erba and β in the neuronal survival after TBI

To assess the impact of Rev-Erba and β modulation on neuronal survival after TBI, brain sections were stained

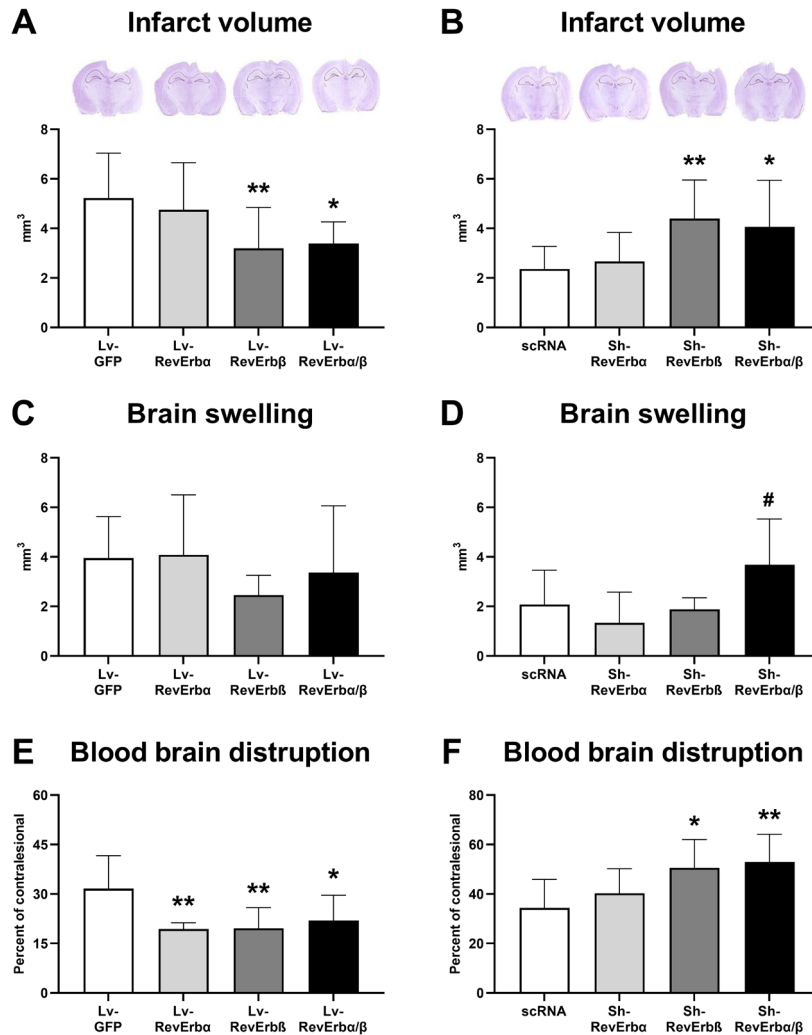


Figure 2. Effects of Rev-Erb proteins on brain infarct volume, swelling, and BBB permeability. Cresyl violet staining was performed to determine brain infarction (A, B) and brain swelling (C, D). To analyze BBB disruption, serum IgG extravasation was performed (E F). Data are represented as mean $\pm$ SD values. ** $p < 0.01$ /* $p < 0.05$ compared with the vehicle (Lv-GFP) for overexpression, scRNA for inhibition groups) treated group. Scale bar, 1000 μ m.

with NeuN. The stained sections were analyzed using a confocal microscope (LSM760, Zeiss). The results were as follows: Lv-GFP $38.83 \pm 7.9\%$, Lv-RevErba $52.93 \pm 10.27\%$, Lv-RevErb β $50.93 \pm 12.02\%$, and Lv-RevErba/ β $58.60 \pm 9.61\%$ (Figure 3A). The η^2 was 0.339, indicating that approximately 33.9% of the variance in neuronal survival can be attributed to the differences between groups. The Cohen's f was 0.716, suggesting a large effect size. These results demonstrate that the Lv-RevErba/ β group had a significantly higher neuronal survival rate compared to the Lv-GFP group. For the inhibition groups, the results were as follows: scRNA $49.37 \pm 9.84\%$, Sh-RevErba $35.83 \pm 4.48\%$, Sh-RevErb β $28.60 \pm 6.42\%$, and Sh-RevErba/ β

$34.43 \pm 7.84\%$ (Figure 3B). The η^2 for this analysis was 0.513, indicating that 51.3% of the variance in neuronal survival was due to group differences. The Cohen's f was 1.027, suggesting a very large effect size. These findings indicate that the Sh-RevErb β group exhibited a significantly lower neuronal survival rate compared to the scRNA group, with the Sh-RevErba/ β group also showing a significant reduction.

4. Discussion

The endogenous circadian clock consists of self-sustained molecular clockwork that includes the transcriptional activators Bmal1, CLOCK, and transcriptional repressors

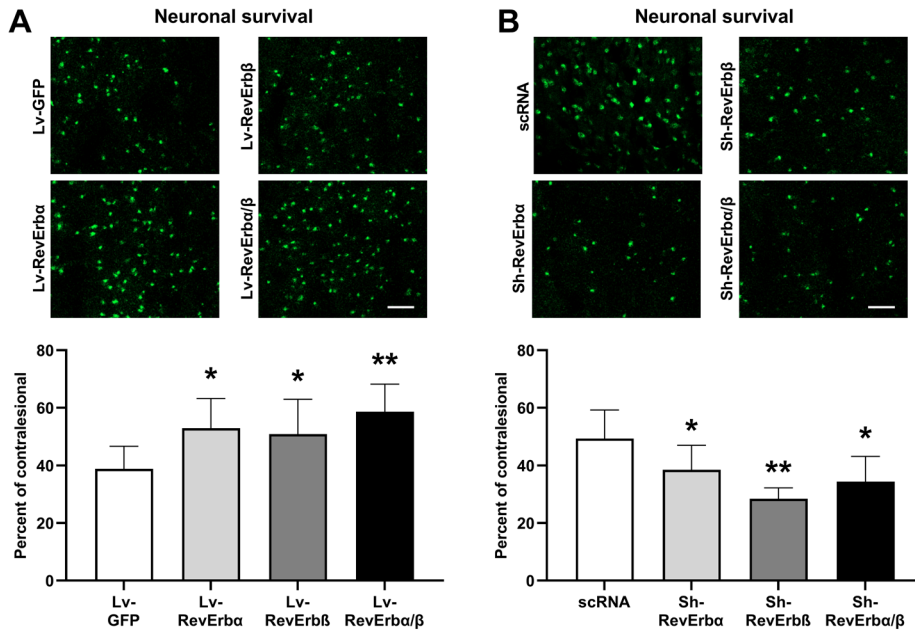


Figure 3. The regulation of Rev-Erb proteins plays an essential role in developing neuronal injury, which was evaluated by NeuN staining after TBI. The effect of overexpression of Rev-Erb (A) and inhibition of Rev-Erb (B) on neuronal survival was calculated as a ratio to the contralateral cortex. Data are represented as mean $\pm$ SD values. ** $p < 0.01$ /* $p < 0.05$ compared with the vehicle (Lv-GFP for overexpression, scRNA for inhibition groups) treated group. Scale bar, 100 μ m.

Cry1, Cry2, Per1, and Per2 [21]. Furthermore, Rev-Erb α/β inhibits the BMAL1 protein by binding to its promoter region, functioning as part of the negative feedback loop within the circadian clock machinery [11]. Although the role of Rev-Erb in the regulation of circadian rhythm is known, the number of studies showing its role in various pathophysiological processes, such as TBI, is limited. In the current study, we increased protein expression of Rev-Erb α , Rev-Erb β , or both (Rev-Erb α/β) by lentivirus to examine the effects of Rev-Erbs on injury processes after TBI. After lentivirus injection, it takes approximately 7–10 days for the viruses to infect the brain region [25]. Therefore, in the present study, intracerebral lentivirus injections were performed 10 days before TBI. Ten days later, a cold-induced TBI model was performed, which is commonly often used to mimic brain injury seen in the clinic in experimental animals [26].

Posttraumatic brain infarction represents a critical secondary insult following TBI. Secondary brain injury processes following TBI, including neuroinflammation, oxidative stress, excitotoxicity, neurodegeneration, BBB disruption, and peripheral immune system activation, are crucial for patient prognosis and the identification of therapeutic targets. Neuroinflammation, especially prominent in the acute phase after injury, frequently leads to necrotic cell death, BBB dysfunction, and subsequent

cerebral edema. Our results demonstrated that Rev-Erb β and Rev-Erb α/β play crucial roles in the development of brain infarct volume following TBI. Notably, another important finding is that inhibition of Rev-Erb α/β significantly reduced brain swelling, highlighting its potential as a therapeutic target. It was observed that neither Rev-Erb α nor Rev-Erb β alone was effective in mitigating brain injury outcomes; however, their combined action exhibited a synergistic effect, suggesting a complex interplay between these proteins in neuroprotection.

It is well known that TBI disrupts the integrity of the BBB, leading to increased permeability and leakage of blood-derived molecules into the brain parenchyma. This compromise of the BBB exacerbates neuroinflammation and contributes to secondary brain damage following the initial injury [29]. We performed IgG staining to determine BBB leakage. It was revealed that nuclear receptors Rev-Erb α and Rev-Erb β have a direct effect on BBB damage after TBI. Increasing Rev-Erb proteins expression separately or together was shown to reduce BBB damage. In contrast, inhibition of the expression of these proteins increased BBB damage.

Rev-Erb has been identified as a significant modulator of neuroinflammation. Inhibiting Rev-Erb increases microglial activation and neuroinflammation, aggravating neuronal damage [30]. Conversely, activating Rev-Erb α/β

with pharmacological agents has been shown to reduce LPS-induced microglial activation by blocking the NF- κ B pathway, thereby decreasing the production of inflammatory mediators and neuronal cytotoxicity [31]. Furthermore, Rev-Erb α/β plays a vital role in controlling peripheral inflammation, notably through its regulatory effects on NLRP3 expression and cytokine release from macrophages, critical components of TBI pathophysiology [32]. Rev-Erb's suppression of TH17 proinflammatory responses and its influence on TLR4-mediated inflammation underscore its therapeutic potential in reducing TBI severity [33]. It is well known that circadian rhythm proteins play an essential role in disseminated neuronal injury after pathophysiological conditions such as cerebral ischemia [19–21]. In the current study, we demonstrated that Rev-Erb proteins play an essential role in the development of neuronal injury in TBI pathophysiology. Inhibition of Rev-Erb proteins increases neuronal damage, while increasing the expression of these proteins decreases neuronal damage after TBI.

Despite the promising findings of the present study, there are several limitations that need to be addressed. First, the use of a cold-induced TBI model, while common in experimental settings, may not fully replicate the complex pathophysiology of TBI seen in clinical scenarios. Second, while the overexpression and inhibition of Rev-Erb proteins were achieved using lentiviral vectors, the efficiency and specificity of these genetic modifications may vary, potentially influencing the observed outcomes. The findings of the current study suggest that targeting Rev-Erb α and Rev-Erb β could be promising therapeutic strategies for TBI. Future research should aim to confirm these findings using additional TBI models and explore alternative methods for modulating Rev-Erb protein levels to ensure the robustness and translational potential of the results. Additionally, conducting long-term clinical trials,

investigating the molecular mechanisms underlying these effects, exploring combination therapies, and developing gene therapy approaches to modulate these receptors in the brain could further validate their therapeutic potential. These efforts could pave the way for novel interventions to improve outcomes for TBI patients.

In conclusion, our findings demonstrate the critical roles of Rev-Erb proteins in modulating brain edema, BBB permeability, and neuronal survival following cold TBI. Overexpression of Rev-Erb proteins, particularly Rev-Erb β , appears to confer protective effects, reducing brain edema, enhancing BBB integrity, and promoting neuronal survival. Conversely, the knockdown of these proteins exacerbates brain edema, disrupts BBB integrity, and decreases neuronal survival. These findings highlight the therapeutic potential of targeting Rev-Erb proteins in the treatment of TBI. Future research should focus on elucidating the molecular mechanisms underlying these effects and exploring the clinical applicability of Rev-Erb modulation in TBI therapy.

Acknowledgment/disclaimers/conflict of interest

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Informed consent

This study has been conducted under the ethics standards of the EU Guidelines on the Care and Use of Laboratory Animals (Directive 2010/63/EU) in agreement with local guidelines and legislation. The study has been approved by the Animal Research Ethics Committee of İstanbul Medipol University (Approval number: 03/02/2021-09).

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