Neuroendocrinology

Research Article

Neuroendocrinology 2024;114:315–330
DOI: 10.1159/000535748

Received: August 11, 2023
Accepted: December 4, 2023
Published online: December 9, 2023

Alterations in Serum miR-126-3p Levels over Time: A Marker of Pituitary Insufficiency following Head Trauma

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Keywords
Traumatic brain injury · miR-126-3p · Pituitary deficiency · Hypothalamus-pituitary-adrenal axis · Mouse model

Abstract
Introduction: Traumatic brain injuries (TBIs) pose a high risk of pituitary insufficiency development in patients. We have previously reported alterations in miR-126-3p levels in sera from patients with TBI-induced pituitary deficiency. ***Methods:*** To investigate why TBI-induced pituitary deficiency develops only in some patients and to reveal the relationship between miR-126-3p with hormone axes, we used mice that were epigenetically modified with miR-126-3p at the embryonic stage. These modified mice were subjected to mild TBI (mTBI) according to the Marmarou's weight-drop model at 2 months of age. The levels of miR-126-3p were assessed at 1 and 30 days in serum after mTBI. Changes in miR-126-3p levels after mTBI of wild-type and miR-126-3p* modified mouse lines validated our human results. Additionally, hypothalamus, pituitary, and adrenal tissues were analyzed for transcripts and associated serum hormone levels. ***Results:*** We report that miR-126-3p directly affects hypothalamus-pituitary-adrenal (HPA) axis upregulation and ACTH secretion in the acute phase after mTBI. We also demonstrated that miR-126-3p suppresses *Gnrh* transcripts in the hypothalamus and pituitary, but this is not reflected in serum FSH/LH levels. The increase in ACTH levels in the acute phase may indicate that upregulation of miR-126-3p at the embryonic stage has a protective effect on the HPA axis after TBI. Notably, the most prominent transcriptional response is found in the adrenals, highlighting their role in the pathophysiology of TBI. ***Conclusion:*** Our study revealed the role of miR-126-3p in TBI and pituitary deficiency developing after TBI, and the obtained data will significantly contribute to

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elucidating the mechanism of pituitary deficiency development after TBI and development of new diagnostic and treatment strategies.

Introduction

Traumatic brain injury (TBI) is a public health problem worldwide and may cause increased morbidity, mortality, and pituitary dysfunction [1]. In cases of TBI, brain function is temporarily or permanently impaired and may worsen over time due to secondary injury [2]. The neuro-inflammatory response and pituitary dysfunction may be related to the secondary injury, which could also occur due to a genetic disposition [3, 4]. It is crucial to prevent secondary damage following TBI to avoid the development of TBI-related conditions such as pituitary dysfunction or neuropsychiatric diseases [5–9].

TBI-induced hypopituitarism has been reported in up to 20–50% of TBI patients, and pituitary hormonal deficits have been detected even after mild TBI (mTBI) [5]. The mechanisms responsible for long-term hypopituitarism following head trauma are not fully understood. Recent prospective clinical studies have demonstrated that pituitary function improves over time in many patients, but in rare cases, it may worsen within 5 years of injury [3, 9–11]. The possible mechanisms underlying the recovery or worsening of pituitary function remain to be discovered and cannot be explained simply by direct injury to the pituitary gland and/or vascular injury [5].

In a previous study, we analyzed the expression profiles of 740 microRNAs (miRNAs) in the sera of TBI patients with and without hypopituitarism at different time points, including 1, 7, 28 days, and 5 years after the injury. We found that alterations in the expression of miR-126-3p and miR-3610 were indicative of the development of pituitary deficiency during the follow-up period of TBI patients compared to the control group [12].

miRNAs are noncoding single-stranded RNA molecules that bind to specific messenger RNAs (mRNAs) and hinder the translation process. miR-126, located within the intron of the epidermal growth factor-like domain 7 (EGFL7) locus on chromosome-9, generates two forms of mature miRNA, namely, miR-126-3p and miR-126-5p. Recent studies show that miR-126 plays an essential role in maintaining vascular integrity and endothelial barrier [13].

In our previous study, we suggested that changes in miR-126-3p expression levels in the blood of patients after TBI can be significantly related to the risk of pituitary dysfunction. Specifically, patients who have elevated levels of miR-126-3p during the chronic 5-year period following TBI are likely to suffer from pituitary insufficiency. These results suggest a relationship between exposure to head trauma and alteration of miR-126-3p [12]. To discover why TBI-induced pituitary deficiency develops only in some patients and reveal the relationship between miR-126-3p with hormonal axes, we used mice epigenetically modified with miR-126-3p at the embryonic stage (zygote) in this study. After that, when the mice were 2 months old, we determined the transcripts linked to hypothalamus-pituitary-adrenal (HPA), hypothalamus-pituitary-gonadal (HPG), and growth hormone-insulin-like growth factor-1 (GH-IGF-1) axes on hypothalamus, pituitary, adrenal tissue, and serum hormone levels before and after mTBI.

The present study investigates the role of miR-126-3p in the mTBI stress response in mice. More specifically, we explore the protective effects of this differential response to stress against the development of pituitary deficiency. Our findings reveal that experimental induction of miR-126-3p upregulation during embryonic development results in altered stress response to mTBI in mice. Notably, we observed that modulation of miR-126-3p expression during the embryonic stage had a significant impact on the levels of miR-126 transcripts in serum as well as on several hormonal profiles and transcript levels in adult tissues. Our study highlights the crucial role of miR-126-3p in modulating the stress response to mTBI and suggests that alterations in miR-126-3p expression levels during embryonic development may have long-lasting effects on the adult brain and metabolism.

Our results provide insight into the dynamic changes in these signaling pathways following mTBI, which may have implications for the regulation of stress, reproductive function, and growth. This study sheds light on the complex interplay between different axes in response to mTBI and contributes to a better understanding of the underlying mechanisms of mTBI-induced pathophysiology.

Materials and Methods

Animals

This study aimed to investigate the role of miR-126-3p in the regulation of the HPA, HPG, and GH-IGF-1 axes before and after TBI in male and female epigenetically modified (miR-126-3p microinjection in the zygotes) and unmodified 8-week-old *Balb/c* mice (n = 48, with equal sex distribution). Six experimental groups were formed, with 4 male and 4 female mice in each group (see the experimental design in Fig. 1). The animals were housed in accordance

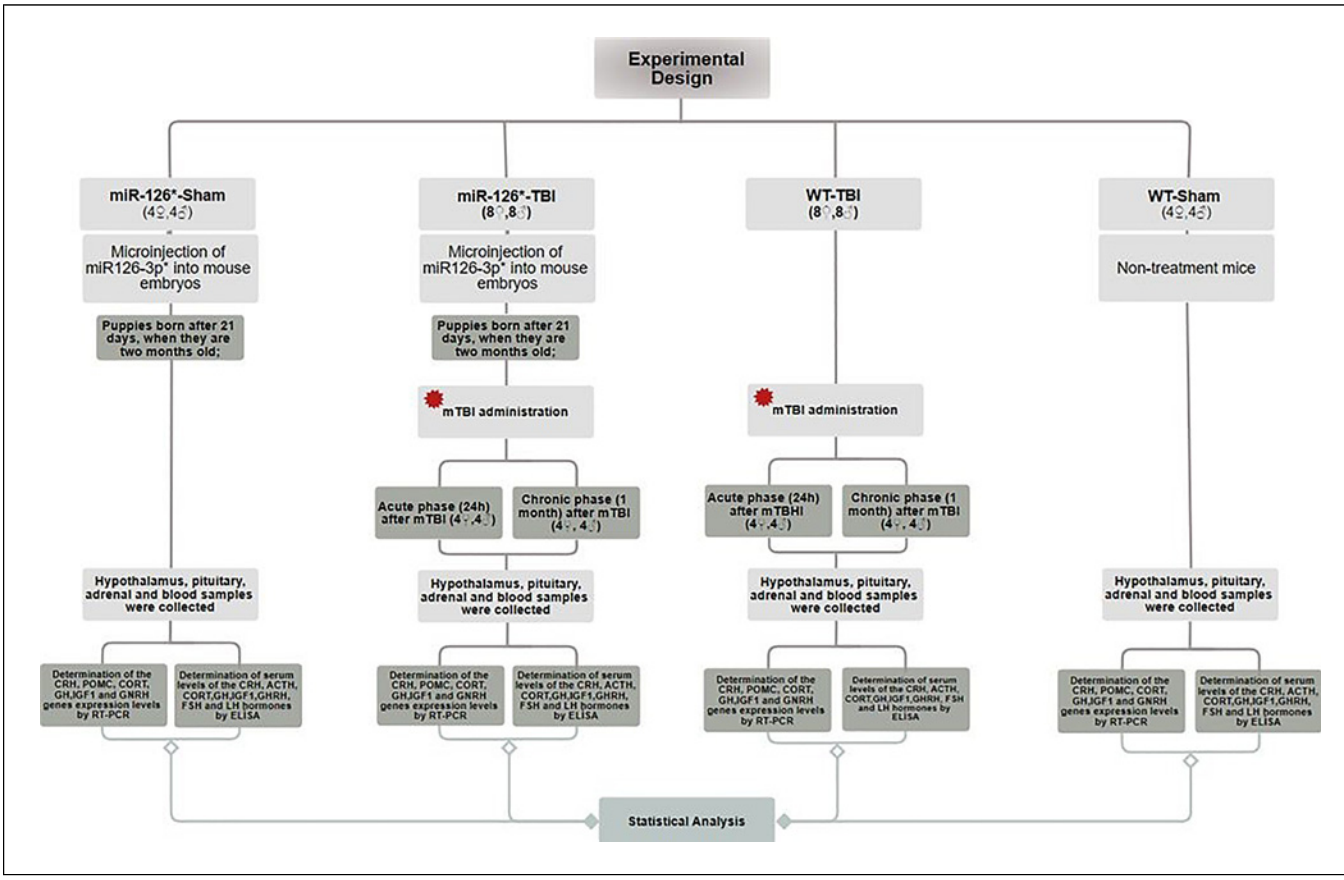


Fig. 1. Experimental design.

with the Principles of Laboratory Animal Care (European rules). The mice were maintained in the Transgenic Animals Department of Erciyes University's Genome and Stem Cell Center in Kayseri, Turkey, under controlled conditions (lights 06:00–18:00, temperature 23°C, and humidity 55%) and provided with food and water ad libitum. Our study design ensured a homogeneous group of animals with consistent housing conditions and minimized the impact of extraneous variables. The mice were the same age, housed in the same facility, and from the same breeding room and cage. Female mice were housed together from birth, which is known to lead to hormonal synchronization, to minimize the impact of hormonal fluctuations such as the estrus cycle on the results [14–16].

Transferring Mimic-miR-126-3p into Mouse Embryos by Microinjection

The procedure for generating epigenetically modified mice by microinjection of miR-126-3p in the mouse embryos (zygote) is given in Figure 2. For microinjection, we used two groups of mice. The first group comprised healthy female mice that mated with the healthy males from which embryos were collected. The second group consisted of healthy females (carrier mothers) that mated with vasectomized males with surgically cut vas deferens ducts. At this stage, we obtained fertilized embryos from the first group of mice and transferred the modified embryos to the carrier mice in the second group that were conditioned for pregnancy. The microinjection of miR-126-3p into fertilized eggs was performed according to established transgenesis methods [17]. The RNA solutions were adjusted to a concentration of 1 μg/mL, and 1–2 pL were microinjected into the pronuclei of *Balb/c* fertilized eggs isolated after normal ovulation and mating. Synthetic miRNA, miR-126-3p (purchased from Sigma, Saint-Louis, MO, USA), was prepared in filtered microinjection buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA) at a concentration of 4,000,000 molecules/pl [17].

Following the microinjection of miR-126-3p into one-cell stage mouse embryos, the surviving embryos were transferred to carrier mothers (Fig. 2). The miR-126-3p generation was born 21 days after the procedure and was fed with breast milk for 21 days. When the mouse groups reached the age of 2 months, they were subjected to mTBI along with the control groups. TBI groups consisted of miR-126-3p*-TBI (acute, chronic), wild-type (WT)-TBI (acute, chronic), miR-126-3p*-sham, and WT-sham (Fig. 1). All groups of mice were housed in the same conditions.

Creating mTBI for Epigenetically Modified and Unmodified (WT) Mouse Groups

The Marmarou's weight-drop model was utilized to induce mTBI in 2-month-old mice [18]. Before the procedure, each mouse was administered general anesthesia via an intraperitoneal injection of 150 μL 2% Rompun (Bayer, America) and 150 μL Ketas (Pfizer, America) dissolved in 2 mL of 0.9% NaCl. Local anesthesia

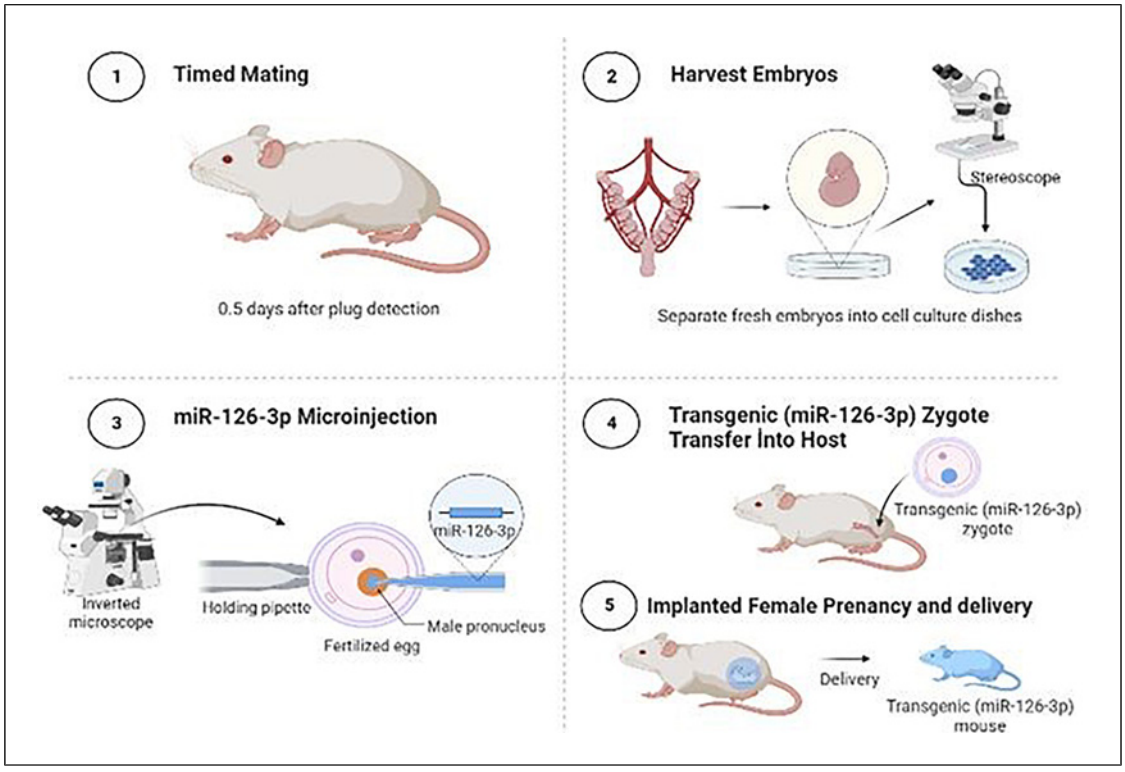


Fig. 2. Transferring mimic-miR-126-3p into mouse embryos by microinjection.

was administered under the skin of mice's heads while under general anesthesia, and the scalp of the mice was opened using a scalpel. The Marmarou's weight-drop model was used to induce mTBI as previously described [18]. The device consisted of a metal tube (inner diameter 13 mm) placed vertically over the mouse's head. To create the mTBI, a 30 g ball was dropped through an 80 cm tube on the surgically opened skull of an anesthetized mouse [18].

Two groups of mice, the acute and chronic phases, were used in this study. In accordance with the literature, 24th hour after TBI was considered as acute phase, and the 28th to 30th days after TBI was considered as chronic phase [12, 19]. The acute phase group was sacrificed by cervical dislocation 24 h after mTBI, as it is widely accepted as the acute phase for mice. The chronic phase group was sacrificed by cervical dislocation 1 month after mTBI creation [20–24]. Hypothalamus, pituitary, adrenal, and serum samples were collected from the sacrificed mice, and whole extracts were used for the analysis. Samples were stored at −80°C in TRIzol (Roche, Germany) until further use (Fig. 1).

RNA Isolation and Real-Time PCR

The RNAs from the serum, hypothalamus, pituitary, and adrenal samples were isolated using TRIzol (Roche, Germany), followed by cDNA synthesis using the EvoScript Reverse Transcriptase cDNA Synthesis Kit (Cat No.:07912374001, Roche, Germany). Initially, miR-126-3p transcript levels were determined in serum samples of mice to confirm the consistency of the human results. Subsequently, the transcript levels of the *Corticotrophin-releasing hormone (Crh)*, *Proopiomelanocortin (Pomc)*, *Corticosterone (Cort)*, *Gonadotropin-Releasing Hormone (Gnrh)*, *Growth Hormone (Gh)*, *and Insulin-Like Growth Factor-1 (Igf-1)* genes were determined using Roche 480 II Real-Time PCR (Roche, Germany) with the Light-Cycler® 480 Probes Master Kit (4707516001, Roche, Germany). The expression of commonly used endogenous reference housekeeping genes (*Gapdh* and *Actb*) for data normalization varies across tissues. All samples were run in duplicate, and values were normalized using the $2^{-\wedge\wedge Ct}$ method [25].

Determination of CRH, ACTH, CORT, GnRH, FSH, LH, GH, and Insulin-Like Growth Hormone-1 (IGF-1) Serum Hormone Levels

After sacrificing the mice, blood samples were collected from the heart using a 1 mL 26 G insulin needle and placed into the biochemistry tubes for serum isolation. The collected blood samples were 20 centrifuged at 3,000 rpm. Serum levels of corticotrophin-releasing hormone (CRH) (YLBiont, Cat. No. YLA0443MO), adrenocorticotropic hormone (ACTH) (YLBiont, Cat. No. YLA1053MO), corticosterone (CORT) (YLBiont, Cat. No. YLA0342MO), gonadotropin-releasing hormone (GnRH) (MyBioSource, Cat No. MBS264939), follicle-stimulating hormone (FSH) (MyBioSource, Cat No. MBS2700327), luteinizing hormone (LH) (MyBioSource, Cat. No. MBS041300), growth hormone (GH) (MyBioSource, Cat No. MBS160945), growth hormone-releasing hormone (GHRH) (MyBioSource, Cat. No. MBS700124), IGF-1 (MyBioSource, Cat No. MBS261121) were quantified using the enzyme-linked immunosorbent assay (ELISA) method [26].

Table 1. Characteristics of mice

Groups	Strain	Age	Male, *n*	Female, *n*	Total, *n*
miR-126* Sham	*Balb/c*	2 months	4	4	8
miR-126* TBI Acute	*Balb/c*	2 months	4	4	8
miR-126* TBI Chronic	*Balb/c*	3 months	4	4	8
WT-TBI Acute	*Balb/c*	2 months	4	4	8
WT-TBI Chronic	*Balb/c*	3 months	4	4	8
WT-sham	*Balb/c*	2 months	4	4	8

Statistical Analysis

The data analysis was performed using GraphPad Prism (version 8.0.1, San Diego, CA, USA) software. The normal distribution of the data was assessed using various methods, including histograms, q-q graphs, Shapiro-Wilk test, and variance homogeneity with Levene's test. Gene expression and hormone levels were compared between multiple groups using a one-way analysis of variance test, followed by post hoc tests as necessary. p value of <0.05 was considered statistically significant. Results were reported as mean values +/− SD. This approach ensured the rigorous statistical evaluation of the data and allowed for precise comparisons among the experimental groups [12, 27].

Results

In our previous study, we found that the transcript levels of miR-126-3p varied between patients with and without hypopituitarism following TBI [27]. To validate the human results, we developed two mouse models of TBI in parallel. Additionally, in WT mice, briefly, a short oligoribonucleotide of miR-126-3p was microinjected into the pronuclei of mouse embryos at the one-cell stage and then transferred to surrogate mothers [17, 28]. Mice in adulthood (2 months) were subjected to mTBI according to Marmarou's weight-drop model [18]. At 2-time points (1 and 30 days) after mTBI, the levels of the miR-126-3p transcript were examined with RT-qPCR techniques in serum samples from groups of mice to confirm the accuracy of the human results (see Figure 1 for the experimental design). To differentiate the groups, wild-type animals are referred to as WT, and mice modified with miR-126-3p microinjection are referred to as miR-126-3p*. The characteristics of the mice belonging to the experimental groups are shown in Table 1.

Microinjection of miR-126-3p into Mouse Embryos at the One-Cell Stage in Adult Mice Protects against a Significant Decrease in miR-126-3p Transcript Levels after TBI

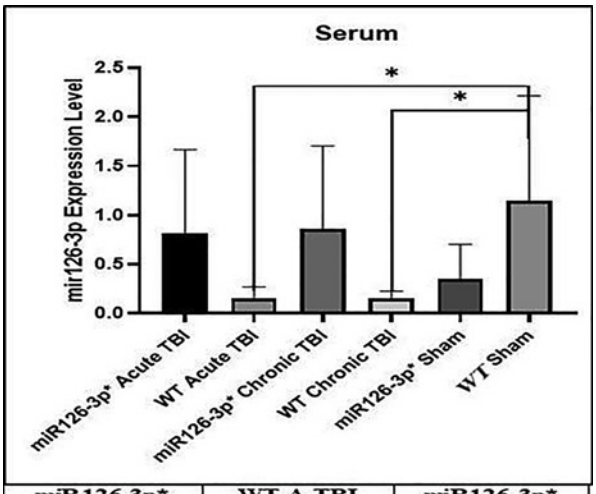
The levels of miR-126-3p were measured by real-time PCR following total RNA isolation from sera of experimental and control groups, as shown in Figure 3. The results demonstrate that there was a significant downregulation of serum levels of miR-126-3p after TBI in WT-acute and WT-chronic groups. These results validate our previous observations in humans and support the relevance of the mouse model to study the pathophysiology of TBI [12].

In contrast to WT groups, miR-126-3p expression levels did not decrease after TBI in the miR-126-3p-acute and miR-126-3p-chronic groups microinjected with miR-126-3p. This result suggests that exposure to a high levels of miR-126-3p at the embryonic stage could impact the levels of corresponding miRNA in the serum of adult mice. Conversely, unlike the control group, the miR-126-3p* groups showed increased serum levels of miR-126-3p transcripts after TBI compared to the sham group (Fig. 3).

Expression levels of miR-126-3p were assessed in mice 24 hours and 1 month after TBI, representing the acute and chronic phases, respectively. The WT-TBI group showed a sustained decrease in miR-126-3p expression levels compared to the WT-sham group due to the fact that 1 month after TBI does not correspond to the chronic phase in mice as in humans which is defined as 5 years after their injury. Additionally, the chronic phase in humans may also be accompanied by additional events during this prolonged period of time. In contrast, the microinjected group (miR-126-3p*-chronic-TBI group) showed increased levels of miR-126-3p after TBI, correlating with the chronic phase observed in humans.

*Evaluation of Transcripts and Hormonal Pathways of HPA, HPG, and GH-IGF-1 Axes in Mouse Groups of WT and Epigenetically Modified with miR-126-3p**

Hypothalamus, pituitary, and adrenal tissues were subjected to transcript analysis at 2 different time points after TBI, and the transcript levels of *Crh*, *Pomc*, *Cort*, *Gnrh*, *Gh*, and *Igf-1* genes were determined by RT-qPCR after isolation of total RNA. Serum hormone levels of CRH, ACTH, CORT, GnRH, FSH, LH, GH, and IGF-1 were determined by ELISA methods at the same time points.



miR126-3p* A-TBI (Day1)	WT A-TBI (Day1)	miR126-3p* Sham
0,815(±0,85)	0,1553(±0,11)	0,3545(±0,35)
miR126-3p* C-TBI (Day 30)	WT C-TBI (Day 30)	WT Sham
0,8582(±0,86)	0,1506(±0,08)	1,149(±1,06)

Fig. 3. Serum miR-126-3p levels in groups of mice after miR-126-3p microinjection (WT-acute: WT mice, 24 h after TBI, WT-chronic: WT mice, 30 days after TBI, miR-126-3p*-acute: miR-126-3p microinjected mice, 24 h after TBI, miR-126-3p*-chronic: miR-126-3p microinjected mice, 30 days after TBI, WT-sham: normal mice, miR-126-3p*-sham: miR-126-3p microinjected mice).

mTBI Affects the Hypothalamus via the HPA Axis Only in the WT Mouse Groups

We reported sex-specific differences in HPA axis-related transcripts during insulin stress (Insulin Tolerance Test) in mice. We also observed sex-specific response patterns in this study (see online suppl. Fig. 1–10; for all online suppl. material, see https://doi.org/10.1159/000535748). In this study, when male and female results were examined in total (Fig. 4a–c), no differences were observed between the experimental and control groups at the levels of any of the transcripts. But in contrast, when the transcript levels of the *Pomc*, *Cort*, and *Crh* genes were analyzed separately in the male and female hypothalamus, male WT mice showed higher *Crh* gene transcript levels than females in the WT-acute-TBI group (online suppl. Fig. 1A).

Additionally, the miR-126-3p*-acute-TBI group showed no significant change in *Cort* transcript levels compared to the WT-sham group, whereas the WT-acute-TBI group showed a decrease in *Cort* transcript levels ($p = 0.027$) (Fig. 4c). Transcript levels of *Cort* were found to be higher in female mice than in male mice in the hypothalamus ($p = 0.029$) (online suppl. Fig. 1C). Our results suggest that after mTBI, only *Cort* transcript levels are decreased in the WT-acute-TBI group compared to the WT-sham group, while miR-126-3p* appears to be protective, with no variation observed in the WT-acute group hypothalamus.

mTBI Affects the Pituitary via the HPA Axis of WT and Epigenetically Modified miR-126-3p Mouse Groups*

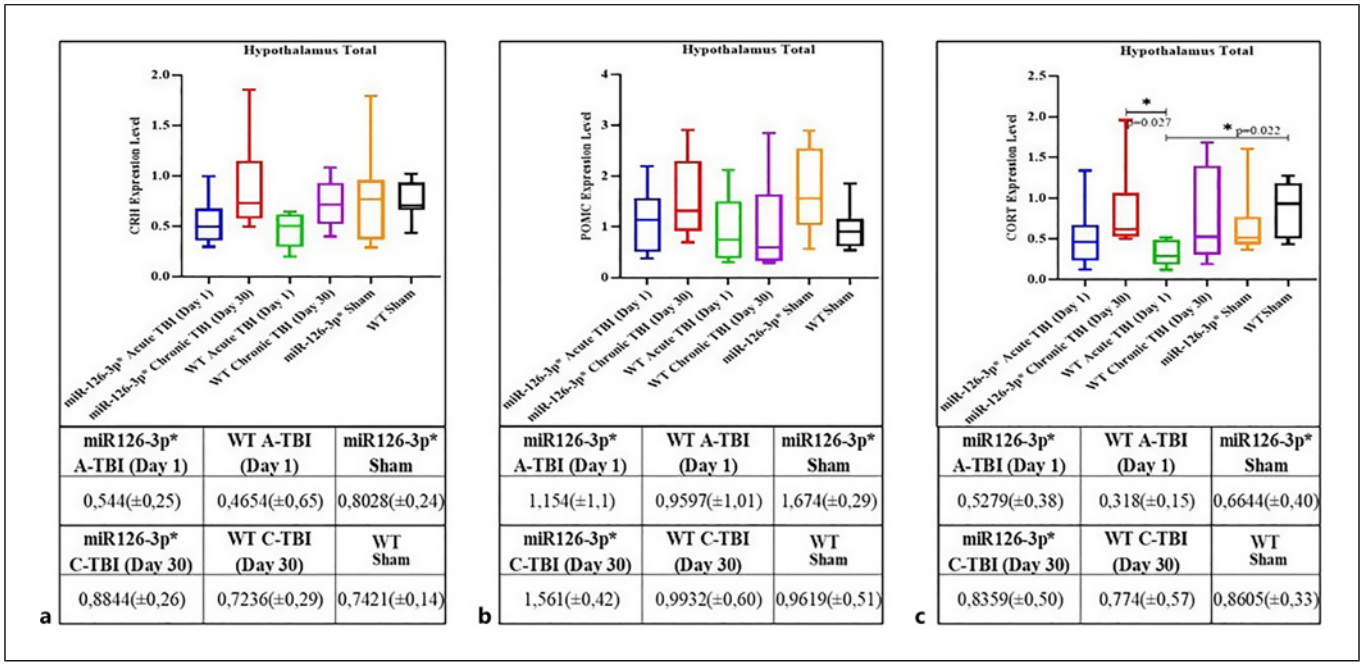
In both groups of male and female mice, mTBI impacted pituitary function. *Crh* transcript levels decreased at both time points after mTBI in all groups, compared to the WT-sham group ($p = 0.029$) (Fig. 5a). Additionally, *Crh* transcript levels were higher in female mice than in male mice in the miR-126-3p*-chronic group after mTBI ($p = 0.016$, $p = 0.004$) (online suppl. Fig. 2A). We found that *Pomc* transcript levels were higher in the miR-126-3p*-sham group compared to other groups (Fig. 5b), especially after mTBI, without any gender difference (online suppl. Fig. 2B). While *Cort* transcript levels decreased in all groups, especially in the WT-acute-TBI group, compared to the WT-sham group ($p = 0.029$) (Fig. 5c). On the other hand, *Cort* transcript levels increased in the WT-sham female group (online suppl. Fig. 2C).

mTBI Affects the Adrenal Glands via the HPA Axis Only in WT Female Mice

In the adrenal glands, the levels of *Crh, Pomc,* and *Cort* transcripts increased only in the WT-chronic-TBI groups (Fig. 6a–c), especially in females 1 month after TBI compared to males and other groups (online suppl. Fig. 3A–C).

mTBI Reverses Sex-Related Hormonal Differences in the HPA Axis of miR-126-3p Modified Mouse Groups

After TBI, we observed comparable levels of hormones CRH, ACTH, and CORT between males and females in the miR-126-3p* groups, which were clearly influenced by gender (online suppl. Fig. 4). Application of mTBI to miR-126-3p* mice resulted in sex-specific differences in hormonal levels. The miR-126-3p*-acute-TBI group showed higher ACTH hormone levels than the other groups (Fig. 7b). In terms of gender differences, miR-126-3p* and WT females had higher ACTH hormone levels in the acute-TBI groups than males (online suppl. Fig. 4B). There were no significant differences in CRH hormone levels between groups (Fig. 7a; online suppl. Fig. 4A). Although CORT hormone levels were higher in the



a

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,544(±0,25)	0,4654(±0,65)	0,8028(±0,24)
miR126-3p* C-TBI (Day 30)	**WT C-TBI (Day 30)**	**WT Sham**
0,8844(±0,26)	0,7236(±0,29)	0,7421(±0,14)

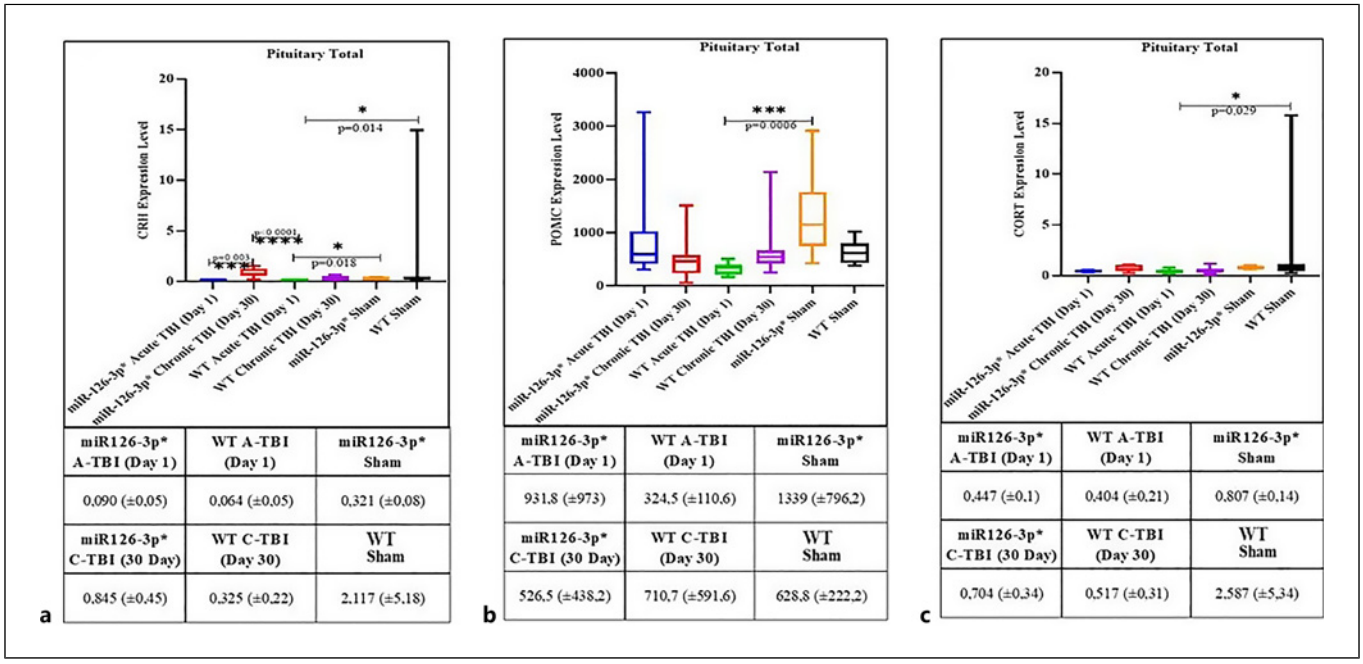
b

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
1,154(±1,1)	0,9597(±1,01)	1,674(±0,29)
miR126-3p* C-TBI (Day 30)	**WT C-TBI (Day 30)**	**WT Sham**
1,561(±0,42)	0,9932(±0,60)	0,9619(±0,51)

c

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,5279(±0,38)	0,318(±0,15)	0,6644(±0,40)
miR126-3p* C-TBI (Day 30)	**WT C-TBI (Day 30)**	**WT Sham**
0,8359(±0,50)	0,774(±0,57)	0,8605(±0,33)

Fig. 4. Levels of *Crh*, *Pomc*, and *Cort* transcripts in the hypothalamus in the acute and chronic phases after mTBI. **a** Levels of *Crh* transcript in the hypothalamus in the acute and chronic phases after mTBI. **b** Levels of *Pomc* transcript in the hypothalamus in the acute and chronic phases after mTBI. **c** Levels of *Cort* transcript in the hypothalamus in the acute and chronic phases after mTBI.



a

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,090 (±0,05)	0,064 (±0,05)	0,321 (±0,08)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
0,845 (±0,45)	0,325 (±0,22)	2,117 (±5,18)

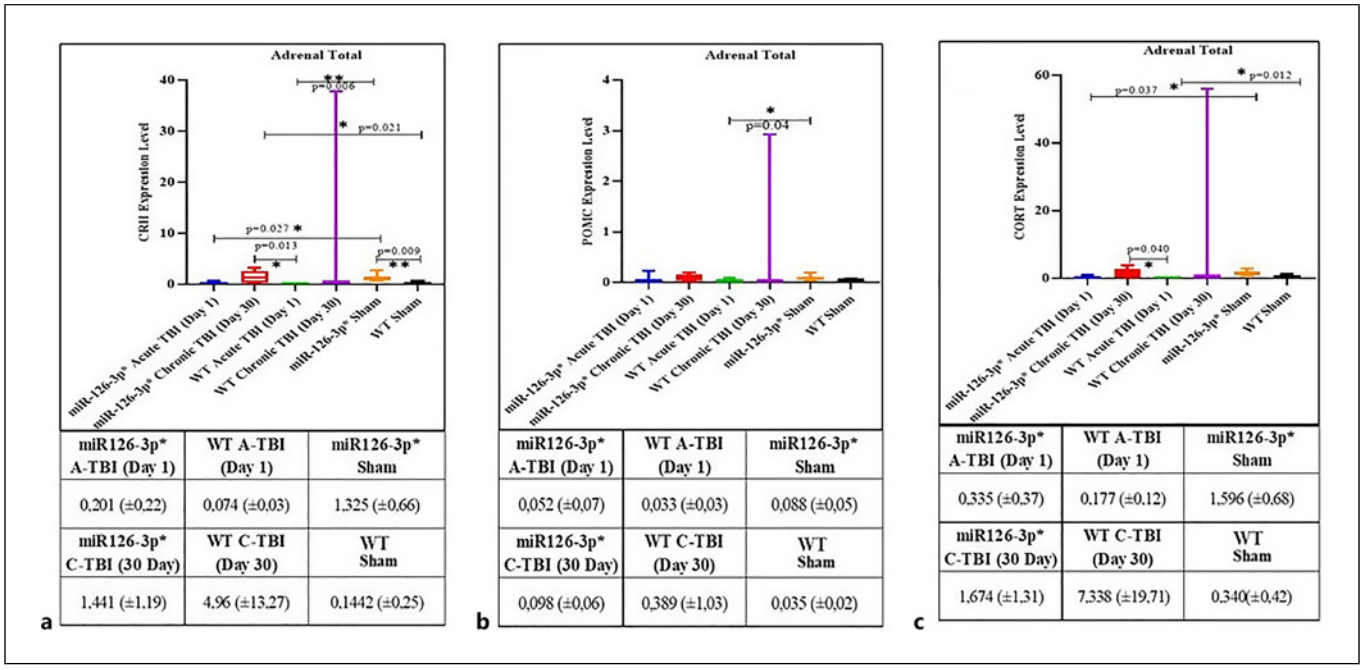
b

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
931,8 (±973)	324,5 (±110,6)	1339 (±796,2)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
526,5 (±438,2)	710,7 (±591,6)	628,8 (±222,2)

c

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,447 (±0,1)	0,404 (±0,21)	0,807 (±0,14)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
0,704 (±0,34)	0,517 (±0,31)	2,587 (±5,34)

Fig. 5. Levels of *Crh*, *Pomc*, and *Cort* transcripts in the pituitary in the acute and chronic phases after mTBI. **a** Levels of *Crh* transcript in the pituitary in the acute and chronic phases after mTBI. **b** Levels of *Pomc* transcript in the pituitary in the acute and chronic phases after mTBI. **c** Levels of *Cort* transcript in the pituitary in the acute and chronic phases after mTBI.



miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,201 (±0,22)	0,074 (±0,03)	1,325 (±0,66)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
1,441 (±1,19)	4,96 (±13,27)	0,1442 (±0,25)

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,052 (±0,07)	0,033 (±0,03)	0,088 (±0,05)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
0,098 (±0,06)	0,389 (±1,03)	0,035 (±0,02)

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
0,335 (±0,37)	0,177 (±0,12)	1,596 (±0,68)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
1,674 (±1,31)	7,338 (±19,71)	0,340(±0,42)

Fig. 6. Levels of *Crh*, *Pomc*, and *Cort* transcripts in the adrenal in the acute and chronic phases after mTBI. **a** Levels of *Crh* transcript in the adrenal in the acute and chronic phases after mTBI. **b** Levels of *Pomc* transcript in the adrenal in the acute and chronic phases after mTBI. **c** Levels of *Cort* transcript in the adrenal in the acute and chronic phases after mTBI.

miR-126-3p*-acute-TBI group than in the other groups (Fig. 7c), we observed no difference in CORT hormone levels between gender groups after TBI (online suppl. Fig. 4C).

Assessment of the HPG Axis Pathways in Groups of WT and Epigenetically Modified Mice with miR-126-3p after TBI

Gnrh transcript levels were measured in the hypothalamus, pituitary, and adrenal of all groups. In the hypothalamus, a significant increase in *Gnrh* transcript levels was observed in the WT-acute-TBI group compared to the other groups except the miR-126-3p*-acute-TBI group (Fig. 8a). This finding suggests that miR-126-3p downregulates *Gnrh* transcript levels in the acute phase after TBI. There was no significant difference when *Gnrh* transcript levels were compared between sexes in the hypothalamus (online suppl. Fig. 5A).

In the pituitary, *Gnrh* transcript levels were low in all groups except the WT-sham group, and they were downregulated with TBI. Interestingly, the miR-126-3p*-sham group exhibited the TBI effect on the expression level of miR-126-3p (Fig. 8b). *Gnrh* transcript levels were determined to be higher in the female pituitary of the WT-chronic-TBI group, but this difference was not found in the miR-126-3p*-chronic-TBI group (online suppl. Fig. 5B).

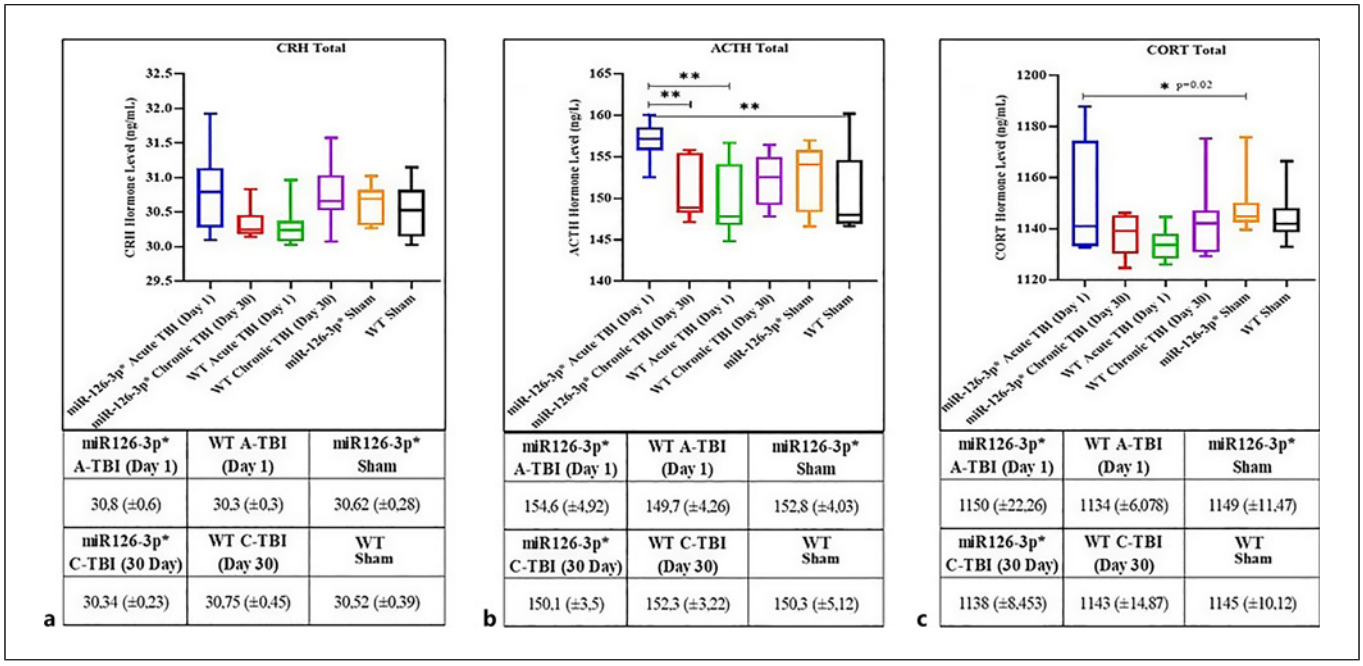
In the adrenal glands, *Gnrh* transcript levels were determined to be increased in the miR-126-Sham group compared to the WT-sham group (Fig. 8c), and the increase was only detected in female mice. However, the increase was not observed in the miR-126-3p*-chronic group (online suppl. Fig. 5C).

Evaluation of HPG Axis-Related Hormones in Groups of WT and Epigenetically Modified Mice with miR-126-3p after TBI

Despite the observed differences in *Gnrh* transcript levels in the hypothalamus, pituitary, and adrenal glands after TBI, these differences did not translate into significant changes in serum levels of FSH/LH hormones (Fig. 8d, E; online suppl. Fig. 6A, B).

Evaluation of GH-IGF-1 Axis Pathways in Groups of WT and Epigenetically Modified Mice with miR-126-3p after TBI

After TBI, we investigated the effects on the GH-IGF-1 axis at transcript and serum hormone levels in mice from WT and miR-126-3p* groups. In the hypothalamus, *Gh*



miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
30,8 (±0,6)	30,3 (±0,3)	30,62 (±0,28)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
30,34 (±0,23)	30,75 (±0,45)	30,52 (±0,39)

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
154,6 (±4,92)	149,7 (±4,26)	152,8 (±4,03)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
150,1 (±3,5)	152,3 (±3,22)	150,3 (±5,12)

miR126-3p* A-TBI (Day 1)	WT A-TBI (Day 1)	miR126-3p* Sham
1150 (±22,26)	1134 (±6,078)	1149 (±11,47)
miR126-3p* C-TBI (30 Day)	**WT C-TBI (Day 30)**	**WT Sham**
1138 (±8,453)	1143 (±14,87)	1145 (±10,12)

Fig. 7. Levels of CRH, ACTH, and CORT hormones in sera of all groups after TBI. **a** Levels of CRH in sera of all groups after TBI. **b** Levels of ACTH in sera of all groups after TBI. **c** Levels of CORT hormones in sera of all groups after TBI.

transcript levels were higher in the miR-126-3p*-sham group than in all other groups, with no significant difference between TBI groups (Fig. 9a). In addition, the transcript levels of *Igf-1* were increased in the miR-126-3p*-acute-TBI group compared with the miR-126-3p*-chronic-TBI group in the hypothalamus, and this effect was observed only in female mice (online suppl. Fig. 7A).

It was determined that the transcript levels of *Igf-1* were increased in the miR-126-3p*-acute-TBI group compared to the miR-126-3p*-chronic-TBI group in the hypothalamus (Fig. 9b). This increase was observed in female mice (online suppl. Fig. 7B). However, *Igf-1* transcript levels were elevated in the WT-sham group, but they were not elevated in the miR-126-3p*-sham group (Fig. 9b).

In the pituitary, we found that *Gh* transcript levels were increased in the miR-126-3p*-chronic-TBI group compared to the WT-sham group, but this increase was not observed in the WT-chronic-TBI group (Fig. 9c). Although *Igf-1* transcript levels were increased in the WT-acute-TBI group, the same increase was not found in the miR-126-3p*-acute-TBI group (Fig. 9d). The increase in *Igf-1* transcript levels in the WT-acute-TBI group was also observed in male mice (online suppl. Fig. 8B).

In the adrenal, we found that *Gh* transcript levels were elevated in the WT-chronic-TBI group compared to all groups (Fig. 9e). This increase was observed in female mice. However, this difference was not observed in the miR-126-3p*-chronic-TBI group (online suppl. Fig. 9A). There were no differences between groups in adrenal *Igf-1* transcript levels (Fig. 9f), but males in the WT-chronic-TBI group had higher levels than females (online suppl. Fig. 9B).

Although levels of *Gh and Igf-1* transcripts in the pituitary, hypothalamus, and adrenal differed between groups after TBI, we found that this difference was not reflected in serum levels of GH/IGF-1 hormones (Fig. 10a–c). However, serum GH levels were significantly higher in females than males in the miR-126-3p*-acute-TBI group (online suppl. Fig. 10A). Although the same difference was observed between males and females in the WT-acute-TBI group, it was not statistically significant (online suppl. Fig. 10A).

Discussion

The study uses epigenetically modified murine models to investigate the mechanisms underlying pituitary insufficiency after mTBI. We aim to explore functional epigenetic variations, associated gene expression, and

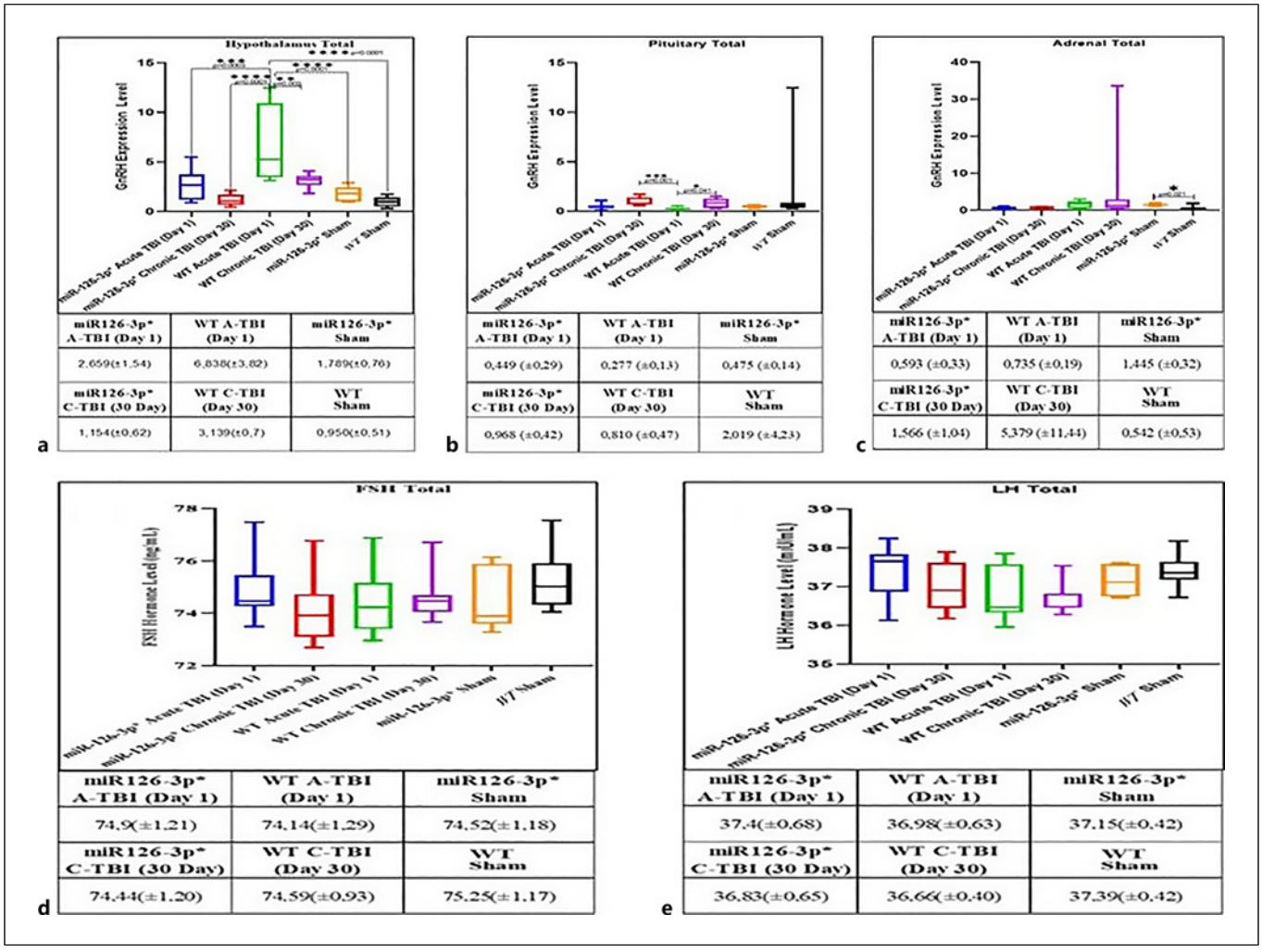


Fig. 8. Levels of *GnRh* transcript in the hypothalamus, pituitary, and adrenal in the acute and chronic phases after TBI. **a** *Gnrh* transcript levels in the hypothalamus. **b** *Gnrh* transcript levels in the pituitary. **c** *Gnrh* transcript levels in the adrenal. **d** Levels of FSH in sera of all groups after TBI. **e** Levels of LH in sera of all groups after TBI.

hormonal consequences on organ interaction following exposure to TBI stress in mice. In our previous study, the expression profiles of 740 miRNAs were determined from serum samples collected from patients with and without hypopituitarism on days 1, 7, 28, and 5 years after TBI. We showed a relationship between miR-3907 and TBI, miR-3610, and miR-126-3p with TBI-induced hypopituitarism. In patients with TBI-induced hypopituitarism, miR-126-3p decreased in the acute phase, while it increased fifteenfold within 5 years after TBI [12]. miR-3907 and miR-3610 are not present in the mouse genome. We therefore investigated the expression of miR-126-3p after mTBI in mice (see the summary of all combined results in Graphical Abstract).

Effect of mTBI on the HPA Axis of Adult Mice in a Controlled Environment

We applied mTBI to adult WT mice (2 months) and analyzed the consequences at 1 and 30 days after trauma. mTBI downregulated miR-126-3p in WT sera, confirming the downregulation observed in acute TBI patients. Although 1 month after TBI in mice is considered in the literature as a chronic phase [29, 30], here in terms of human results the downregulation is maintained after 30 days. In other words, an upregulation observed after 5 years in patients is not observed after 1 month in mice. We hypothesize that prolonged reduction in miR-126-3p expression may play a role in hypopituitarism and HPA axis regulation.

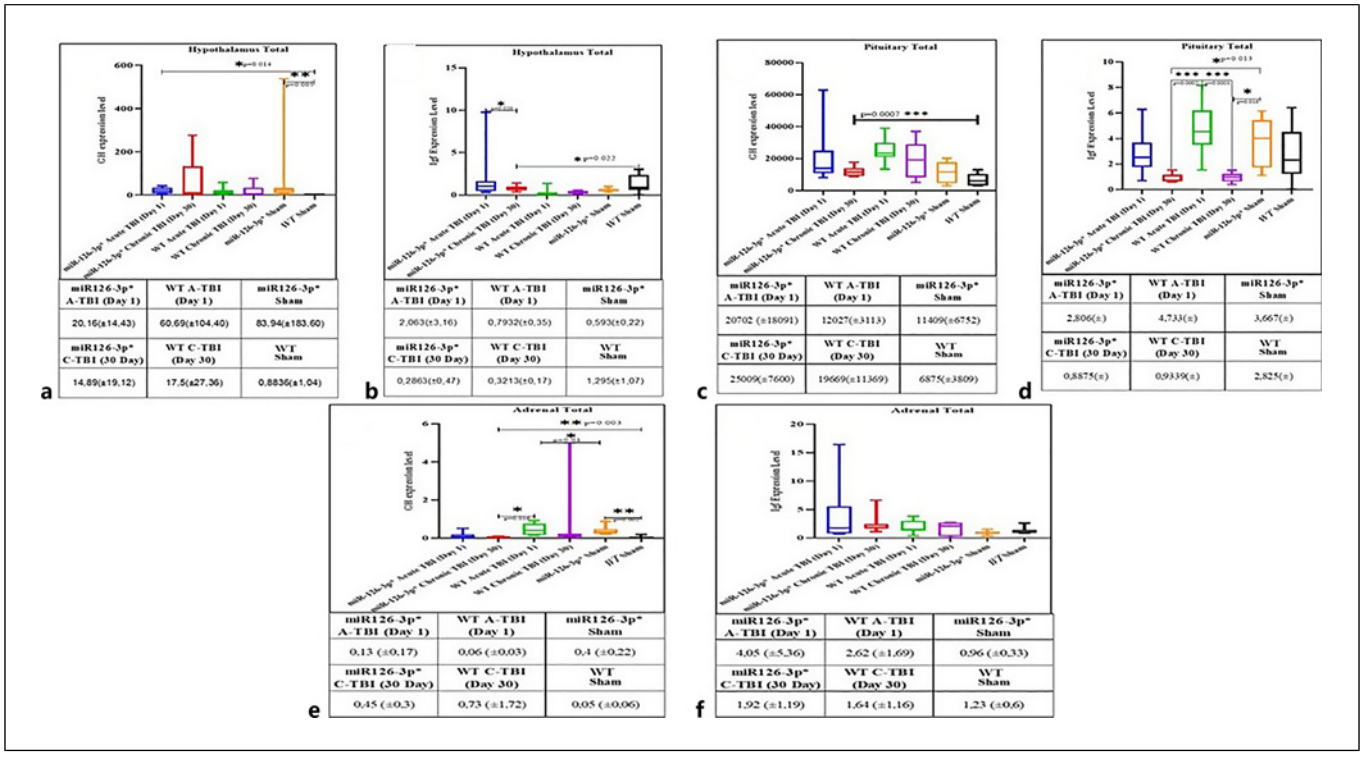


Fig. 9. Levels of *Gh* and *Igf-1* transcripts in the hypothalamus, pituitary, and adrenal in the acute and chronic phases after TBI. **a** Levels of *Gh* transcript in the hypothalamus in the acute and chronic phases after TBI. **b** Levels of *Igf-1* transcripts in the hypothalamus in the acute and chronic phases after TBI. **c** Levels of *Gh* transcript in the pituitary in the acute and chronic phases after TBI. **d** Levels of *Igf-1* transcript in the pituitary in the acute and chronic phases after TBI. **e** Levels of *Gh* transcript in the adrenal in the acute and chronic phases after TBI. **f** Levels of *Igf-1* transcript in the adrenal in the acute and chronic phases after TBI.

Crh, *Pomc*, and *Cort* transcripts are not the intended targets of miR-126-3p. The subtle effects observed on these transcripts may not be the result of direct targeting by miR-126-3p on post-transcription expression but rather an alteration of other unidentified involved targets. Hormones play an important role in the regulation of the HPA, HPG, and GH-IGF-1 axes by supporting organ communication requiring activation of the pathways. Likewise, they can have an indirect effect on the brain and adaptive responses. In the absence of stress, there are already differences in transcription levels between the sexes. Our results suggest that even subtle aberrations in transcript levels in male and female mice could potentially impact hormone levels.

The role of miRNA levels in regulating the HPA axis after TBI has yet to be investigated. However, many studies have demonstrated that miRNA levels are altered in various brain regions after TBI, indicating their crucial role in TBI response [31, 32].

For example, Ge et al. [33] demonstrated that miR-21 was upregulated in traumatic brain areas, and angiogenesis was promoted, while apoptosis was inhibited by miR-21. In another study, inhibition of miR-155 was shown to protect mice from neuro-inflammatory responses after TBI [21]. However, a limited number of studies have focused on the role of miRNAs in the pathophysiology of secondary damage, particularly in the chronic phase after TBI [12, 34].

In our previous human study, we found that the serum expression levels of miR-126-3p decreased during the acute phase (1, 7, and 28 days) and increased in the chronic phase in patients with induced hypopituitarism after TBI [12]. In the present study, the expression levels of miR-126-3p decreased over a period of 1 to 30 days after TBI in the WT mouse group compared to the sham group.

The Typical Response of Epigenetically Modified miR-126-3p-Adult Mice*

The miR-126* mouse line was created through the induction of an epigenetic variation in miR-126 levels in the zygote. This was achieved by microinjecting miR-126-3p into fertilized eggs, which resulted in downregulation of

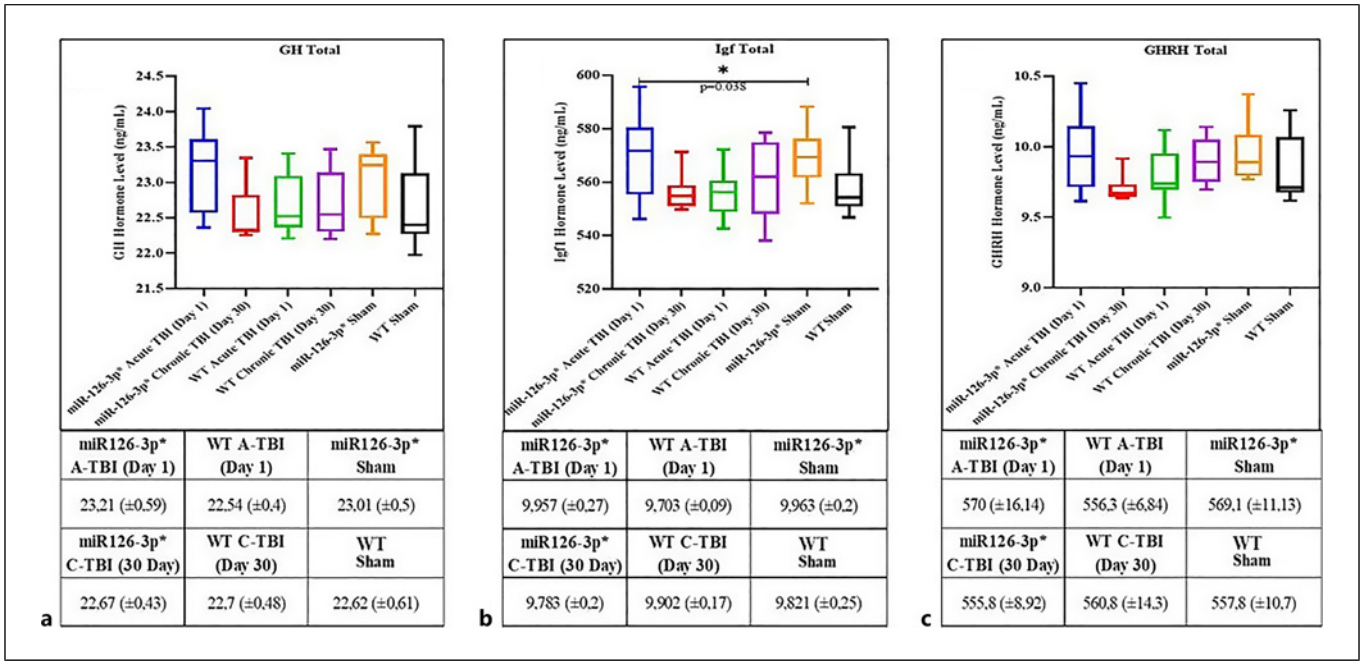


Fig. 10. Levels of GH, IGF-1, and GHRH in sera of all groups after TBI. **a** Levels of GH in sera of all groups after TBI. **b** Levels of IGF-1 in sera of all groups after TBI. **c** Levels of GHRH in sera of all groups after TBI. Graphical abstract: summary scheme of transcript levels changing in the hypothalamus, pituitary, and adrenal tissues and miR-126-3p* and hormone levels' expression changing in serum in all groups after TBI.

miR-126-3p in adult tissues and sera. Interestingly, we found that an acute phase equivalent to TBI in mice was achieved without mTBI and that miR-126-3p levels increased from 1 to 30 days after TBI. These results demonstrate that modification of the miRNA content of the mouse zygote by RNA microinjection leads to reprogramming of the zygote, which in turn modifies hormonal regulation in the HPA axis. This study provides the first evidence of such a phenomenon.

Transcripts and Hormones Related to HPA Axis

We examined whether modification of miR-126-3p had an effect on the transcript levels of *Crh*, *Pomc*, and *Cort* in the HPA axis. First, we found that miR-126-3p had no significant effect on the transcript levels of *Crh*, *Pomc*, and *Cort* genes in the hypothalamus. However, in the pituitary gland, after TBI, we found that *Crh* and *Cort* transcript levels decreased in all groups, including the sham miR-126-3p* group, compared to the sham WT group. *Pomc* transcript levels were unchanged in the pituitary in all groups except in the miR-126-3p*-sham group. In the adrenal, we found that the levels of *Crh*, *Pomc*, and *Cort* transcripts were higher within 30 days of the WT-mTBI group compared to the other groups. Furthermore, this increase was solely observed in WT female mice, and no difference was observed in transcript levels between males and females in the miR-126-3p*-chronic-TBI group.

Although microinjection of miR-126-3p had a limited effect on HPA axis-linked transcripts, we observed significant changes in hormone levels after TBI. After TBI, when the levels of hormones CRH, ACTH, and CORT were compared between groups, we found that ACTH levels were higher than other groups in the miR-126-3p*-acute (1 day) mTBI group. Additionally, when ACTH levels were compared between the sexes, we found that ACTH levels were higher in females. However, the difference in ACTH levels observed during the acute phase (1 day) was not maintained during the chronic phase (30 days). Despite this, this finding remains significant, as it suggests a potential protective effect of miR-126-3p after TBI, notably by increasing ACTH levels during the acute phase. CORT hormone levels were higher in the miR-126-3p*-acute-TBI group than in the other groups. However, we did not observe a significant difference in CORT levels between other groups after TBI. There was also no significant difference in CRH levels between the groups.

Atypical mTBI Stress Response in miR-126-3p Mice*

In response to mTBI, miR-126-3p* mice showed a significant increase in miR-126-3p transcript levels, whereas WT mice showed a significant decrease. This observed effect could be attributed to the epigenetic alteration of the locus, which could provide protection against downregulation through an adaptation mechanism described in various circumstances.

Notably, a distinctive pathway was impacted, whereby transcripts were exclusively upregulated in stress-exposed mice. Overexpression of RNA and hormones have been described in TBI, although their roles beyond organ communications are not well understood. There is still a lack of research linking early miRNA overexpression and developmental disorders. Recently, Demin et al. described how chronic stress due to social defeat leads to anxiety and depression in male mice and is accompanied by upregulation of many genes in the hypothalamus [35].

Effect of miR-126-3p on the HPG Axis after mTBI

Gonadotropin deficiencies, particularly FSH/LH, are frequently observed after TBI. The secretion of FSH/LH by the pituitary gland is stimulated by the hypothalamic hormone GnRH, which also promotes the secretion of sex hormones by the gonads [36].

Under normal conditions, activation of the HPA axis during stress, such as trauma or hypoglycemia, suppresses the HPG axis. There is an inverse correlation between the HPA and HPG axis [27]. Our results demonstrate that although *Gnrh* transcript levels were increased in the hypothalamus of WT mice after acute TBI, this increase was not observed in the miR-126-3p*-acute-TBI group. Thus, miR-126-3p may be involved in modulating *Gnrh* transcript levels. Regarding the pituitary gland, all groups except the WT-sham group had lower *Gnrh* transcript levels. This increase in the WT-sham group was particularly observed in female mice. However, this increase was not observed in the miR-126-3p*-sham group, indicating the role of miR-126-3p in modulating *Gnrh* transcript levels. The downregulation of *Gnrh* transcripts in miR-126-3p* and WT groups after TBI also suggests the negative impact of TBI, which is a stressful condition for the body, on the HPG axis.

In the adrenal glands, *Gnrh* transcript levels were increased in the WT-chronic-TBI group, again only in female mice, but this was not the case in the miR-126-3p*-chronic-TBI group.

Our results indicate that miR-126-3p is involved in the alteration of *Gnrh* transcripts. However, although miR-126-3p reduces *Gnrh* transcript levels, this is not reflected in serum FSH/LH levels. This indicates that other miRNAs (miR-3610, miR-3907) might play a role in regulating the HPG axis after TBI [12].

Effect of miR-126-3p on GH-IGF-1 Axis after mTBI

As is known, GH deficiency takes the first place in hormonal deficits that develop after TBI. Although there was no difference in *Gh* transcript levels in the hypothalamus after TBI, it was observed that *Gh* transcript levels were higher in the miR-126-3p*-sham group than in the WT-sham group. In addition, *Gh* transcript levels were higher in the miR-126-3p*-chronic-TBI group in the pituitary of female mice and also in the WT-chronic-TBI group in the adrenal.

In the hypothalamus, *Igf-1* transcript levels increased in the miR-126-3p*-acute-TBI group but not in the WT-acute-TBI group. Conversely, in the pituitary, Igf-1 transcript levels were increased in the WT-acute-TBI group but not in the miR-126-3p*-acute-TBI group. In the adrenal glands, no difference was observed between groups in terms of *Igf-1* transcript levels.

Of note, changes in *Gh* and *Igf-1* transcript levels in the hypothalamus, pituitary, and adrenal glands were not reflected in serum levels of GH, IGF-1, and GHRH.

While hormones are secreted from a tissue during the body's response to a stimulus, the relevant transcript can be synthesized at variable levels from all tissues. Additionally, transcripts (RNAs) may not always turn into proteins. It is a collective response that the body may give from all its cells to any stimulus. Furthermore, recent studies reveal additional layers of functional complexity between RNA and proteins: some long noncoding RNAs have been redefined as transcripts encoding micropeptides, and conversely, some RNAs encoding proteins are bifunctional and display noncoding functions. Coding RNAs (mRNAs) and noncoding RNAs together provide regulation in response to any stimulus [37]. In our 2022 study, we showed that during the process of HPA axis activation in hypoglycemia, different transcript profiles are formed in the hypothalamus, pituitary, adrenal, and blood. In other words, just as there are hormonal networks in an organism, there are also transcript networks that participate in tissue regulation [27]. We suggest that the body creates a RNA response just as it creates a protein/hormonal response to any stimulus, and these RNAs function at different stages of regulation like proteins.

Limitations

The lifespan of mice is 2 years on average, considerably shorter than that of humans. It is therefore not possible to observe the equivalent of 5 years of human life in mice. Nevertheless, we found that miR-126-3p levels are

consistently reduced in WT mice after 1 and 30 days of mTBI, corresponding to the defined human acute phase (1, 7, and 30 days). Conversely, in miR-126-3p* mice, we observed an increase in miR-126-3p levels immediately after mTBI, which is strikingly similar to the increase observed in humans during the 5-year chronic phase.

Furthermore, our results suggest that miR-126-3p is involved in the downregulation of *Gnrh* transcripts. However, despite the downregulation of *Gnrh* transcripts by miR-126-3p, serum FSH/LH levels were not affected, indicating that other miRNAs, such as miR-3610 and miR-3907, could also play a role in the regulation of the HPG axis after TBI.

Despite the limited opportunities for research and sample collection in human subjects, it is essential to understand the importance of altered transcripts induced during the early stages of development.

To solve this problem, we propose microinjection into the embryo to establish the same signals as in mTBI. In fact, as in humans after 5 years, miR-126-3p* mice develop a comparable phenotype despite an already negative regulatory response. This highlights the broad genetic and epigenetic variations that already exist in patients. Additionally, some trends may represent the tissue specificity of the mouse brain. Finally, our study shows that modifying the content of miRNAs at the zygote stage has an effect on adult mice, thus raising questions about potential epigenetic alterations in patients.

Conclusion

In light of the data we obtained after TBI in WT-TBI mouse groups with homogeneous genetic structure, any changes likely occurring after TBI are due to the downregulation of miR-126-3p levels in WT groups. Our study suggests a miRNA-related epigenetic mechanism involving miR-126-3p in the development of pituitary deficiency. We propose that downregulation of miR-126-3p may indirectly contribute to resilience after mTBI, potentially serving as an adaptive mechanism allowing appropriate adverse conditions.

Transparency, Rigor, and Reproducibility Summary

This study was not preregistered. Statistical power and sample size calculations were based on the expected effect size at day 1 and day 30 after TBI relative to previous studies with *Balb/c* mice. Single-cell embryos (zygotes) were microinjected with mimic-miR-126-3p and transferred to the surrogate mothers. The mTBI mouse model was established after these mice were 8 weeks old. WT and miR-126-3p* mice groups were sacrificed on day 1 and day 30 after mTBI, and then the hypothalamus, pituitary, adrenal tissues, and serum samples were collected. To determine the transcript and hormone levels associated with the HPA, HPG, and GH-IGF-1 axis from the tissue and serum samples taken, 6 groups of 4 males and 4 females were studied in each group. Subjects were randomly assigned to groups, and mice and tissues were processed in multiple batches due to the scale of the project. Experimental manipulations were carried out between 09:00 and 10:00 with fed subjects. Care has been taken to include more than one group per lot whenever possible. The experimental analyses were analyzed by researchers who were blind to the relevant characteristics of the subjects. While detailed in the method sections, more information on the equipment and analytical reagents used to perform the experimental manipulations is available on request. All analyzed data during this study are included in this published article. The study's raw data are available from the corresponding author on request.

Statement of Ethics

All animal experimental protocols were approved by the Local Ethics Committee for Animal Experiments of Erciyes University (No. 17/002).

Conflict of Interest Statement

The authors declare no competing interests.

Funding Sources

This work was supported by the Erciyes University Scientific Research Project Unit (Grant No. # TCD-2013-4604).

Author Contributions

S.T., F.K., M.R., Z.K., F.T., and K.U. conceived and designed the experiments and the primary research hypothesis. E.T., H.U., K.K., and S.T. created the TBI model and collected tissue from mice. E.T. performed the experiments; E.T., E.M., and Z.Y.Ş. performed the statistical analysis and data representation; Z.K., F.T., M.R., K.U., and S.T. analyzed the draft manuscript and

corrected the style and content of the manuscript; and F.K., K.U., M.R., Z.K., F.T., and S.T. supervised all steps of this work, including the experiments. All the aforementioned authors fully contributed to the reading, writing, and approval of the final version of this manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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