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# The Role of Apoptosis and Autophagy in the Hypothalamic-Pituitary-Adrenal (HPA) Axis After Traumatic Brain Injury (TBI)

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# The Role of Apoptosis and Autophagy in the Hypothalamic-Pituitary-Adrenal (HPA) Axis after Traumatic Brain Injury (TBI)

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**Short Title:** Apoptosis or autophagy in the HPA axis after TBI

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**Abstract**

Traumatic brain injury (TBI) is one of the major health problems affecting millions of people worldwide leading to death or permanent damage. TBI affects the hypothalamic-pituitary-adrenal (HPA) axis either by primary injury to the hypothalamic-hypophyseal region or by secondary vascular damage, brain, and/or pituitary edema, vasospasm, and inflammation. Neuroendocrine dysfunctions after TBI have been clinically described in all hypothalamic-pituitary axes. In this study, we focused on the HPA axis-related tissues in the acute and chronic phases of TBI to determine the role of autophagy and apoptosis in the development of endocrine disturbances after TBI.

We established a mild TBI (mTBI) in rats using the controlled cortical impact (CCI) model. The hypothalamus, pituitary, and adrenal tissues were collected in the acute (24 hours) and chronic (30 days) groups after TBI; and we investigated autophagy *(Lc3, Bcln1, P150, Ulk*, and *Atg5*) and apoptosis (pro-caspase-3, cleaved caspase-3) markers in the HPA axis-related tissues using RT-PCR and Western blotting assays.

The present data showed that after mTBI, *P150* and *Atg5* transcripts showed a stable decrement from the acute to chronic phases in the hypothalamus, pituitary, and adrenal glands. In contrast, *Bcln1* transcripts were found to be only affected in the adrenal glands, with a prominent increase during the chronic phase. We also evaluated the levels of TNF-α and corticosterone by western blot in the HPA axis-related tissues in the acute and chronic phases after TBI. The increased corticosterone levels in the adrenal glands pointed out the activation of the HPA axis, while high TNF-α levels in the hypothalamus, pituitary, and adrenal glands suggested neuroinflammation.

As a result, transcripts related to autophagy were reduced in the hypothalamus, pituitary, and adrenals after TBI; but this was not reflected in autophagy-related protein levels. In contrast, protein markers related to apoptosis increased in the adrenal glands during the acute phase and in the pituitary during the chronic phase. TBI seems to trigger apoptosis by inducing inflammation that can also affect the adrenals in the acute phase; and this situation may lead to an impairment of communication through the hypothalamus, pituitary and adrenal glands. This may explain the permanent damage to the pituitary with increased apoptosis and inflammation in the chronic phase. These findings may contribute to the elucidation of the underlying mechanisms of endocrine dysfunctions such as pituitary and adrenal insufficiency that occur after TBI. Although adrenals are not directly affected by TBI, we suggest that the role of the adrenals together with the hypothalamus and pituitary should not be ignored in the acute phase after TBI.

**Keywords:** Traumatic brain injury (TBI), neuroinflammation, HPA axis, autophagy, apoptosis

## 1. Introduction

Traumatic brain injury (TBI) is an important health problem worldwide that affects many people. Brain functions can be temporarily or permanently impaired during TBI and a secondary injury may arise which is usually not detectable (Simon et al. 2017). The secondary injury could be associated with neuroinflammatory response and/or with a genetic predisposition (Tanriverdi et al. 2008b). The hypothalamic–pituitary–adrenal (HPA) axis, which consists of the hypothalamus, pituitary, and adrenal glands, is responsible for providing an adequate cortisol response to maintain the homeostasis of organisms in stressful situations, such as in TBI (Karaca et al. 2021). TBI affects the HPA axis either by direct injury to the hypothalamic-hypophyseal region or by secondary vascular damage, brain and/or pituitary edema, vasospasm, and inflammation (Özben 1998; Hiebert et al. 2015; Mckee and Daneshvar 2015). Endocrine dysfunctions after TBI have been clinically described in all hypothalamic-pituitary axes. Besides frequently seen neuropsychiatric symptoms including irritability, depression, and anxiety, one of the most important neuroendocrine disorders emerging after TBI is pituitary insufficiency (Tanriverdi et al. 2015; Karaca et al. 2016). Although these abnormalities that emerge after TBI are temporary in most patients, they can be permanent in some (Tanriverdi et al. 2015).

The controlled cortical impact (CCI) animal model is mainly used to mimic human severe head trauma resulting in extensive cortical loss, hippocampus and thalamic damage, cytotoxic and vasogenic brain edema, and increased intracranial pressure, leading to focal damage. A significant objective of TBI models is to generate injuries with gradual modifications in morphology and behavior, where the functional result is either enhanced or worsened by interference. The application of many TBI animal models is critical for developing preventative measures and reducing the impact of various types of primary injury (Taylor et al. 2008).

The mechanism(s) of pituitary dysfunction, which can be seen in 27% of the survivors (6-36 months) after moderate to severe TBI, has/have not yet been fully elucidated (Tanriverdi et al. 2010). Although some neuroendocrine diseases associated with abnormalities in the regulation of the HPA axis have been identified after TBI, it is not yet known which mechanisms play a role in the emergence of these disorders in the HPA axis. The role of autophagy and apoptosis in the emergence of HPA axis dysfunction after TBI is not known. It has been reported that TBI produced in a moderate CCI model, causes long-term dysregulation of the neuroendocrine stress response (Taylor et al. 2008).

Secondary neuronal injury is linked to a neuroinflammatory response characterized by the generation of reactive oxygen species (ROS) and inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL1), and interleukin-6 (IL6) after primary head trauma. TNF-α, in particular, is important in the neuroinflammation that occurs after TBI. TNF-α is a downstream

inflammatory signaling pathway initiator, and its activation can start a cascade of inflammatory reactions(Tapp et al. 2019). Many studies have shown increased secretion of TNF-α, a neuroinflammatory marker from active astrocytes, and microglia in the brain after TBI. Prolonged TNF-α production is harmful to neurons and leads to apoptosis and autophagy (Wolf et al. 2019; Wu and Lipinski 2019).

Apoptosis is defined as a programmed cell death mediated by mitochondria in particular and results in nucleosomal DNA fragmentation. There are 14 caspases known to be involved in the process of apoptosis in mammals. The initiator caspases (caspases 8, 9, 10) are activated as a result of apoptotic stimulation, which leads to the activation of more effector caspases. It has been shown that active cleaved caspase-3 is required to stimulate apoptosis in many studies.

Firstly, caspase-3 is synthesized as a 32-kilo Dalton (kDa) pro-enzyme, and then it is cleaved into 12 and 17 kDa subunits. Two subunits (12 k Da and two 17 k Da) are reassembled to create the functionally active cleaved-caspase-3 enzyme. After the formation of the active cleaved caspase-3 enzyme, the effector caspases, which include caspase-3, -6, and -7, initiate the degradation of many important cellular proteins. After the degradation of important proteins essential for the cell, finally, chromatin condense and DNA is fragmented (Tan et al. 2017; Ye et al. 2017; Li et al. 2019).

Basal levels of autophagy provide the removal of abnormal proteins and aged or damaged organelles in the cell in the normal state. However, when the autophagy mechanism is over-activated, it can trigger apoptosis. Autophagy is a very complicated process regulated by the transcription of at minimum 15 genes and occurring at different well-coordinated stages, including initiation, nucleation, elongation, and fusion with the lysosome. In these stages, varied autophagy-related (Atg) genes/proteins, including Beclin-1 *(Bcln1*) and microtubule-associated light chain-3 play a major role and are frequently accepted as potential markers of autophagic activity (Ozpolat and Benbrook 2015; Zhang and Wang 2018). Beclin-1 and LC3 proteins serve in different stages of the autophagy mechanism. Beclin-1 is involved in the nucleation phase, the very early stage of autophagosome formation, and is considered an essential component for the initiation of autophagy. LC3 protein has two forms, LC3-I and LC3-II. LC3-I protein is localized in the cytoplasm under normal cell conditions. When the autophagy mechanism is triggered by various stresses, such as hypoglycemia, hypoxia, and inflammation, a cytosolic form of LC3-I is transformed to LC3-II by conjugation of a lipid molecule called phosphatidyl-ethanolamine (PE) for incorporation into the membrane of autophagosomes. Therefore, LC3 is considered one of the important markers of autophagy (Ozpolat and Benbrook 2015; Klionsky et al. 2016; Saha et al. 2018).

Although the role of apoptosis and autophagy have been investigated in different regions of the brain after TBI (Liu et al. 2008), evaluation of autophagy and apoptosis in the HPA axis after TBI may explain the mechanism of endocrine disturbances

such as pituitary insufficiency after TBI. Therefore, in order to further characterize the effects of TBI on the HPA axis, the present study was to investigate the markers of apoptosis and autophagy in the acute and chronic phases after mild-CCI to reveal the underlying mechanism and the targeted glandes in the HPA axis dysfunction.

## 2. Materials and Methods

### 2.1. Animals

A total of 42 healthy 8-week-old adult male Sprague–Dawley rats weighing 250–300 g, were used in this study. These rats were housed under a 12 h light/dark cycle at 23℃ and allowed ad libitum access to food and Erciyes University, GenKok, Transgenic Department, Kayseri, Turkey. The animals were cared for and treated under the Laboratory Animal Care Principles (European rules). All experiments were performed on randomized and controlled double-blind. All animal experimental protocols were approved by The Local Ethics Committee for Animal Experiments of Erciyes University (No: 16/103).

### 2.2. Models of TBI

CCI techniques, parameters, and postoperative care have all been thoroughly reported previously (Bilgen 2005; Taylor et al. 2008). Rats were sedated with isoflurane (2.0–2.5 percent in 100% O2, 2.0 mL/min flow rate) and put in a stereotaxic frame with the head in a horizontal plane about the interaural line. The spontaneous respiration of the rats was not suppressed during the experiment. Depth of anesthesia was monitored by the chin and skeletal muscle tone. Every surgical procedure was carried out in an aseptic environment. In this method, a craniotomy is performed, and head trauma is created in the area of interest with a computerized pneumatic system at the desired speed, depth, and duration. After anesthesia, the scalp of the rat, which was fixed in the prone position with a standard nailed frame, was shaved and the area was cleaned with 10% povidone-iodine, and the periosteum was dissected by cutting the skin and fascia with a 10 mm median linear incision. The skin and galea were fixed to the laterals with a bulldog clip to provide an adequate visual field. Then, in the left parasagittal region, a craniectomy defect was produced by drilling the cranium with a 3 mm diameter dental drill 2 mm lateral to the sagittal suture and 2 mm posterior to the bregma. By varying the velocity with which the exposed dura was struck and the depth of tissue compression, a mild injury was induced (mild CCI: 20 psi, 1.5 mm). Finally, the scalp was sutured with 3/0 silk and the wound was cleaned again with 10% povidone-iodine. No operation was performed on the Sham group. Following the injury, the animals were observed daily. Rats that showed signs of pain (frozen, stooped posture, or vocalization) or infection (swelling, redness, or discharge) were eliminated from the experiment, as were those that lost more than 20% of their body weight (Bilgen 2005; Taylor et al. 2008).

### 2.3. Experimental Design

As a result of the power analysis, the amount of Type I error (alpha) was 0.05, the power of the test (1-beta) was 0.95, and the effect size was 0.82 while using the Independent Samples One-Way ANOVA test for minimum sample size required a significant difference of 9 for each group; a total of 27 male rat was used. G-power (v3.1) was used for power analysis.

The male rats were randomly divided into three groups, and each group included 14 rats. The details were as follows: (1) control group: sham- no operated group; (2) acute TBI: rats were sacrificed at 24 hours after TBI; (3) chronic TBI: rats were sacrificed 30 days after TBI. mRNA expression analyses were performed on 14 rats. Western blot analyses were performed on 9 rats because enough protein was not obtained. All of the findings were derived from the entire total tissue of the hypothalamus, pituitary and adrenals. Tissue samples were stored in Trizol (Thermo Fisher Scientific, USA) at -80°C until use. The experimental design is provided in Figure 1.

### 2.4. Western Blot Analysis

Standard procedures were used to extract total proteins from dissected hypothalamus, pituitary tissues, and adrenal glands. (Hamurcu et al. 2016). A detergent-compatible protein assay kit was used to quantify the total protein concentration in each sample (DC kit; Bio-Rad, Hercules, CA). For protein separation, aliquots comprising 40 g of total protein from each sample were electrotransferred to polyvinylidene difluoride membranes using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 4–20 percent gradient. The membranes were blocked with a blocking buffer (0.1 Triton X-100 in Tris-buffered saline–Tween 20) [TBS-T] for 60 minutes. The membranes were probed with the primary antibodies listed following after being washed with TBS-T: Tnf-α (Proteintech, Catalog no: 17590-1-AP), Corticosterone (MyBioSource, Catalog no: MBS2006378), Lc3 (Cell Signaling, Catalog no:2775S), Beclin-1(Cell Signaling, Catalog no:3738S), pro-caspase-3 (Cell Signaling, Catalog no:9662S), cleaved-caspase-3 (Cell Signaling, Catalog no:9661S), β-actin (Proteintech, Catalog no:60008-1-Ig). The membranes were treated with horseradish peroxidase-conjugated anti-rabbit (Bio-Rad, Catalog no:170-6515) or anti-mouse secondary antibody (Bio-Rad, Catalog no:170-6516) after being rinsed with TBS-T. TBS-T containing 5% dry milk was used to dilute all antibodies. Clarity Western ECL Substrate (Bio-Rad) was used for chemiluminescence detection, and the blots were viewed with a Chemidoc MP Imaging System (Bio-Rad) (Hamurcu et al. 2019). All antibodies were diluted in TBS-T containing 5% dry milk. The membranes were incubated with primary antibodies overnight at 4 °C, then with secondary antibodies for 1 hour. Chemiluminescence detection was performed with Clarity Western ECL Substrate (Bio-Rad) and the blots were visualized with a Chemidoc MP Imaging System (Bio-Rad) and quantified with a densitometer using the imager application

program (Bio-Rad Image Lab 5). All western blot experiments were independently repeated at least twice. All of the antibodies were validated and made ready for use by the supplier company "according to the antibody validation principle of Uhlen et al. (You can check manufactory web-site for antibody validation princible: https://www.cellsignal.com/about-us/cst-antibody-validation-principles) (Bordeaux et al. 2010; Uhlen et al. 2016; Hamurcu et al. 2018, 2019; Pillai-Kastoori et al. 2020).

### 2.5. RNA Isolation and Real-Time PCR

Total RNA isolation was made from tissues using Trizol (Thermo Fisher Scientific, USA) and cDNA was synthesized from the RNA samples using an Evo Script cDNA synthesis kit (Roche, Germany). The cDNA procedure was conducted according to the manufacturer`s protocol and samples were quantified using a Roche 480 Real-Time PCR (Roche, Germany). Transcript levels of *Ulk, P150, Atg5* and *Bcln1* genes in the hypothalamus-pituitary-adrenal of all groups were determined. *Actb* gene was used as a housekeeping gene. All PCR experiments were repeated twice. The Ct values were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Taheri et al. 2021).

### 2.6. Statistical analysis

The compliance of the data to normal distribution was evaluated by the histogram, q-q graphs, and Shapiro-Wilk test. For determination of the differences in gene expression data, independent samples were analyzed by ordinary one-way ANOVA or by Kruskal Walls test according to their distribution. If samples had normal distribution, parametric ordinary one-way ANOVA test was used with Tukey post-hog test. If samples didn't have normal distribution non-parametric Kruskal Walls test with Dunnett's post hog test was used.

Differences in protein expression were analyzed by unpaired student t-test or by Mann Whitney-U test according to their distribution. If samples had normal distribution, parametric unpaired student t-test was used. If samples didn't have normal distribution non-parametric Mann Whitney-U test was used

Graph-Pad Prism 8 packages were used for statistical analysis. Results with p values $<0.05$ were considered statistically significant. All data are expressed as the mean with SD. The sample size was calculated with power analysis, the amount of Type I error (alpha) was 0.05, the power of the test (1-beta) was 0.95, and the effect size was 0.82 while using the Independent Samples One-Way ANOVA test for minimum sample size required a significant difference of 9 for each group; a total of 27 male rat was used for G-power (v3.1) power analysis.

## 3. Results

TBI was generated in rats using the controlled cortical impact (CCI) model. The hypothalamus, pituitary, and adrenal tissues were removed in the acute (24 hours) and chronic phase (30 days).

The corticosterone levels were found to be increased in the adrenal tissue indicating HPA axis activation in the acute and chronic phases of TBI. In addition, TNF-α protein levels were found to be increased in the hypothalamus, pituitary, and adrenal tissues indicating neuroinflammation in the acute and chronic phases of TBI (Figure 2A-E). TNF-α protein was not detectable in the hypothalamus of the sham group (Figure 2A, 2D).

*Bcln1, P150, Ulk, and Atg5 mRNA* and Lc3, Beclin-1, pro-caspase-3, cleaved caspase-3 (Clv-caspase-3) protein levels were determined in the hypothalamus, pituitary and adrenal glands in the acute (24 hours) and chronic (30 days) phases after TBI.

### 3.1. The decrease in the levels of transcripts initiated by TBI in the hypothalamus and pituitary from the acute phase worsens during the chronic phase.

Among the four transcripts tested, the *Bcln1* transcript levels were slightly, albeit not significantly lower in the hypothalamus and pituitary during the acute (respectively, dF=2,2; p=0,41 and p>0,99) and chronic phases (respectively, dF=2,2; p=0,73 and p>0,15) of TBI than the sham group (Figures 3A and 3B). The *Ulk* transcript levels were unchanged in the hypothalamus in the acute (dF=2; p=0,27) and chronic phases (dF=2; p=0,43) of TBI (Figure 3A); but in the pituitary during the acute phase *Ulk* transcripts were reduced (dF=2; p=0,049), which was not maintained during the chronic phase (dF=2; p>0,99) (Figure 3B). In contrast, two other transcripts, P150 (dF=2, p=0.0004; dF=2, p<0.001) and Atg5 (dF=2, p=0.0012; dF=2, p=0.001) were significantly decreased from the acute phase and further decreased in the chronic phase after TBI in the hypothalamus (Figure 3A) and pituitary compared to the sham group (Figure 3B).

### 3.2. TBI induces distinct transcript profiles in the adrenals compared to the hypothalamus and pituitary from the acute phase to the chronic phase.

*Bcln1* was the only transcript to increase in the adrenal tissue of the chronic group compared to the acute group of TBI and the sham group (dF=2, p=0.007) (Figure 3C). Three other transcript levels (*Ulk, P150,* and *Atg5*) were significantly decreased compared to the sham group in the acute phase of TBI (respectively, dF=2, p= 0.011; dF=2, p= 0.031; dF=2, p= 0.023) (Figure 3C). The levels of the *Ulk* and *Atg5* transcripts were decreased compared to the sham group in the acute and chronic phases of

TBI. The decrease was significant in the adrenal only in the acute phase (*Ulk*: dF=2, p=0.025; *Atg5*: dF=2, p=0.023) (Figure 3C).

### 3.3. TBI increases apoptosis in the adrenal and pituitary but the transcript levels of the autophagy markers are not changed.

When autophagy is induced, cytosolic Lc3-I is transformed to Lc3-II and localized in autophagosome membranes by the addition of PE. As a result, Lc3-II expression is widely considered to be a sign of autophagy induction (Lee et al. 2016). When autophagy is induced, cytosolic Lc3-I is transformed to Lc3-II and localized in autophagosome membranes by the addition of PE. As a result, Lc3-II expression is widely considered to be a sign of autophagy induction. Both Beclin-1 and Lc3 proteins are crucial autophagy mediators and are involved in distinct phases of the autophagic process (Ueno et al. 2016). Western blot analysis showed that Beclin-1 protein levels were similar in the hypothalamus, pituitary, and adrenal glands in the acute and chronic TBI groups compared to the sham group (Figure 3D, Supplementary Figure 1).

To evaluate the occurrence of apoptosis after TBI in the hypothalamus, pituitary, and adrenal in the acute and chronic phases, we assessed the expression of total caspase-3 and levels of Clv-caspase-3 (active form of caspase 3) using Western blot analysis. There was no detectable level of Clv-caspase-3 protein in the hypothalamus in the acute and chronic phases after TBI (Figure 4A). We found that the induction of Clv-caspase-3 showed a marked increase in the acute phase in the adrenal and in the chronic phase in the pituitary after TBI (Figure 4B, 4C).

## 4. Discussion

TBI leads to two types of injury, the primary and secondary injury which includes edema, disturbed blood flow, and neuroinflammation, and has been implicated in the development of post-injury neuroendocrine diseases such as neuropsychiatric disorders and pituitary insufficiency (Tanriverdi et al. 2010). The HPA axis is responsible for providing an adequate cortisol response to maintain the homeostasis of organisms in stressful situations, such as TBI. Endocrine dysfunctions after TBI have been clinically described in all hypothalamic-pituitary axes. In addition, hypopituitarism has been detected in 27% of the survivors (6-36 months) after moderate to severe TBI. Although these abnormalities that emerge after TBI are temporary in most patients, they are permanent in some (Tanriverdi et al. 2015).

Based on the evidence so far, it's tempting to speculate that chronic neuroinflammation plays a role in the development of long-term endocrine dysfunctions following TBI, especially in people who have a hereditary susceptibility (Tanriverdi et al. 2008a, b, 2010; Kasturi and Stein 2009). It is very important to investigate cellular mechanisms that may be beneficial for the development of new treatments that can provide protection in secondary injury cases after TBI.

We investigated whether autophagy or apoptosis was involved in the regulation of vital cell functions in the hypothalamus, pituitary, and adrenal tissues of rats after the mild controlled cortical impact (CCI) model and we focused on the transcript and protein markers associated with autophagy and apoptosis.

The HPA axis is activated acutely after a TBI as a result of the stress of the damage. Following the injury, TBI raises serum corticosterone levels, which peak at 3 hours after TBI compared to controls and return to normal by 5 hours after TBI in rats. This same increase in corticosterone levels together with the HPA axis activation is also seen in patients in the first 1–2 days following mild and moderate TBI (Taylor et al. 2008). On the other hand, TBI may result in the HPA axis dysfunction characterized by decreased corticosterone levels. Generally, corticosterone and TNF-α protein levels are measured from the sera to detect the activation and neuroinflammation in the HPA axis (Taylor et al. 2008). In this study, we determined the corticosterone and TNF-α protein levels in tissues-specifically by western blotting assay in order to show the effect of TBI only in the target tissues. We have shown that the corticosterone levels, as a sign of the HPA axis activation in the adrenals, in the acute and chronic phases after mild CCI model, increase as expected. In addition, increased TNF-α protein levels in the hypothalamus, pituitary and adrenals in the acute and chronic phase after mild CCI were also evidence of increased neuroinflammation.

Regarding autophagy transcripts, we demonstrated that the levels of *P150* and *Atg5* transcripts are significantly decreased during the chronic phase in the hypothalamus and pituitary. In contrast, in the adrenals, the levels of *P150* and *Atg5* transcripts were only decreased in the acute phase. The decrease in P150 and Atg5 protein levels in the hypothalamus is a sign of damage to the neurons (Wu and Lipinski 2019). Here, we suggest that a decrease in *P150* and *Atg5* transcript levels in the adrenal tissue may be associated with an increase in apoptosis ratio as well independently from autophagy.

We found that the levels of *Ulk* transcripts are decreased in the acute phase in the pituitary and adrenals after TBI. In the adrenal tissue, in contrast to the hypothalamus and pituitary, the levels of *Bcln1* transcripts were increased in the chronic phase. Altogether, more robust alterations in the levels of transcripts were observed in the adrenal than in the hypothalamus and pituitary in response to TBI. Previous studies have demonstrated that autophagy is activated post-TBI, and the investigators thought that inhibition of the autophagic pathway might improve neurological deficits (Clark et al. 2008; Gao et al. 2017; Zhang and Wang 2018). However, none of these studies focused on the HPA axis-related tissues after TBI. According to our results, although the transcript levels of autophagic markers showed significant changes in the hypothalamus, pituitary and adrenal in the acute and chronic phase after TBI, these changes were not reflected in the autophagy related-protein levels of Lc3 and Beclin-1. In other words, there was no change in the autophagic activity in the HPA axis-related tissues in the acute or chronic phase after TBI in

terms of protein expression. Although the transcript levels of autophagic markers are significantly lower in all tissues after TBI, especially during the chronic phase these changes were not reflected in the tissue protein levels of these transcripts. There appears to be a role for autophagic transcripts in regulating the HPA axisafter TBI.

In the pituitary, the marker of apoptosis "Clv-caspase-3" expression was only detected in the chronic phase after TBI. Remarkably, in the adrenal tissue, Clv-caspase-3 expression was increased in the acute phase, but it was not expressed in the chronic phase. The transient increase in apoptosis during the acute phase after TBI in the adrenal may correspond to an active regenerative process in the tissues. To date, no studies in the literature have investigated the response and situation of the adrenal after TBI, therefore, more investigation is required on this issue which could provide information to prevent consequences from secondary injury.

In 2011, Chen et al. created TBI in rats using the fluid percussion injury model and found that the rate of apoptosis increased in the hypothalamus and pituitary in the 7th and 14th days after TBI (Chen et al. 2011). Tan et al. developed an intracranial hypertension model to investigate its effects on the HPA axis caused by TBI. They found that 24-hour intracranial hypertension increased the rate of apoptosis, particularly in the hypothalamus and pituitary (Tan et al. 2017). However, we found that the ratio of apoptosis markers in the hypothalamus and pituitary did not change in the acute phase after TBI. In contrast, we found increases in apoptosis marker Clv-Caspase-3 expression in the adrenal glands as the target organ in the acute phase of the HPA axis.

Adrenal insufficiency due to suppressed activation of the HPA axis emerges in 11-13 percent of all TBI cases, according to clinical evidence (Tanriverdi et al. 2015). After TBI, corticosterone (cortisol in human) levels are usually increased due to the stress of the injury. On the other hand, TBI can also result in the HPA axis dysfunction characterized by decreased cortisol levels (Taylor et al. 2008). The present study revealed the increased ratio of apoptosis in the acute phase of TBI in the adrenal glands, in contrast, corticosterone levels were increased both in the acute and chronic phases after TBI (Garrahy and Agha 2016; Taheri et al. 2021).

After TBI, although the literature has generally focused on the hypothalamus and pituitary (Tan et al. 2017; Zhang and Wang 2018; Wu and Lipinski 2019), we did not detect any changes in Clv-Caspase-3 protein expression in the hypothalamus in the acute and chronic phases after TBI, which may be associated with differences in the model systems used. Many studies have declared that apoptosis increases in the hypothalamus especially in the acute phase after TBI (Chen et al. 2011; Tan et al. 2017). We found that the increase in Clv-caspase-3 protein expression was only detected in the acute phase in the adrenal tissue, and subsequently only in the chronic phase in the pituitary. We speculated that neuroinflammation in the brain after TBI may trigger

changes in the adrenal glands. In addition, an increased ratio of apoptosis in the adrenals in the acute phase may also disrupt communication throughout the HPA axis and lead to permanent damage in the pituitary in the chronic phase. Finally, these results may help to explain at least one of the underlying mechanisms of endocrine disturbances that occur after TBI, especially the mechanism of pituitary insufficiency after TBI. Although adrenals are not directly affected by TBI, we suggest that the role of the adrenal glands together with the hypothalamus and pituitary should not be ignored in the acute phase after TBI.

Determining which molecular mechanisms are playing a role in the HPA axis after TBI is crucial for both TBI treatment and prevention of injuries (Tanriverdi et al. 2008a, 2010, 2015; Taheri et al. 2016). After TBI, HPA axis dysfunction can arise at any level of the HPA axis and this situation may prevent the suppression of neuroinflammation by preventing the appropriate response to stress.

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**Author Contributions**

FK**,** KU, MR, ZK, FT, HU and ST conceived and designed the experiments and the primary research hypothesis; EM**,** ZY, FD and ST created the TBI model and collected tissue from mice. ST, FD, VC and EM performed the experiments; EM and ZY performed the statistical analysis and data representation; ZK, FT, HU and ST analyzed the draft manuscript and corrected the style and content of the paper; and FK, KU, MR, ZK, HU and ST 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.

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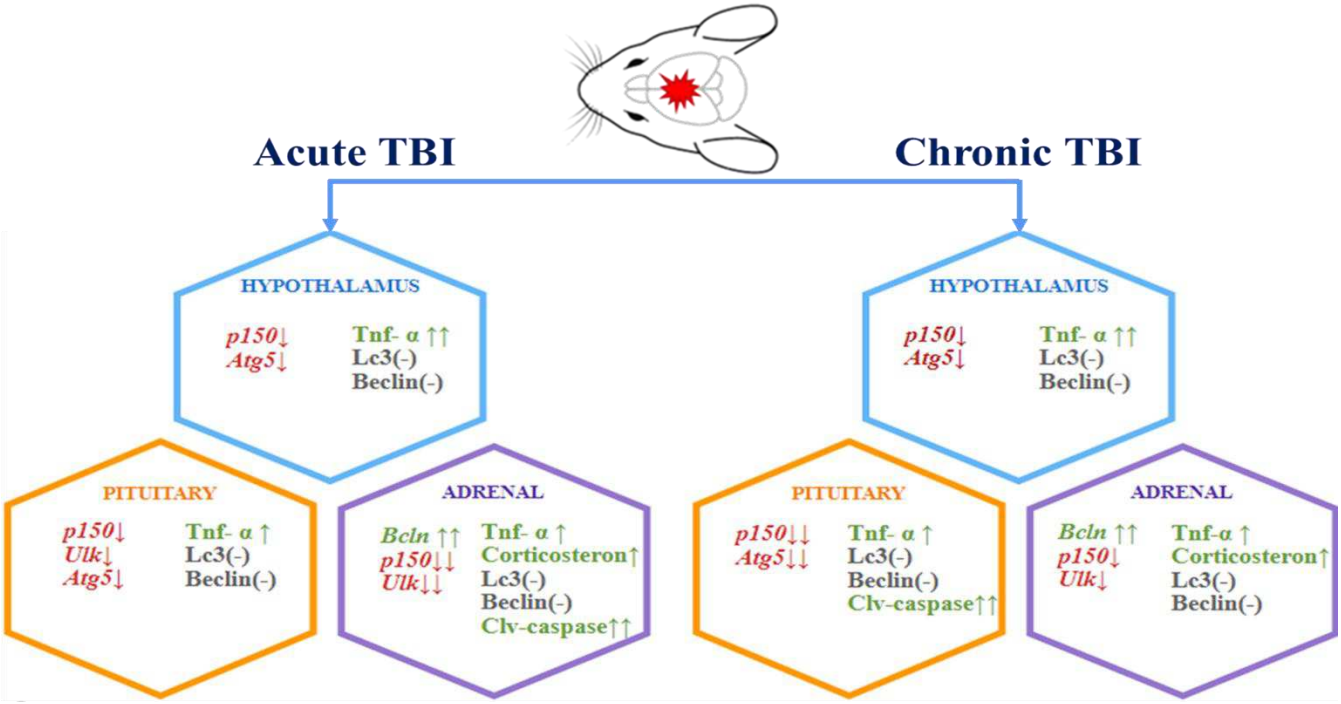
## Graphical Abstract

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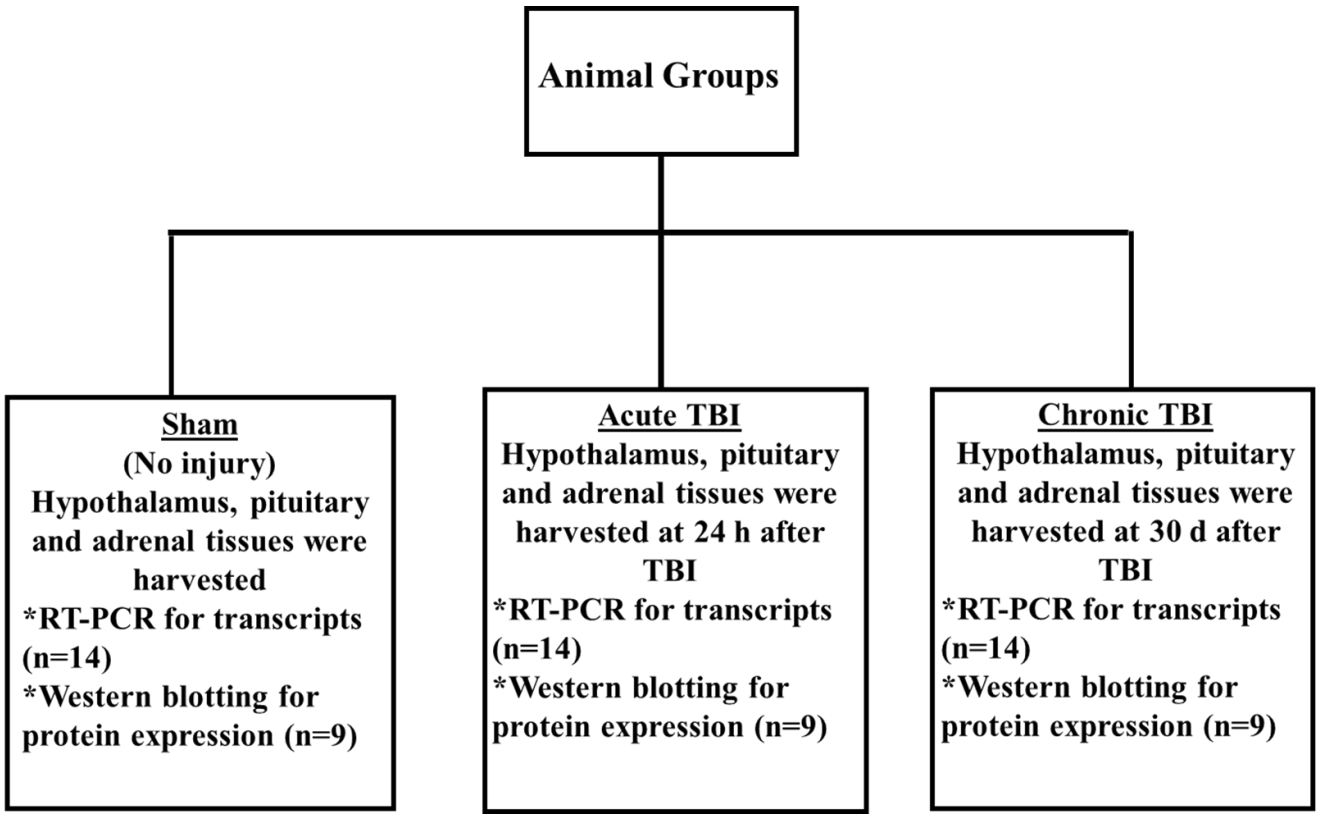


**Fig. 1** Experimental Design. Anesthetized male rats were subjected to TBI with the The controlled cortical impact (CCI) or sham procedure (without CCI). Sham animals were sacrificed directly without any operation. Acute TBI group was sacrificed at 24 h after TBI with CCI. Chronic TBI group animals were sacrificed at 30 days after TBI with CCI. The male rats were randomly divided into three groups, and each group included 14 rats. The details were as follows: (1) control group: sham- no operated group; (2) acute TBI: rats were sacrificed at 24 hours after TBI; (3) chronic TBI: rats were sacrificed 30 days after TBI. Gene expression analyses were performed on 14 rats and western blot analyses on 9 rats. Biological and technical replicates for each experiment were added included in both the manuscript and figure legends as below. Gene expression analyses were performed on 14 rats and western blot analyses on 9 rats. All PCR experiments and western blot analyses for detecting transcript and protein levels were repeated at least twice independently and blindly.

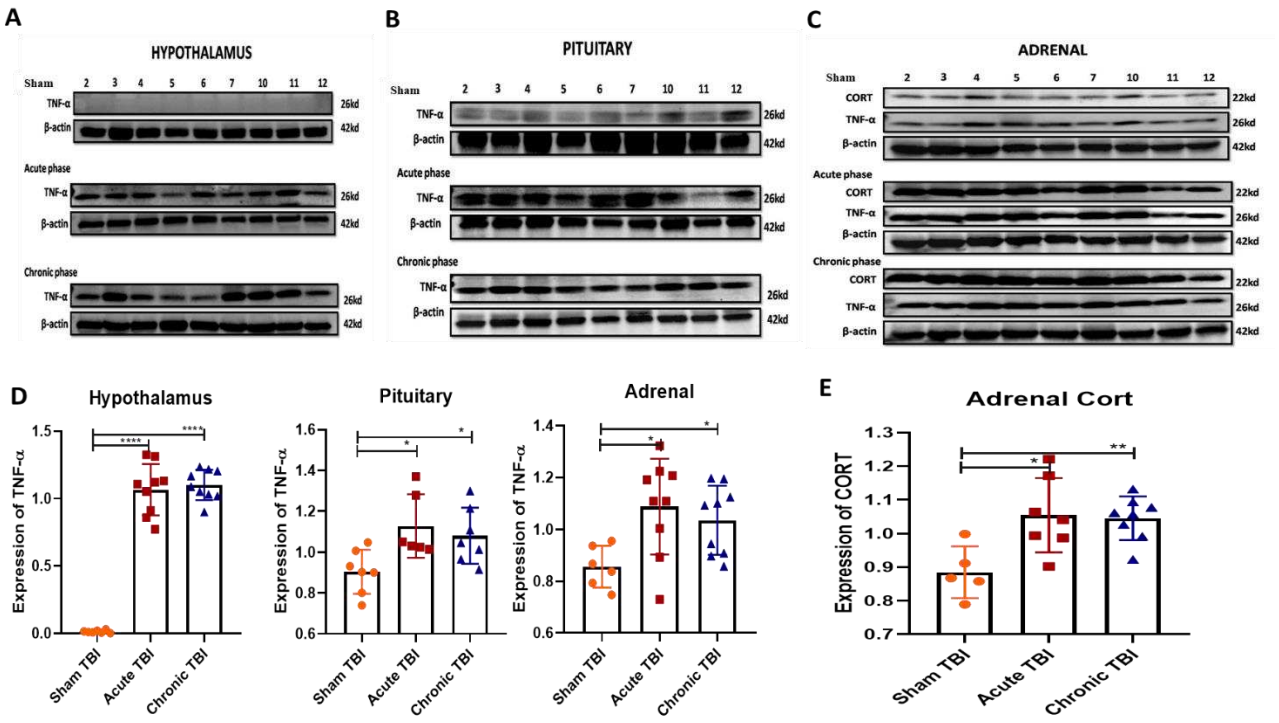


Fig. 2

**Fig. 2** Western blot images of Tnf-α and Corticosterone (Cort) levels in the acute and chronic phase after TBI A. Tnf-α protein levels in the hypothalamus in the acute and chronic phases after TBI. β-Actin was used as a loading control. Data are presented as mean ± SD (n = 9 rats in each group). B. Tnf-α protein levels in the pituitary in the acute and chronic phases after TBI. C.Tnf-α and Cort levels in the adrenal in the acute and chronic phases after TBI. β-Actin was used as a loading control. Data are presented as mean ± SD (n = 9 rats in each group). D. The graph showing Tnf-α protein levels in the hypothalamus, pituitary, and adrenal in the acute and chronic phases after TBI. β-Actin was used as a loading control. Data are presented as mean ± SD (n = 9 rats in each group). (*p<0.05, ** p<0.001, **** p<0.0001). All western blot analyses were independently repeated at least twice.

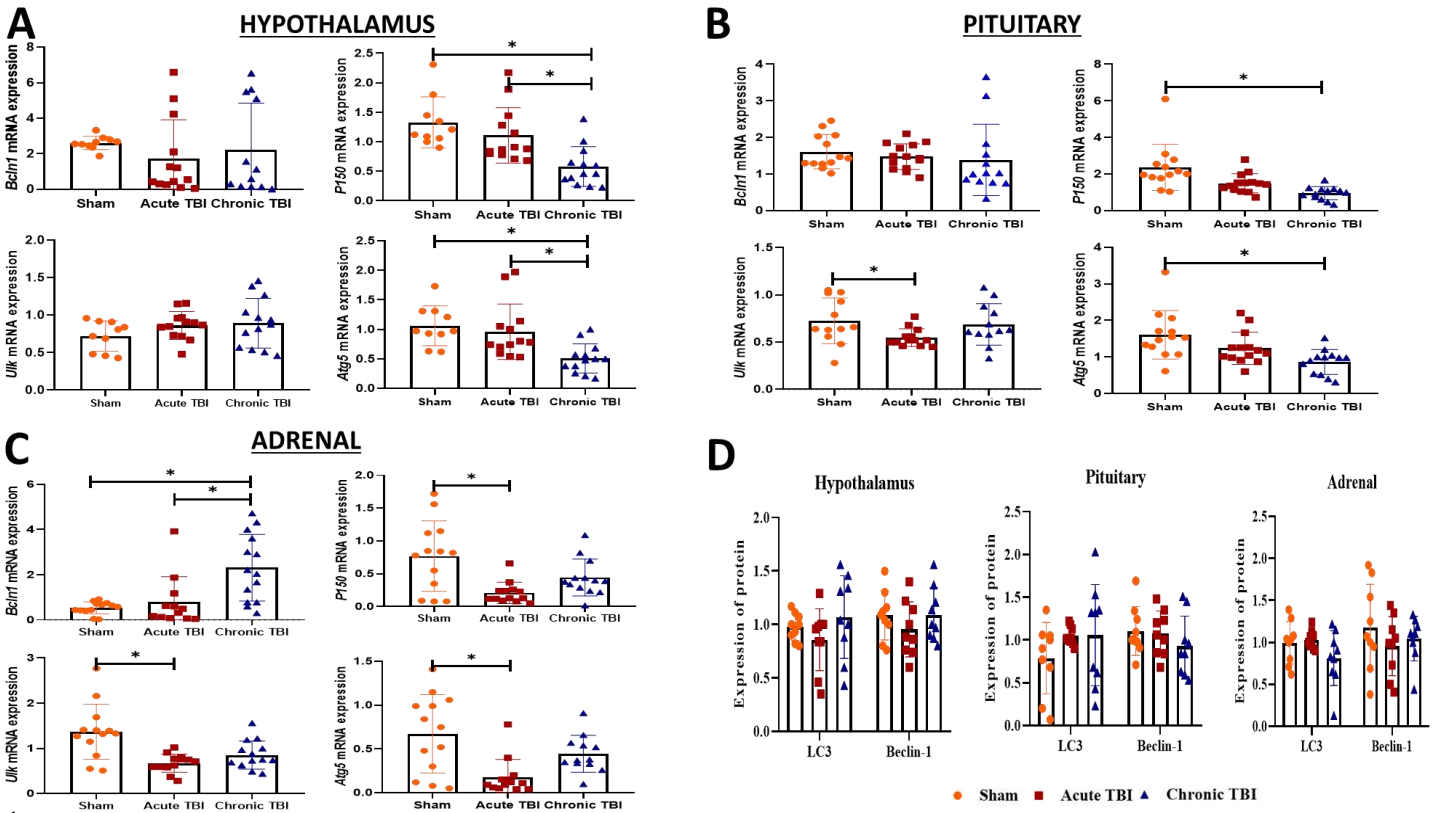


**Fig 3. A.** The transcript levels of *Bcln1*, *P150*, *Ulk* and *Atg5* genes in the hypothalamus after TBI (A, B, C and D) (Bar Graph) (*p<0.05). Box plots are expressed as mean ± SD (n = 14 rats in each group). **B.** The transcript levels of *Bcln1*, *P150*, *Ulk* and *Atg5* genes in the pituitary after TBI (A, B, C and D) (Bar Graph) (*p<0.05). Box plots are as mean ± SD (n = 14 rats in each group). **C.** The transcript levels of *Bcln1*, *P150*, *Ulk* and *Atg5* genes in the adrenal after TBI (A, B, C and D) (Bar Graph) (*p<0.05). Box plots are expressed as mean ± SD (n = 14 rats in each group). All PCR experiments for detecting transcript levels were repeated at least twice independently and blindly. **D.** The graph showing LC-3 and Beclin-1 protein levels in the hypothalamus, pituitary, and adrenal in the acute and chronic phases after TBI. Data are presented as mean ± SD (n = 9 rats in each group). All western blot analyses were independently repeated at least twice

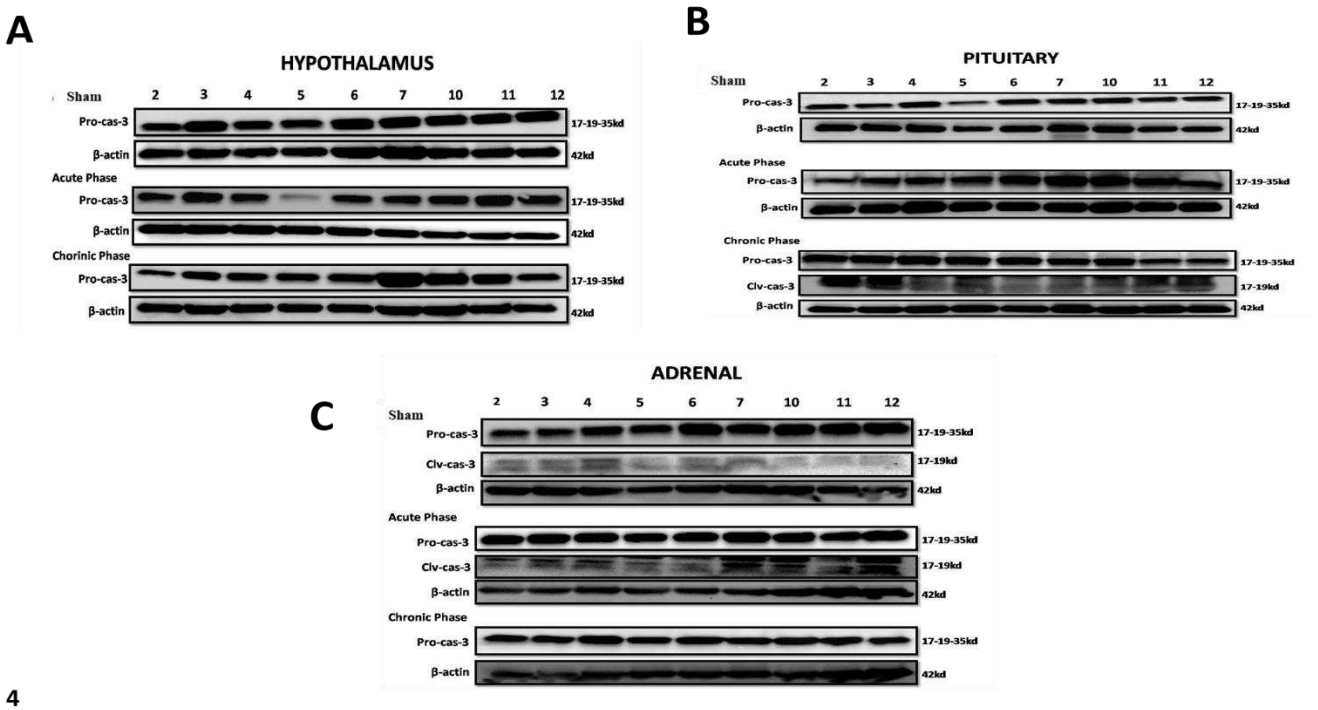


Fig. 4

**Fig. 4** Western blot images of Clv-caspase-3 in the acute and chronic phase after TBI in the hypothalamus, pituitary, and adrenal **A.** Clv-caspase-3 protein levels in the hypothalamus in the acute and chronic phases after TBI. **B.**Clv-caspase-3 protein levels in the pituitary in the acute and chronic phases after TBI. **C.**Clv-caspase-3 protein levels in the adrenal in the acute and chronic phases after TBI. β-Actin was used as a loading control. Data are presented as mean ± SD (n = 9 rats in each group). All western blot analyses were independently repeated at least twice.

## Supplementary Files

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- supplementfigure2.pptx