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The Dose-dependent Dual Effects of Corticosterone on Spatial Memory Ability After Traumatic Brain Injury Are Mediated by Two Corticosteroid Receptor Systems

Bin Zhang
Beijing Tiantan Hospital
Mengshi Yang
Beijing Tiantan Hospital
Qiongyu Yan
Beijing Tiantan Hospital
Xiaojian Xu
Beijing Neurosurgical Institute
Fei Niu
Beijing Neurosurgical Institute
Jinqian Dong
Beijing Tiantan Hospital
Yuan Zhuang
Beijing Tiantan Hospital
Shenghua Lu
Beijing Neurosurgical Institute
Qianqian Ge
Beijing Tiantan Hospital
Baiyun Liu (✉ liubaiyun12121963@163.com)
Beijing Neurosurgical Institute

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Abstract

Background: Our recent studies reported the opposite effects of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) on neuron survival after traumatic brain injury (TBI). However, as a mixed agonist for MR and GR, whether short term use of high-dose endogenous glucocorticoids exerts neurotoxic effects by excessive activation of GR, what is the set-point, and the possible signaling pathways remain unclear. This study examined the dose-dependent dual effects of corticosterone (CORT) on the spatial memory, the survival of hippocampal neurons and the possible receptor-mediated downstream signaling pathways after TBI.

Methods: Based on controlled cortical impact (CCI) and CORT treatments, Sprague-Dawley rats (n=168) were randomly divided into the sham, CCI, CCI + CORT1 (0.3 mg/kg), CCI + CORT2 (3 mg/kg), CCI + CORT3 (30 mg/kg), CCI + CORT1 + spirolactone (spirolactone: 50 mg/kg/d), and CCI + CORT3 + RU486 (RU486: 50 mg/kg/d) groups. Brain tissues were collected on postinjury day 3 and processed for histology and western blot analysis.

Results: On postinjury day 3, we tested the learning and memory ability, neuronal apoptosis in the hippocampus, activation levels of MR and GR, Bcl-2 family proteins, and apoptosis-related intracellular signaling pathways. We found that different doses of CORT exhibited dual effects on the survival of hippocampal neurons and the spatial memory. Lower doses of CORT (0.3, 3 mg/kg) significantly increased the activation of MR, upregulated the phosphorylation of Akt/CREB/Bad and the Bcl-2 expression, reduced the number of apoptotic neurons, and subsequently improved the spatial memory. In contrast, higher dose of CORT (30 mg/kg) exerted opposite effect by over activating GR, upregulating the expressions of P53/Bax, and inhibiting the Erk/CREB activities.

Conclusion: The results suggest that there is a threshold between the neuroprotective and neurotoxic effects of endogenous GC, higher dose of which, even for short-term use, should also be avoided after TBI.

Introduction

Glucocorticoids (GCs), especially the synthetic GCs, are widely used in a large range of diseases since their discovery in the 1950s. However, their clinical use is limited by severe adverse effects, such as diabetes mellitus, osteoporosis, hypertension, increased risk of infection [1-3]. In recent years, the central side effects of synthetic GCs, including cognitive, mental, and stress dysfunction, are of serious concerned [4,5]. In central nervous system, the effects of GCs are mediated by two receptors: the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) [6,7]. Endogenous GCs (cortisol in human/ corticosterone (CORT) in rodent) are mixed agonists for both MR and GR, but has a 10-fold higher affinity for the MR, while synthetic GCs (methylprednisolone (MP) and dexamethasone (DEX)) with higher affinity for GR than MR are more potent anti-inflammatory and immunosuppressive agents [8]. Experimental studies have revealed that the sufficient activation of MR is crucial for the normal functions

of many structures in the brain including hippocampus and hypothalamus, while excessive or prolonged activation of GR caused by long-term synthetic GR agonist treatment or excessive endogenous secretion (chronic stress/Cushing syndrome) cause severe cognitive, stress, and mood disorder [8-12].

The secondary brain injury, including brain edema, increased intracranial pressure, hemorrhage/ischemia, apoptosis, inflammation, excitotoxicity, oxidative stress, calcium dysregulation, and axonal degeneration, occurs after primary brain injury [13,14]. Apoptosis is a major form of cell loss after traumatic brain injury (TBI) leading to severe neurological and psychiatric complications which seriously influence the life quality of the survivors or even threaten their lives [15]. Synthetic GCs have been commonly used in TBI patients to alleviate the secondary brain injury. However, the outcomes of the previous clinical trials were inconsistency or even opposite [16,17]. One study reported the detrimental effects of MP on TBI patients [18]. Our recent findings showed that the deleterious effects of high-dose GCs might be central origin. Excessive activation of GR by MP and DEX increased the neuronal apoptosis by activating GR in hippocampus and hypothalamus, aggravated spatial memory and stress dysfunction, and increased the mortality. In contrast, low-dose corticosterone (CORT) replacement restored the plasma CORT, refilled MR, and promoted the survival of neurons, but the dose-dependent effects of CORT and the receptor-mediated downstream mechanisms remain unclear [19-21]. cAMP-responsive element-binding protein (CREB) and several intracellular pathways have been demonstrated to be crucial for regulating the apoptotic-related proteins and neuronal apoptosis after TBI. [22, 23]. However, as a mixed agonist for both MR and GR, whether short term use of high-dose CORT has deleterious effects on hippocampal neurons, at which dose level CORT activates GR, and the crosstalk between CORT and apoptosis-related signaling pathways in the hippocampus after TBI remain unclear.

In this study, we hypothesize that different doses of CORT play opposite roles in hippocampal cell survival ang spatial memory after TBI, lower doses of CORT restore the activation of MR, promote the survival of neurons, whereas higher doses of CORT increase neuronal apoptosis by activating GR. CREB and Bcl-2 family proteins might be involved in the dose-dependent effects of CORT. By using a rat TBI model and different doses of CORT, we tested our hypothesis and found a proper dosage that could fully activate MR but avoid overactivation of GR.

Materials And Methods

Animal preparation and controlled cortical impact (CCI)

Adult male Sprague-Dawley rats (weighing 300–320 g) were used in the present study. The rats were housed individually under controlled conditions (temperature, 22±1°C; humidity, 60%) with a 12-hr light/dark cycle. food and water were available ad libitum. All protocols were approved by the Capital Medical University Institutional Animal Care and Use Committee. Controlled Cortical Impact (CCI) was performed as previous described [19]. Briefly, all Rats were anesthetized by isoflurane inhalation and the head was fixed in a stereotaxic frame. A 6mm craniotomy was made in the middle of the right parietal bone, leaving the underlying dura intact. Then rats were then subjected to impact with parameters we

have described before (moderate TIB model: velocity 2.8 m/s; compression time 85 ms; and depth 2 mm; diameter of impactor tip 5 mm). Sham operated rats underwent the same procedure without percussion.

Experimental groups and CORT treatments

All 168 rats (n=24 per group) were grouped by CCI and treatments into: 1) sham control group (Sham); 2) CCI + dimethyl sulfoxide (DMSO, with a final concentration <1%) group (CCI); 3) CCI + CORT (0.3, 3, 30mg/kg, sigma) group (CCI+CORT1; CCI+CORT2; CCI+CORT3), 4) CCI + Spironolactone (50mg/kg, ab141289, Abcam) + CORT (0.3mg/kg) group (CORT1+SPIRO); and 4) CCI + Mifepristone (50mg/kg, ab141289, Abcam) + CORT group (30mg/kg) (CORT3+RU486). Drugs were administered intraperitoneally for 3 days after CCI. To effectively block MR and GR, SPIRO and RU486 (twice a day) were given for 2 days before CCI and 3 days after CCI. All drug dosages were chosen based on pilot experiments from our laboratory and our previous study [19].

Morris water maze (MWM)

MWM test was used to test the spatial memory ability in this study. Rats (n=24 per group) were trained using MWM before injury according to the protocol described before [20]. Each rat received 4 trials per day for 5 consecutive days (8-4 days before CCI) to find the platform submerged below the water (20±2°C, with nontoxic black ink). The pool (150 cm in diameter) was divided into four equal quadrants (northwest, northeast, southwest, southeast). Each trial (with 5 min interval) was started from different positions (north, east, southeast, and northwest) and lasted at most 120 s. if the animal reached the platform within 120 s, the time was recorded as the latency time. If it failed to find the platform, the trial was terminated and the animal was placed on the platform for 15 s, the latency time was recorded as 120 s. To assess the spatial memory, probe trial was conducted 1 day before and 3 days after CCI. The hidden platform was removed, and the rats started at a northeast position. The percentage of time spent in the goal quadrant during the 30-s (total) swimming period was recorded.

TUNEL assay and H&E staining

The rats (n=8 per group) were sacrificed by decapitation on day 3 after CCI, and then 5 μm-thick coronal paraffin sections at the level of the hippocampus were made according to the Paxinos atlas of the rat brain [24]. To assess the survival apoptosis of neurons in the hippocampus, H&E staining and TUNEL assay (Roche, Germany) were carried out as we previously described [19]. Briefly, after rehydration, tissue sections were incubated with proteinase K working solution (20 mg/ml in 10 mM Tris-HCl buffer, pH 7.5–8.0) for 10 min at 37°C. They were rinsed twice with 0.01 M PBS (pH 7.4) and subsequently incubated at 37°C with the TUNEL reaction mixture for 1 h. Finally, color development was conducted for 10 min using 3, 3-diaminobenzidine (DAB) substrate. A MIDI FL (3D Histech, Hungary) system was used to obtain the digital images of the brain sections. A digital image analysis system (3D Histech, Hungary) was used to count the number of neurons and apoptotic cells in the ipsilateral hippocampus (three sections) as previously described [16]. Data are presented as the number of hippocampal neurons per mm and the total number of TUNEL-positive cells. All analysis was performed in a blinded fashion.

Immunofluorescence staining

Immunofluorescence staining for cleaved-caspase 3 were performed as we previously

described [21]. Briefly, the brain sections were incubated in 3% hydrogen peroxide for 0.5 h and then blocked with normal bovine serum for 0.5 h. The brain sections were washed three times and incubated with rabbit polyclonal anti-cleaved caspase-3 (1:400, CST, #9664), overnight at 4°C. The slices were then washed with PBS and incubated with Alexa Fluor 647-conjugated donkey anti-rabbit IgG at room temperature for 2 h. Finally, the samples were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO) for 10 min. Digital images of the whole brain sections were obtained by a MIDI FL (3D Histech, Hungary) system.

Western blot

The rats (n=16 per group) were sacrificed by decapitation on day 3 after CCI and the right hippocampus was removed at 4°C. Total (n=8 per group) and nuclear protein (n=8 per group) extraction were performed as previously described [25]. Equal amounts of proteins (30 μg) were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. Blots were incubated in 5% nonfat milk or BSA for 2 hr and then reacted overnight at 4°C with primary antibody P-Akt (ser 473, 1:5000; ab81283), P-Ert (1:1000, CST, #9010), P-CREB (ser133, 1:5000; ab32096), P53 (1:1000, ab131442), Bax (1:1000, ab32503), P-Bad (S136, 1:1000, ab28824), cleaved caspase-3 (1:1000, #9664, CST), Bcl-2 (1:1000; ab196495), MR (1:400, ab2774), GR (1/200 dilution; ab3578), β-Actin (1:5000; ab179467, Abcam, UK), and Histone H3 (1:400; Millipore Co.), followed by incubation with secondary antibodies for 2 hr at room temperature. Blots were visualized using chemiluminescence (Bio Spectrum 500 Imaging System; UVP Co., Upland, CA, USA) and ImageJ software determined the intensity of each band. The percent expression compared with that of the sham controls was calculated for each sample.

Co-immunoprecipitation

Co-immunoprecipitation was performed to assess protein-protein interaction among Bcl-2, Bad, and Bax. According to previously described [26], an aliquot of 500 μg total protein was first pre-treated with either rabbit polyclonal anti-Bcl-2 (1:1000; ab196495, Abcam, UK) or rabbit polyclonal anti-Bad (1 μg/ml, ab90435, Abcam). A total of 20 μL Protein A/G agarose (Sigma) was added to each sample, and the mixture was incubated overnight at 4 °C, and then centrifuged for 1 min at 12,000g. To remove non-specifically bound proteins, the precipitates were rinsed with NP-40 buffer four times. Agarose-bound immunocomplexes then were released by resuspension in a loading buffer containing denaturing solution. IgG was used as a negative control for precipitation.

Statistical analysis

All data are depicted as the mean ±SDs. Data were analyzed using SPSS 22.0 (IBM Corporation, USA). The percentage of time spent in the goal quadrant, cell counts, and western blot were statistically

analyzed using one-way analysis of variance (ANOVA). Repeated ANOVA was used to analyze the Escape latency. A P-value < 0.05 was considered statistically significant.

Results

The dose-dependent effects of CORT on spatial memory in rats after TBI

To assess the baseline spatial learning and memory ability, latency time and probe trials was tested before TBI. Escape latencies in all experimental groups were significantly reduced (P<0.05) from 1 days to 5 days, while no significantly differences were observed among different groups at each time point [Repeated ANOVA, F (4,644) = 8365.233 for time, F (6,161) = 0.866, P>0.05 for treatments] **(Fig. 1A)**. In this study, we used probe trials to evaluate the effects of dose-dependent effects of CORT treatment on spatial memory ability. We found that there was no statistic difference in the percent time among all groups before TBI [one-way ANOVA, F (6,167) = 1.289, P>0.05], but it was significantly reduced after TBI. lower doses of CORT (0.3 and 3 mg/kg) improved the recovery of spatial memory and significantly increased the percent time in the goal quadrant compared with CCI control group. On the contrary, a high dose of CORT (30 mg/kg) significantly reduced the percent time in the goal quadrant compared with CCI group. [one-way ANOVA, F (6,167) = 129.755, P< 0.05] **(Fig. 1B)**. The opposite effects of low-dose and high-dose CORT were counteracted by SPIRO and RU486 respectively.

The dose-dependent effects of CORT on the survival of hippocampal neurons after TBI

In accordance with previous studies, our recent studies found that secondary injury, including inflammation, edema, as well as apoptosis peaked at about 48-72 hrs after TBI. In the present study, we tested the number of apoptotic and survival neurons in the ipsilateral hippocampus on post-injury day 3 using TUNEL assay **(Fig. 2A)** and H&E staining **(Fig. 3A)**. Our results showed that the number of TUNEL-positive cells was significantly increased (P 0.05) and the number of survival neurons was significantly reduced (P 0.05) in the ipsilateral hippocampus on post-injury day 3 **(Fig. 2B, 3B)**. Accordingly, the level of cleaved caspase-3 was significantly (P0.05) increased **(Fig. 4)**. Lower doses of CORT (0.3 and 3mg/kg) significantly (P0.05) reduced the level of cleaved caspase-3 and the number of apoptotic neurons, and subsequently increased the number of survival neurons compared with CCI control rats (P0.05), whereas high-dose CORT (30mg/kg) significantly increased the level of cleaved caspase-3 and the number of TUNEL-positive cells, and accordingly reduced the number of neurons (P0.05). In addition, the dose-dependent effects of CORT on cell survival were counteracted by SPIRO and RU486 pretreatment respectively [One-way ANOVA, F (6,55) =124.749 for apoptosis; F (6,55) =271.516 for cell count, P<0.05; F (6,55) =130.236 for c-caspase 3].

The dose-dependent effects on the survival of neurons were mediated by two receptor systems-MR and GR

The nuclear translocation of receptors is an important parameter for assessing the activation level of MR and GR. Our recent studies have proved that CORT insufficiency, inadequate activation of MR and

imbalance of MR/GR are important factors leading to apoptosis of hippocampal neurons in the acute phase after TBI [20]. In this study, we showed that nuclear MR and the ratio of MR/GR were significantly (P□0.05) reduced in the ipsilateral hippocampus after TBI. CORT treatments significantly (P□0.05) increased nuclear MR compared with TBI control group, but only lower doses of CORT (0.3mg/kg and 3mg/kg) significantly increased the ratio of MR/GR compared with CCI group. When the dose of CORT was increased to 3 mg/kg, nuclear GR level began to increase and peaked at 30mg/kg (P□0.05). However, a significantly (P□0.05) reduced ratio of MR/GR was only found in the high-dose CORT group compared with CORT1 group. The SPIRO and RU486 pretreatment inhibited the increased activation of MR and GR respectively [One-way ANOVA, F (6,55) = 96.631 for MR; F (6,55) = 238.763 for GR; F (6,55) = 53.239 for MR/GR] **(Fig. 5)**.

The dose-dependent effects of CORT on the expressions and interactions of Bcl-2 family proteins

Both extrinsic and intrinsic apoptosis are regulated by Bcl-2 family proteins, which include anti-apoptotic (Bcl-2) and pro-apoptotic (Bax and Bad) proteins. The ratio of Bax/Bcl-2 and the interactions between these proteins are important factors determining whether apoptosis occurs. **Fig. 6** showed the levels of Bcl-2, Bax, and P-Bad. Bcl-2 and P-Bad in the ipsilateral hippocampus were significantly (P□0.05) reduced at 3 days after TBI, whereas the expression of Bax and the ratio of Bax/Bcl-2 were significantly (P□0.05) increased. Low doses of CORT (0.3 and 3mg/kg) treatment significantly (P□0.05) increased the Bcl-2 and P-Bad, and reduced the ratio of Bax/Bcl-2 compared with the CCI group. In contrast, high-dose CORT treatment significantly (P□0.05) reduced the level of P-Bad and increased the ratio of Bax/Bcl-2 by upregulating the expression of Bax. [one-way ANOVA, F (6,55) = 135.379, P< 0.01 for Bcl-2; F (6,55) = 77.776, P< 0.01 for Bax; F (6,55) = 41.326, P< 0.01 for P-Bad; F (6,55) = 81.04, P< 0.01 for Bax/Bcl-2]. The dose dependent effects of CORT on the BCL-2 family proteins were counteracted by RU486 and SPIRO, respectively, which demonstrated that these effects were GR- and MR-mediated.

In addition, the interactions among Bad, Bcl-2 and Bax directly reflected their activities. Bad can combine with Bcl-2 and inhibits its activity, which exhibits anti-apoptotic effect by binding to Bax. The Co-IP results showed that the level of Bcl-2 combining with Bad was significantly (P□0.05) increased, and the level of Bax combining with Bcl-2 was significantly (P□0.05) reduced on post-injury day 3. low dose of CORT (0.3) significantly (P□0.05) reduced the interaction between Bad and Bcl-2 and increased the level of Bax combining with Bcl-2. On the contrary, high dose of CORT (30mg/kg) significantly (P□0.05) increased the level of Bcl-2 combining with Bad and reduced the level of Bax combining with Bcl-2 **(Fig. 7)** [one-way ANOVA, F (6,35) = 107.86, P< 0.01 for Bcl-2; F (6,35) = 96.17, P< 0.01 for Bax; F (6,35) = 136.94, P< 0.01 for P-Bad/Bad].

The opposite effects of MR and GR on apoptosis were mediated by different downstream signaling pathways

The PI3K/Akt, MAPK/Erk and P53 pathways are crucial for the survival of neurons by activating CREB and regulating the expression of Bcl-2 protein family after TBI. Our results showed that the levels of P-Akt, P-CREB, and P-Ert in the ipsilateral hippocampus were significantly reduced, whereas the expression of

P53 was significantly increased at 3 days after TBI. Lower doses of CORT (0.3, 3 mg/kg) significantly (P□0.05) increased p-Akt and p-CREB rather than P-Erk and P53, while high-dose CORT significantly (P□0.05) inhibited the activation levels of p-Erk, p-CREB and P53 but not p-Akt as compared to that in the low-dose CORT group [One-way ANOVA, F (6,55) = 108.698 for p-Akt; F (6,55) = 99.531 for p-CREB; F (6,55) = 59.883 for P-53; F (6,55) = 29.990 for p-Erk]. The opposite effects of different doses of CORT were counteracted by RU486 and SPIRO, respectively **(Fig. 8)**.

Discussion

We previously found that continuous and full activation of MR by low-dose CORT protected hippocampal neurons from apoptosis in the acute phase after TBI, while overactivation of GR by DEX promoted apoptosis [19,20]. In the present study, we examined the dose-dependent dual effects of CORT on the activation of MR and GR, the survival of hippocampal neurons, the spatial memory ability, and the receptor downstream signaling pathways after TBI. We found that lower doses of CORT (0.3, 3 mg/kg) replacement significantly increased the activation of MR, upregulated the phosphorylation of Akt/CREB/Bad and the expression of Bcl-2, reduced the number of apoptotic neurons, and subsequently improved the spatial memory. While higher dose of CORT (30 mg/kg) exhibited the opposite effect by over activating GR, upregulating the expressions of P53/Bax, and inhibiting the activity of Erk/CREB pathway.

Due to the potent anti-inflammatory and immunosuppressive effects, synthetic GCs rather than endogenous GCs were widely used in TBI patients for decades. However, the effectiveness and safety of synthetic GCs in TBI patients have been the focus of discussion and of huge controversial [27,28]. Until now, corticosteroids, especially synthetic GCs are still not recommended for TBI patients, because short-term use of MP have been found to be harmful to TBI patients [29]. However, due to the inadequate understanding of the effects of GC and its two receptors on CNS, the previous clinical studies have obvious limitations. For example, synthetic GCs (MP) with higher affinity for GR than MR or GR specific agonist (DEX) were selected in most of these studies. Additionally, the GR-induced anti-inflammatory effect of synthetic GCs were used to reduce brain edema and increased ICP after TBI, whereas GR-mediated neurotoxic effects of synthetic GCs and MR-mediated neuroprotective effects of endogenous GCs were overlooked.

Both our previous experimental studies and other clinical trials have revealed the protective effects of endogenous GCs after TBI [19,20,30]. In rat CCI model, we investigated the corticosteroid receptor mechanism and found that inadequate activation of MR was directly associated with increased neuron apoptosis in the ipsilateral hippocampal in the acute phase after TBI. Therefore, a proper dose of endogenous GCs replacement might be an effective neuroprotective agent for TBI patients. However, long-term exposure to high levels of endogenous GCs have been reported to be deleterious to the brain. A majority of the previous studies have revealed that chronic increased plasma CORT level induced by chronic stress or long-term CORT treatment impaired hippocampal structural integrity and function, including dendritic retraction, reduced neurogenesis in the dentate gyrus, and increased cell loss, which

were associated with increased depressive-behaviors and impaired spatial memory. A proper ratio of MR/GR activation by slightly increased CORT level induce neuroprotective effects, whereas low or high level of CORT breaks the balance of MR/GR leading to neurotoxic effects [30-33]. However, the set-point of GC balance may significantly vary under distinct pathological conditions and be affected by the age of individuals. As a mixed agonist for both MR and GR, whether short term use of high-dose CORT has deleterious effects on hippocampal neurons after TBI, and at which dose level CORT activates GR and breaks the balance of MR/GR remain unclear.

In the present study, we showed that CORT exhibited dose-dependent effects by activating MR and GR and their balance. Low-dose CORT treatment (0.3mg/kg) fully activated MR in the ipsilateral hippocampus, and the activation levels had no differences among the three CORT treatment groups. Lower doses of CORT (0.3mg/kg, 3mg/kg) alleviated the apoptosis in the ipsilateral hippocampus and promoted the recovery of spatial memory by increasing the activation level of MR and the ratio of MR/GR. The activation level of GR was significantly increased at the dose 3mg/kg, and peaked at 30mg/kg. However, only high dose of CORT (30 mg/kg) reduced the ratio of MR/GR, increased the neuronal apoptosis and aggravated the spatial learning impairment. Both CORT at 0.3mg/kg and 3mg/kg restored the ratio of MR/GR and exhibited protective effects. The balance of MR/GR but not the single MR or GR determined the dose-dependent effects of CORT, only when the activation of GR reached a certain level leading to a reverse of MR/GR, the protective effects of CORT were counteracted by excessive activation of GR.

As a main target of GCs, hippocampus is one of the most common injury structures and is vulnerable to the secondary injuries such as apoptosis [34]. Apoptosis is an important form of cell death contributing to one to two third of cell loss after TBI [15]. Our previous studies found that apoptosis mainly occurred in the ipsilateral hippocampus at 24 h, and peaked at 48-72h after TBI [19,20]. Bcl-2 family proteins include pro- and anti-apoptotic members. The homodimerization of pro-apoptotic proteins such as Bax can promote the formation of mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c from mitochondria, whereas the anti-apoptotic proteins such Bcl-2 exert anti-apoptotic effects by binding to Bax. Additionally, some BH3-only proteins such as Bad can bind to Bcl-2 family members and hence inhibit its anti-apoptotic ability [35-37]. Therefore, only the expression levels of pro- or anti-apoptotic proteins can’t reflect the real status of apoptosis, but the ratio of Bax/Bcl-2 and their protein-protein interactions seem to determine whether a cell survive or death. Although we have reported the MR-mediated anti-apoptotic effects of CORT treatment after TBI, but the dose-dependent effects of CORT and the downstream mechanisms remain unclear. In the present study, we found that both Bax expression the ratio of Bax/Bcl-2 were significantly increased on postinjury day 3. Low doses of CORT (0.3mg/kg, 3mg/kg) reduced the ratio of Bax/Bcl-2 by upregulating the expression of Bcl-2. In contrast, the expression of Bax and the ratio of Bax/Bcl-2 were increased by high-dose CORT treatment (30mg/kg). In addition, low doses of CORT reduced the level of Bcl-2 binding to Bad and accordingly increased the level of Bax binding to Bcl-2, whereas high dose of CORT exerted the opposite effects. The data suggested that different doses of CORT exhibited opposite effects on the ratio of Bax/Bcl-2 in the hippocampus after TBI. The dose-dependent effects of CORT were mediated by two receptor system, MR

and GR. However, their target protein was different, MR increased the Bcl-2 expression, whereas GR upregulated the expression of Bax.

Several intracellular signaling pathways are found to be involved in neuronal apoptosis after TBI [38-40], such as Akt, the activation of which exhibits anti-apoptotic effects by inactivation of Bad and phosphorylation of CREB [41]. As a key transcription factor, CREB plays important roles in neuronal survival and neurogenesis in many conditions by regulating the gene expressions, including brain-derived neurotrophic factor (BDNF) and Bcl-2 [42,43]. In addition, the crosstalk between these apoptosis-related pathways is very common. For instance, CREB has been reported to be activated by several signaling pathways after TBI, including IP3K/Akt, cyclic AMP-dependent protein kinase/protein kinase-A, as well as MAPK/Erk pathway [44-46]. However, the effect of a given signaling pathway on CREB remains controversial, because the overall activation level of CREB is generally determined by the interactions of multiple signaling pathways but not a single one. In addition, even the same protein is differentially activated depending on the region of brain and timing after TBI.

Previous studies have found that chronic stress or prolonged exogenous GCs administration aggravate neuronal apoptosis by suppressing CREB activation [47], whereas transient elevation of GC exhibits neuroprotective effects by upregulating CREB activation and BDNF expression [48,49]. However, the receptor mechanism of the contradictory results remains unclear, and the dose-dependent effects of CORT on CREB need further study. In the present study, we showed that the activity of the Erk, Akt and CREB were reduced on post-injury day 3 when the neuronal apoptosis peaked in the ipsilateral hippocampus. Lower doses of CORT (0.3mg/kg, 3mg/kg) upregulated the activation levels of p-Akt and p-CREB. However, when the dose increased to 30mg/kg, short use of CORT exerted the opposite effects by inhibiting the activities of Erk and CREB and upregulating the expressions of P53 and Bax. The GR-specific inhibitor RU486 and MR specific inhibitor SPIRO counteracted the opposite effects of high and low doses of CORT on the above proteins, which proved the pro-apoptotic effect of GR and the anti-apoptotic effect of MR were mediated by different downstream signaling pathways.

In conclusion, our study showed the dose-dependent effects of short-term CORT treatments on spatial memory, cell apoptosis in the ipsilateral hippocampus, and the different receptor downstream signaling pathways after TBI. Our study is of important clinical significance. On the one hand, we reveal a new neuroprotective mechanism of endogenous GC in modulation of apoptosis after TBI, which may therefore provide important therapeutic targets for the modulation of cognition and mood. On the other hand, our results suggest that lower doses of endogenous GC maintaining a proper activation ratio of MR/GR is beneficial and should be recommended for TBI patients. However, although the safe dose range of CORT is very wide (0.3-3mg/kg), there is a set-point at which the role of CORT shifts from anti-apoptosis to pro-apoptosis. A much higher dose (30mg/kg) can also exhibit neurotoxic effects, even short-term use.

Abbreviations

CORT: corticosterone; CREB: cAMP-responsive element-binding protein; CCI: controlled cortical impact; DEX: dexamethasone; DG: dentate gyrus; GCs: glucocorticoids; GR: glucocorticoid receptor; MP: methylprednisolone; MR: mineralocorticoid receptor; PKB (Akt): protein kinase B; Erk: extracellular regulated protein kinases; TBI: traumatic brain injury

Declarations

Ethics approval and consent to participate

All experimental procedures were carried out in accordance with the guidelines approved by the Capital Medical University Institutional Animal Care and Use Committee.

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Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Liu B and Zhang B conceived and designed the experiments. Yang M, Yan Q and Dong J performed CCI model and WB. Zhuang Y, Ge Q and Lu S performed the histological analysis. Zhang B, Xu X and Niu F analyzed the data. Zhang B and Liu B wrote and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests

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Figures

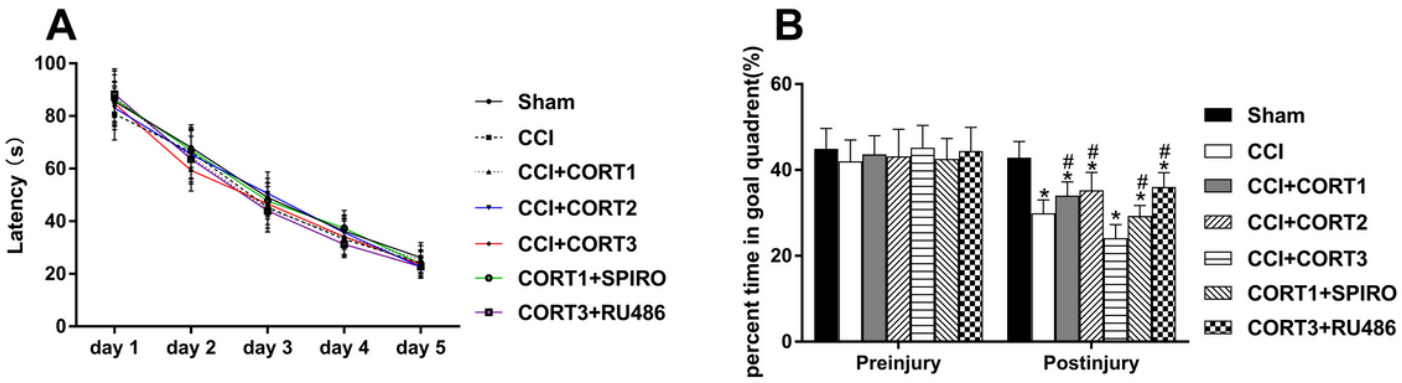

Figure 1

The dose-dependent effects of CORT treatments on spatial memory after CCI. (A) Quantification of the escape latency of the MWM test before CCI. (B) The percentage of time in the goal quadrant during the probe trial at before and after CCI (n=24 per group). *P<0.05 versus the sham control group; #P<0.05 versus the CCI control group. Data are presented as the means ± SDs.

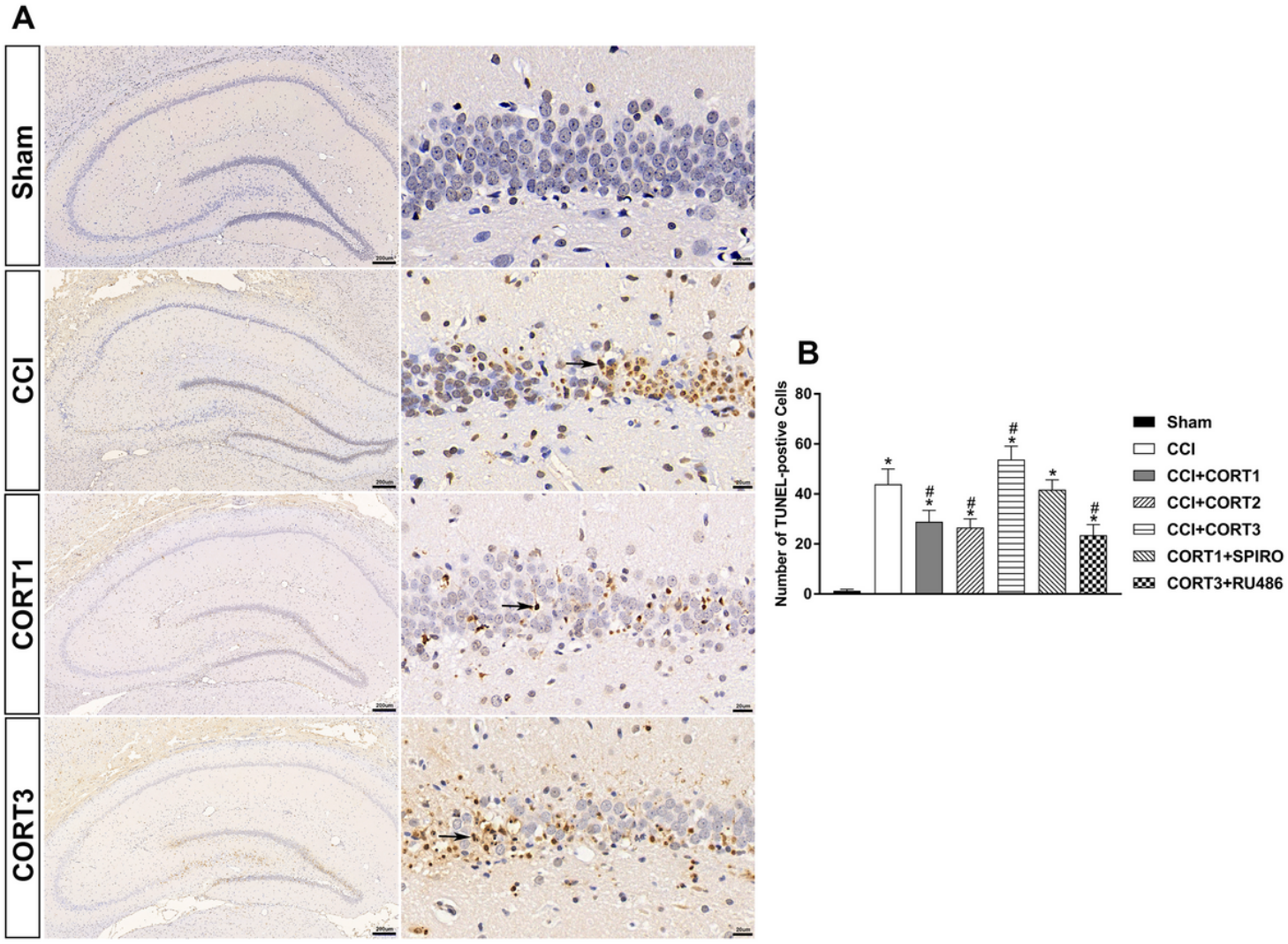

Figure 2

The dose-dependent effects of CORT treatments on the apoptosis of ipsilateral hippocampal neurons after CCI. (A) Representative images of TUNEL-positive cells (black arrow) in the ipsilateral hippocampus at 3 days after CCI. (B) Quantification of apoptotic neurons in the ipsilateral hippocampus. *P<0.05 versus the sham control group; #P<0.05 versus the CCI control group. Data are presented as the means ± SDs.

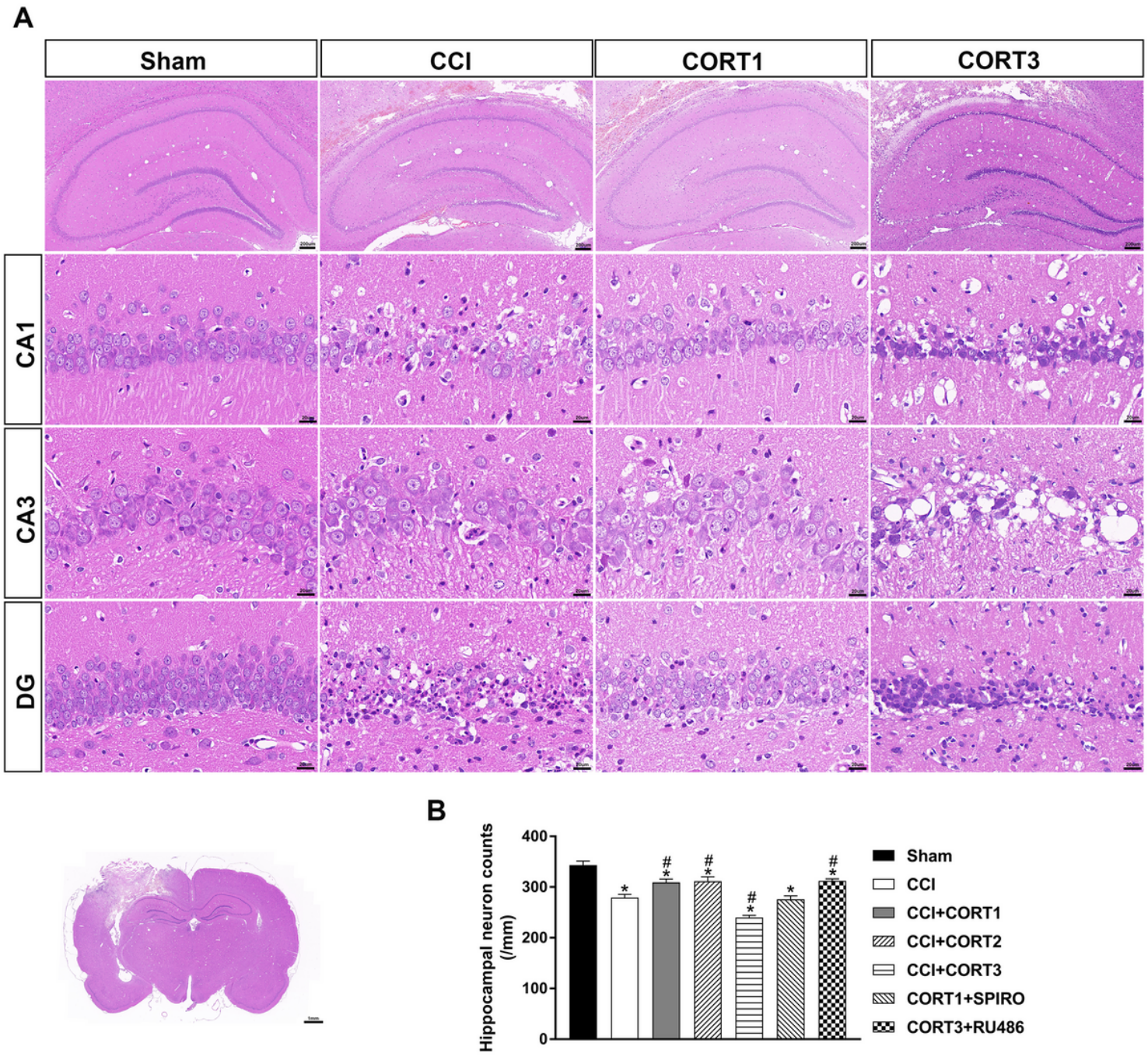

Figure 3

The dose-dependent effects of CORT treatments on the survival of neurons in the ipsilateral hippocampus after CCI. (A) Representative images of H&E staining showing ipsilateral hippocampal neurons in subareas. (B) Quantification of neurons in the ipsilateral hippocampus. *P<0.05 versus the sham control group; #P<0.05 versus the CCI control group. Data are presented as the means ± SDs.

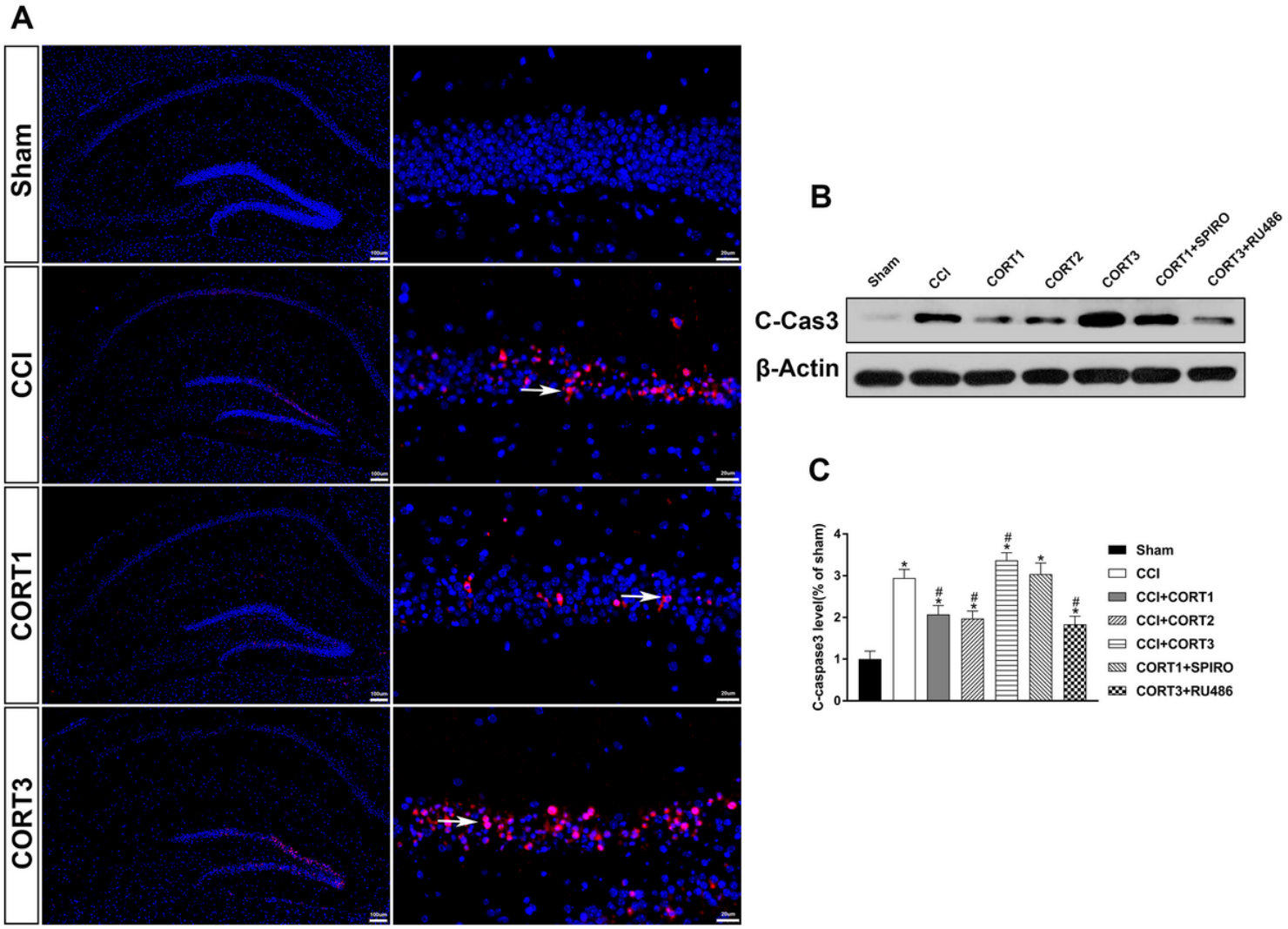

Figure 4

The dose-dependent effects of CORT treatments on the level of cleaved caspase-3 after CCI. (A) Representative images of cleaved caspase-3 (red, white arrow) immunofluorescence in the ipsilateral hippocampus. (B) Representative western blot images of cleaved caspase-3. (C) Quantification of the cleaved-caspase 3 expression. The relative band density was measured with ImageJ (1.49 V) and normalized to that of β-actin, and the percent expression compared to that of sham controls was calculated for each sample. *P□0.05 compared to the sham group; and #P□0.05 compared to the CCI control group. The data are presented as the means ± SDs of 8 animals per group.

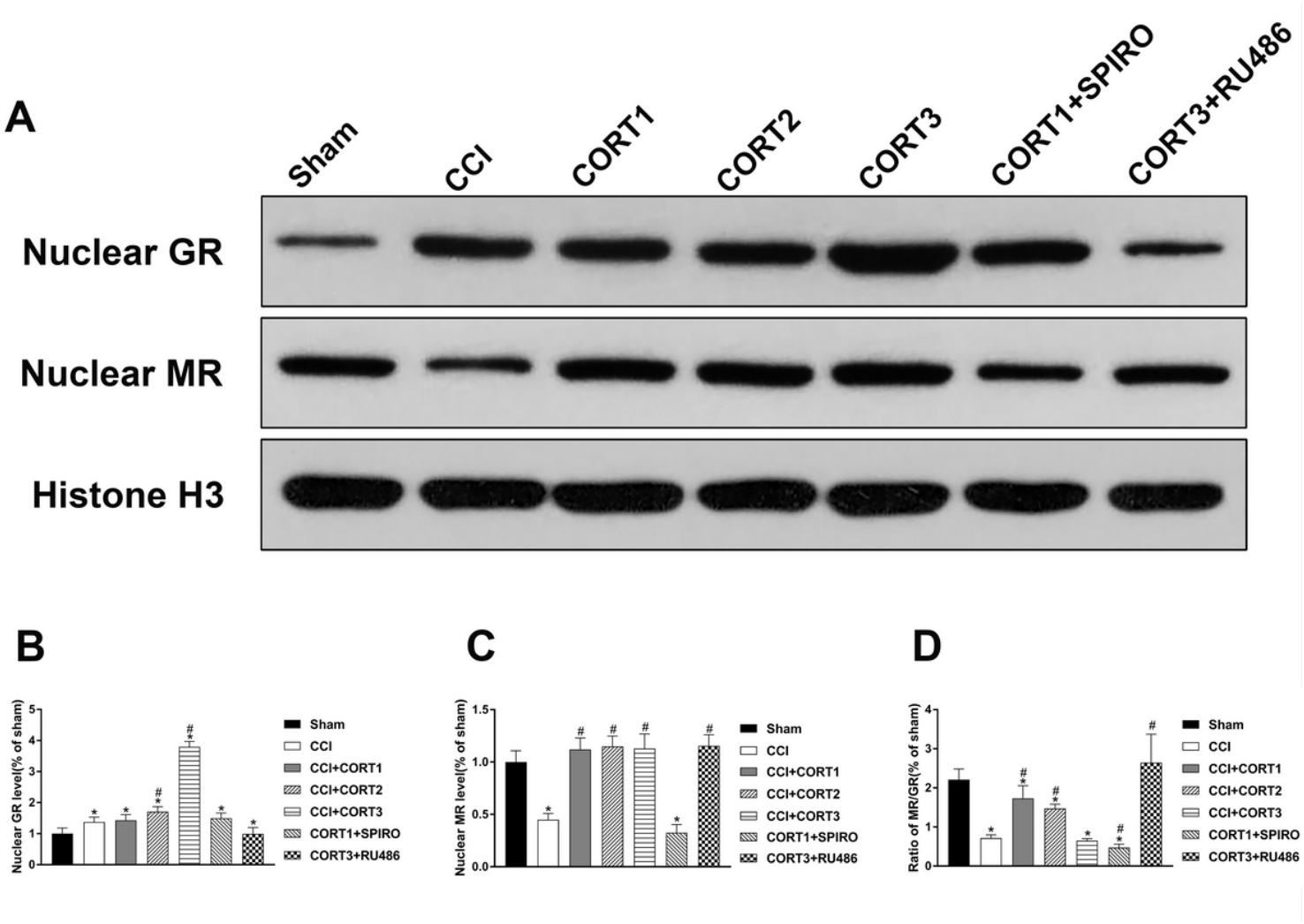

Figure 5

The dose-dependent effects of CORT treatments on activations of MR and GR. (A) Representative western blot images of nuclear MR and GR. (B) Quantification of nuclear MR; (C) Quantification of nuclear GR. The relative band density was measured with ImageJ (1.49 V) and normalized to that of histone H3, and the percent expression compared to that of sham controls was calculated for each sample. *P□0.05 compared to the sham group; and #P□0.05 compared to the CCI control group. The data are presented as the means ± SDs of 8 animals per group.

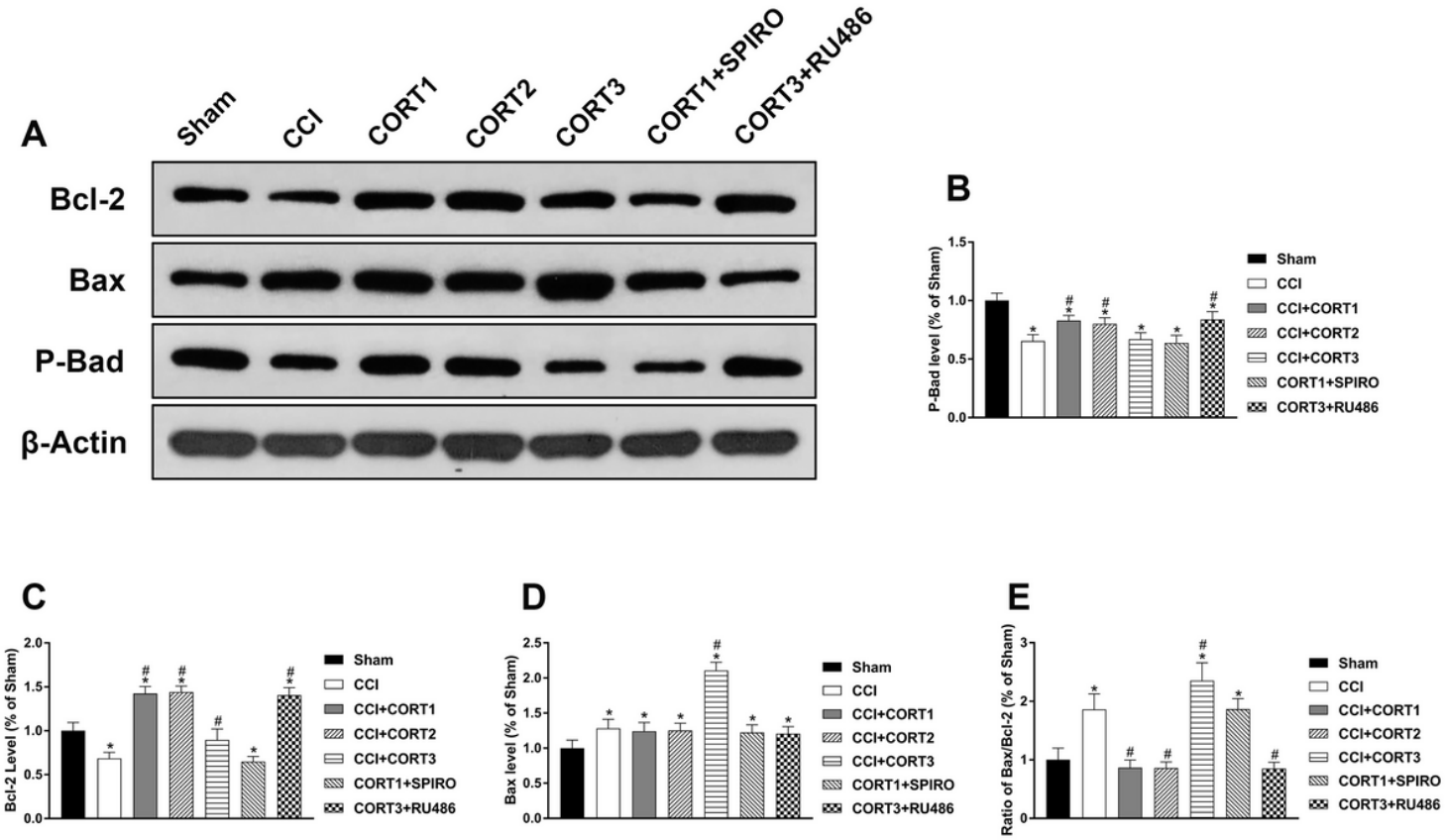

Figure 6

The dose-dependent effects of CORT treatments on the activation and expressions of Bcl-2 family proteins. (A) Representative western blot images of Bcl-2, Bax and p-Bad expressions. (B-D) Quantifications of p-Bad, Bcl-2, Bax and Bax/Bcl-2. *P<0.05 versus the sham control group; The relative band density was measured with ImageJ (1.49 V) and normalized to that of β-actin, and the percent expression compared to that of sham controls was calculated for each sample. *P□0.05 compared to the sham group; #P<0.05 versus the CCI control group. The data are presented as the means ± SDs of 8 animals per group.

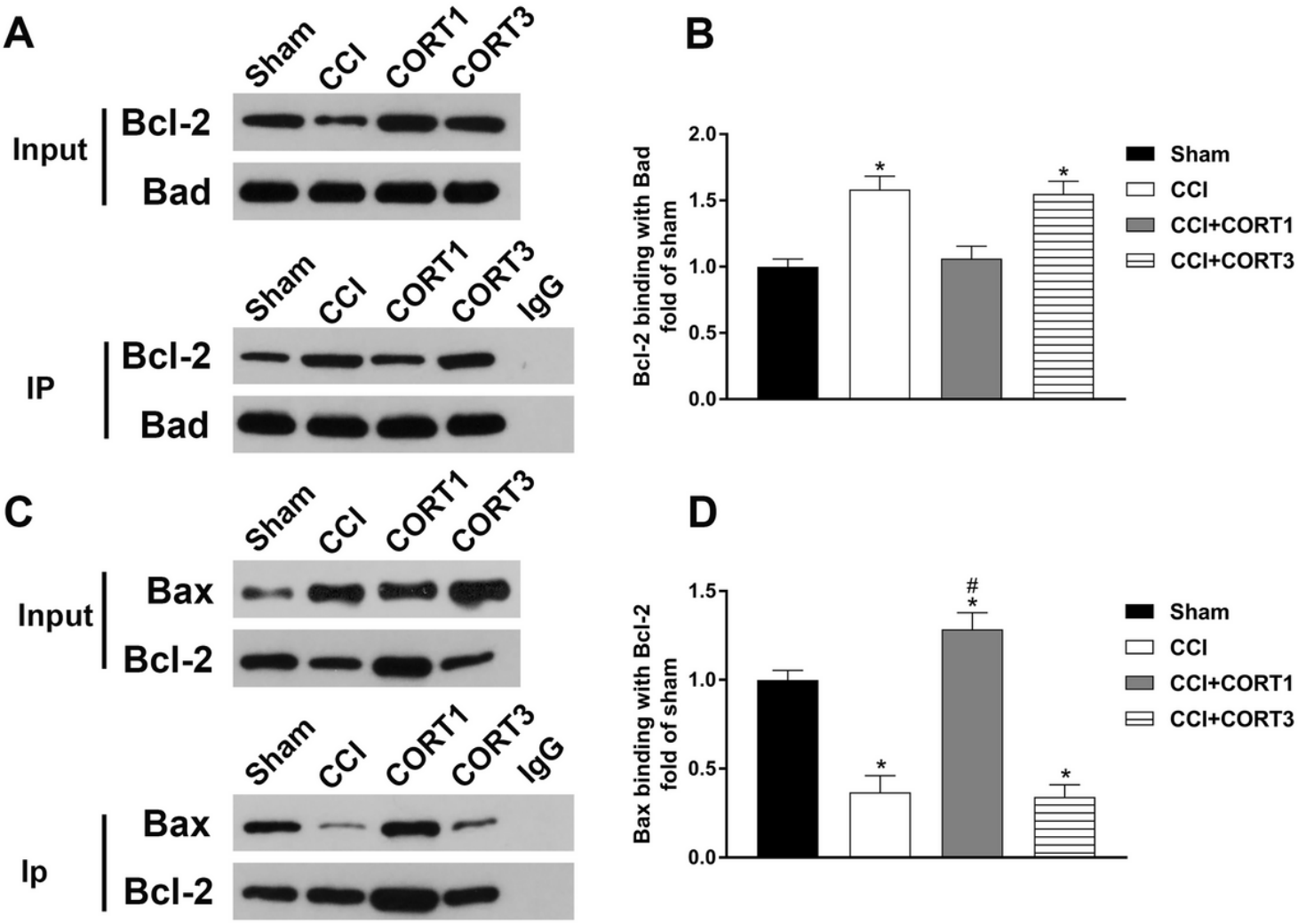

Figure 7

The dose-dependent effects of CORT treatments on the interactions between Bcl-2 family proteins. (A, B) Representative Western blot images and quantification for Bcl-2 level (binding with Bad) after Co-IP with Bad antibody. (C, D) Representative Western blot images and quantification for Bax level (binding with Bcl-2) after Co-IP with Bcl-2 antibody. The percent expression compared to that of sham controls was calculated for each sample. *P□0.05 compared to the sham group; #P<0.05 versus the CCI control group. The data are presented as the means ± SDs of 8 animals per group.

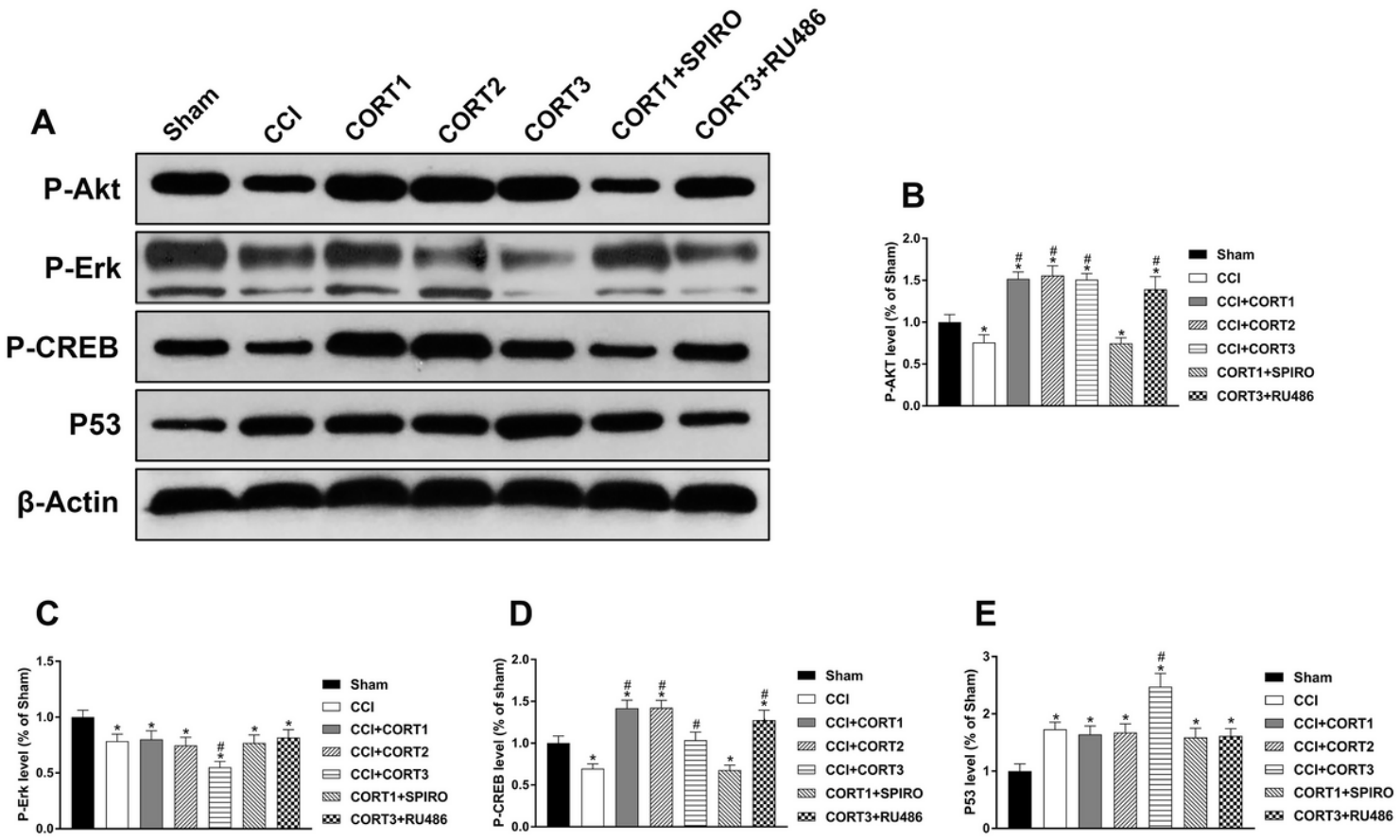

Figure 8

The dose-dependent effects of CORT treatments on apoptosis were mediated by different signal pathways. (A) Representative western blot images showing the levels of p-Akt, p-Ert, p-CREB, and P53 expressions. (B-E) Quantifications of p-Akt, p-Ert, p-CREB, and P53 in the ipsilateral hippocampus on post-injury day 3. The relative band density was measured with ImageJ (1.49 V) and normalized to that of β-actin, and the percent expression compared to that of sham controls was calculated for each sample. *P□0.05 compared to the sham group; and #P□0.05 compared to the CCI control group. The data are presented as the means ± SDs of 8 animals per group.